

Novel selective MMP-13 inhibitors reduce collagen degradation in bovine articular and human osteoarthritis cartilage explants

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Received: 3 August 2009 / Revised: 12 October 2009 / Accepted: 13 October 2009 / Published online: 10 November 2009
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Abstract

Objective MMP-13 is highly upregulated in arthritis and therefore strongly implicated in the pathogenesis of osteoarthritis (OA). Selective inhibition of MMP-13 may provide the desired cartilage degradation protection, while overcoming the musculoskeletal toxicity seen with nonselective inhibition of MMPs.

Methods Activity and selectivity of novel MMP-13 inhibitors were determined in enzymatic and collagenase assays. Inhibition kinetics and competitive binding experiments were performed. The inhibition of collagen degradation was studied in cartilage explants from OA patients and in bovine and human articular cartilage systems.

Results We have identified a new class of very potent and highly selective non-zinc-binding MMP-13 inhibitors.

Selective MMP-13 inhibitors completely blocked type II collagen degradation in bovine explants and showed up to 80% inhibition in human OA cartilage.

Conclusions These results indicate MMP-13 as the primary collagenase in the human OA cartilage and in the IL-1/OSM-induced cartilage degradation process and suggest that selective MMP-13 inhibitors may be a potential treatment of OA.

Keywords Osteoarthritis · Cartilage · Collagen degradation · Matrix metalloproteinase · Collagenase · MMP-13 · Inhibitor

Introduction

Osteoarthritis (OA) is a degenerative joint disease that involves not only the articular cartilage but also the synovium,

Responsible Editor: J. Di Battista.

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the joint capsule, and the subchondral bone [1]. The disease is characterized by a high degree of clinical and pathophysiological heterogeneity. The destruction of cartilage in arthritic conditions is believed to be catalyzed by matrix metalloproteases (MMPs), aggrecanases, and probably cathepsins [2–7].

MMP-13, the major type II collagenase, is highly upregulated in arthritic diseases and therefore strongly implicated in the pathogenesis of OA. The imbalance of collagen synthesis and degradation has been shown in humans to correlate with disease progression as assessed by biomarkers, arthroscopy, and radiographic analysis [8, 9]. Furthermore, MMP-13 transgenic mice develop cartilage defects similar to human OA [10]. An MMP inhibitor that preferentially inhibits MMP-13 has been shown to block the degradation of explanted human osteoarthritic cartilage [11]. Recently published results showed the efficacy of a selective MMP-13 inhibitor to reduce cartilage degradation in the rabbit ACL model [12] and in some models of rheumatoid arthritis [13]. The selective MMP-13 inhibitor investigated in this study showed chondroprotective effects in the rat model of monoiodoacetate (MIA)-induced OA and in a surgical medial meniscus tear model [14].

Broad spectrum MMP inhibitors have long been considered to be excellent drugs for treatment of rheumatoid arthritis and OA. However, the clinical utility of broad spectrum MMP inhibitors, mostly hydroxamic acids, has been restricted by a toxicity described as musculoskeletal syndrome (MSS) [2]. To avoid MSS in clinical studies, broad spectrum inhibitors were administered at lower doses, which resulted in a lack of efficacy. The molecular mechanism of MSS is not known, but it is largely believed that the toxicities seen were either due to the nonselective inhibition of multiple, critical MMPs or to liabilities associated with the hydroxamic acid moiety (reactive site zinc-binding group). Therefore, the design of highly selective MMP-13 inhibitors without a hydroxamic acid moiety represents the best strategy to avoid the limitation in the therapeutic index and to obtain a new high potential option for the treatment of arthritis. Accordingly, an orally active and fully selective MMP-13 inhibitor did not induce MSS-like side effects in a rat model of MSS [12, 14, 15].

Proinflammatory cytokines, such as interleukin-1 (IL-1), tumor necrosis factor α (TNF- α), and oncostatin M (OSM) are upregulated in OA and contribute to cartilage damage by induction and activation of MMPs and aggrecanases [16, 17]. In order to evaluate the potential of selective MMP-13 inhibitors to block excessive cartilage degradation in OA cartilage, human cartilage explants were cultured in the presence of the inhibitor, while the release of collagen fragments was measured and compared to untreated cartilage. Additionally, inhibition of collagen degradation stimulated with IL-1/OSM in bovine articular

and human osteoarthritic cartilage explants was studied using fully selective MMP-13 inhibitors. The aim of this study was to validate MMP-13 as a primary collagenase in cartilage destruction and to emphasize the potential role of selective MMP-13 inhibitors for the therapy of OA.

Materials and methods

Activity assays

MMP-13 activity and selectivity assays were performed using the catalytic domains of recombinant human MMPs (MMP-1, -2, -3, -7, -9: Biomol, Hamburg, Germany; MMP-8: Calbiochem, Schwalbach, Germany; MMP-13, -14: Invitek, Berlin, Germany). MMP-13 activity was tested using the specific MMP-13 substrate MCA-Pro-Cha-Gly-Nva-His-Ala-Dpa-NH₂ (Calbiochem). MMP-3 was tested using the NFF3 substrate MCA-Arg-Pro-Lys-Pro-Val-Glu-NvaTrp-Arg-Lys(DNP)-NH₂ (Calbiochem), and all remaining MMPs were tested using OmniMMP substrate MCA-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂*AcOH (Biomol). TACE activity was measured using the recombinant human TACE enzyme (R&D Systems, Wiesbaden, Germany) and the specific peptide substrate MCA-Pro-Leu-Ala-Gln-Ala-Val-Dpa-Arg-Ser-Ser-Ser-Arg-NH₂ (Calbiochem).

Recombinant bovine MMP-13 catalytic domain construct encompassing Tyr 104-Tyr 274 was generated by RT-PCR using bovine chondrocyte mRNA. The protein was expressed in *Escherichia coli* and purified from inclusion bodies as previously described [18].

The MMP-3 assay was performed in 50 mM MES buffer, pH 6.0, 10 mM CaCl₂, and 0.05% Brij-35. All other MMP assays were performed in 50 mM Tris-HCl buffer, pH 7.4, 150 mM NaCl, 5 mM CaCl₂, and 0.05% Brij-35. TACE assay was run in 25 mM Tris-HCl, pH 9, 1.25 μ M Zn-acetate, and 0.05% Brij-35. Concentrations of enzymes and substrates were optimized for each assay and ranged from 1 to 10 nM for the enzyme and 4 to 10 μ M for the substrate. The enzyme activity was measured after 10 min preincubation of the enzyme with differing concentrations of the inhibitor. The IC₅₀ values were calculated using Life Science Workbench (LSW) Data Analysis Plugin for Microsoft Excel. The data were fitted into the formula: $y = V_{\max}/(1 + ([I]/IC_{50}))$.

Aggrecanase activity was determined using the EnviLISA Kit (Invitek). Human recombinant aggrecanases (0.75 nM ADAMTS-4 and 0.5 nM ADAMTS-5) were incubated with different concentrations of compounds and the recombinant interglobular domain of human aggrecan (100 nM aggrecan-IGD) as substrate for 15 min at 37°C. After EDTA termination, the generated aggrecan cleavage

neopeptides (ARGSVIL) were detected and quantified by ELISA.

Collagenase cleavage assay

Inhibition assays of the MMP-13-catalyzed hydrolysis of bovine type II collagen (from bovine trachea, Sigma-Aldrich, Taufkirchen, Germany) were performed in 50 mM Tris-HCl buffer, pH 7.4, 5 mM CaCl₂, 150 mM NaCl, and 0.05% Brij-35. Full length MMP-13 (10 nM; Invitex), activated with 1 mM APMA (*p*-aminophenyl mercuric acetate, Sigma-Aldrich) for 90 min at 37°C, was incubated with 0.5 mg/mL soluble type II collagen and different concentrations of inhibitor at 27°C for 4 h. The reaction was stopped by adding an equal volume of 2× SDS sample loading buffer. The proteins were separated by 10% SDS-PAGE and visualized by Coomassie staining. The 3/4-cleavage protein bands were scanned and quantified using DIANA II detection system (Raytest, Straubenhardt, Germany). The IC₅₀ was defined from a plot of the percent (%) of control 3/4-protein band versus inhibitor concentration.

Fluorogenic assay format was used for the inhibition of MMP-13-catalyzed hydrolysis of DQ type I collagen from bovine skin (Invitrogen, Karlsruhe, Germany). A 10 nM sample of activated MMP-13fl was incubated with 20 µg/mL type I collagen and different concentrations of inhibitor. Reaction rates were measured by detection of the fluorescent signal generated by cleavage of the substrate. For the IC₅₀ calculation, the relative fluorescence light units after 50 min reaction are plotted versus the concentration of the inhibitor.

Inhibition kinetics of MMP-13

To determine the kinetic mechanism of inhibition by ALS 1-0635, 1 nM recombinant catalytic MMP-13 domain was assayed in reaction mixtures containing 0–25 µM MMP-13 peptide substrate 5-FAM-Pro-Cha-Gly-Nva-His-Ala-Dap(QXL520)-NH₂ (Tebu-bio, Offenbach, Germany) and various concentrations of the inhibitor. Kinetic analysis and comparison of different models were performed using SigmaPlot Enzyme Kinetics module (SigmaPlot 10.0, Systat Software).

Competitive binding experiment

The objective of this study was to determine if ALS 1-0635 binds to the catalytic zinc moiety of the MMP-13 enzyme. The IC₅₀ value determinations of ALS 1-0635 with MMP-13 were performed in the presence or absence of a fixed concentration of the zinc-binding agent acetohydroxamic acid (AcNHOH, 15 mM) according to the

protocol of the MMP-13 activity assay. For Dixon plot analysis, reciprocal velocity was calculated. Linear fitting of the experimental data was performed as $[I]$ versus $1/v$, where $[I]$ is the concentration of inhibitor and v is the initial velocity.

Human OA cartilage explant degradation assay

Human femoral and tibial cartilage was obtained from total knee arthroplasty of seven patients (from the Orthopaedic University Hospital, Heidelberg, Germany, and Aeirtec, Newcastle upon Tyne, UK). For each experiment, the obtained cartilage was cut into small pieces. Approximately 60 mg of the cartilage was randomly distributed into each culture well (24-well plate, 4 wells/condition) and cultured in 1 mL DMEM, Pen/Strep, 50 µg/mL ascorbic acid, 0.1 mg/mL BSA, ITS (5 µg/mL insulin, 5 µg/mL transferrin, 5 ng/mL sodium selenite), and 50 mg/mL gentamicin. Media were replaced and collected every 3–4 days over a period of 3 weeks. The conditioned media from OA cartilage without inhibitor and cartilage cultured in presence of 5 µM ALS 1-0635, 5 µM ALS 1-1087, or 500 nM GM6001 were analyzed for collagen degradation product release using the C1,2C ELISA (IBEX, Montreal, Canada) [11, 19, 20].

IL-1/OSM-stimulated cartilage explant degradation assay

Bovine articular cartilage was freshly isolated from the first phalanges of 16- to 18-month-old cows shortly after slaughter, cut into ~3 mm² pieces, and randomly distributed into a 48-well plate (~30 mg/well; 6 wells/condition). After 48 h preincubation in culture medium, the cartilage explants were cultured for 12 days in the presence of 5 ng/mL IL-1 α (Tebu-bio) and 50 ng/mL oncostatin M (OSM; Tebu-bio) and inhibitor at different concentrations. Media were replaced and collected every 3–4 days.

Human OA cartilage was cut into small pieces, randomly distributed (48-well plate; ~30 mg/well; 6 wells/condition), and cultured for 21 days in the presence of 5 ng/mL IL-1 α and 50 ng/mL OSM and inhibitor at various concentrations. Conditioned media were analyzed for the release of collagen degradation products using the C1,2C ELISA, C2C ELISA (IBEX), and hydroxyproline determination [21]. Briefly, 50 µL of conditioned cell culture media was hydrolyzed with concentrated HCl over 18 h at 100°C. The hydrolysate was dried, washed, and redissolved in citric acid buffer, pH 6. Chloramin T (Sigma-Aldrich) and DMBA (dimethylaminobenzaldehyde, Sigma-Aldrich) were added and incubated for 20 min at 70°C. Absorbances were determined at 570 nm on a Bio-Tek EL808 microplate reader. Standard curve was measured with hydroxyproline ranging from 20 to 2,000 ng/well.

Cartilage penetration assay

Isolated cartilage slices (5–10 mg each) were incubated with either 1 μ M ALS 1-0635 or ALS 1-1087 in PBS buffer containing no HSA or 1% (w/v) HSA for 48 h at room temperature. The cartilage slices were carefully dried from buffer and weighed. The cartilage was digested by freshly prepared 3 U/mL papain solution (Roche Diagnostics, Mannheim, Germany) and 0.35 mg/mL cysteine (Sigma-Aldrich) at 65°C for 3 h by shaking. The digested cartilage proteins were precipitated with acetonitrile and the compounds were quantified by HPLC-MS (HPLC, Agilent, Waldbronn, Germany; Quattro Ultima triple-quadrupole mass spectrometer, Waters, Eschborn, Germany).

Results

In vitro potency and selectivity of MMP-13 inhibitors

Alantos Pharmaceuticals has synthesized several structural classes of potent MMP-13 inhibitors [22] with IC_{50} values in the low nM and pM range in the activity assay utilizing the catalytic domain of MMP-13 enzyme and a specific peptide substrate. Activities of two different inhibitors, ALS 1-0635 and ALS 1-1087, are presented in Table 1. Remarkably, these highly potent MMP-13 inhibitors show no activity against other tested MMPs, TACE, or aggrecanases up to micromolar concentration. In contrast to this

Table 1 Selectivity of ALS 1-0635, ALS 1-1087, and GM6001

Zn-metalloproteinase	IC_{50} (nM)		
	ALS 1-0635	ALS 1-1087	GM6001
MMP-1cat	>20,000	>20,000	1.4
MMP-2cat	>20,000	>20,000	0.6
MMP-3cat	18,000	15,000	6.8
MMP-7cat	>20,000	>20,000	NT
MMP-8cat	>20,000	>20,000	0.39
MMP-9cat	>20,000	>20,000	0.5
MMP-12cat	>20,000	>20,000	1.1
MMP-13cat	2.1	1.3	0.36
MMP-14cat	>20,000	>20,000	2.5
ADAM17	>20,000	>20,000	40
ADAMTS-4	>20,000	>20,000	547
ADAMTS-5	>20,000	7,000	NT

The inhibitors were used at the highest concentration of 20 μ M, and >20,000 means no inhibition observed at the highest concentration used

NT Not tested

high selectivity, the hydroxamic acid-containing broad spectrum inhibitor GM6001 (Ilomastat) inhibits all tested zinc-metallo proteinases.

The potency of ALS 1-0635 and ALS 1-1087 to inhibit the collagenase activity of the full length MMP-13 enzyme was tested using bovine type I and type II collagen as native substrates. Both compounds potently inhibited the MMP-13-catalyzed collagen hydrolysis (Table 2; Fig. 1). No relevant differences were detected between the inhibition of human and bovine MMP-13 catalytic domain or by inhibiting collagen type I or type II hydrolysis by these two compounds (Table 2).

Biochemical characterization of the selective MMP-13 inhibitor

MMP-13 enzyme activity was measured in the presence of increasing substrate concentrations and increasing ALS 1-0635 concentrations. The initial velocity (picomoles of cleaved substrate per minute, pmol/min) was calculated from the slope of the initial reaction curve. For determination of the kinetic constants, the data were plotted in a linear form (Lineweaver-Burk plot; Fig. 2). The best fit of the experimental data was achieved for a mixed-type inhibitor. Kinetic models were ranked by correlation coefficient R^2 , which quantifies the goodness of fit. The best fit, highest value for R^2 (0.98152) was achieved for the mixed-type inhibitor model with $K_i = 0.78$ nM, $V_{max} = 430$ pmol/min, $K_m = 5.5$ μ M, and $\alpha = 16.3$.

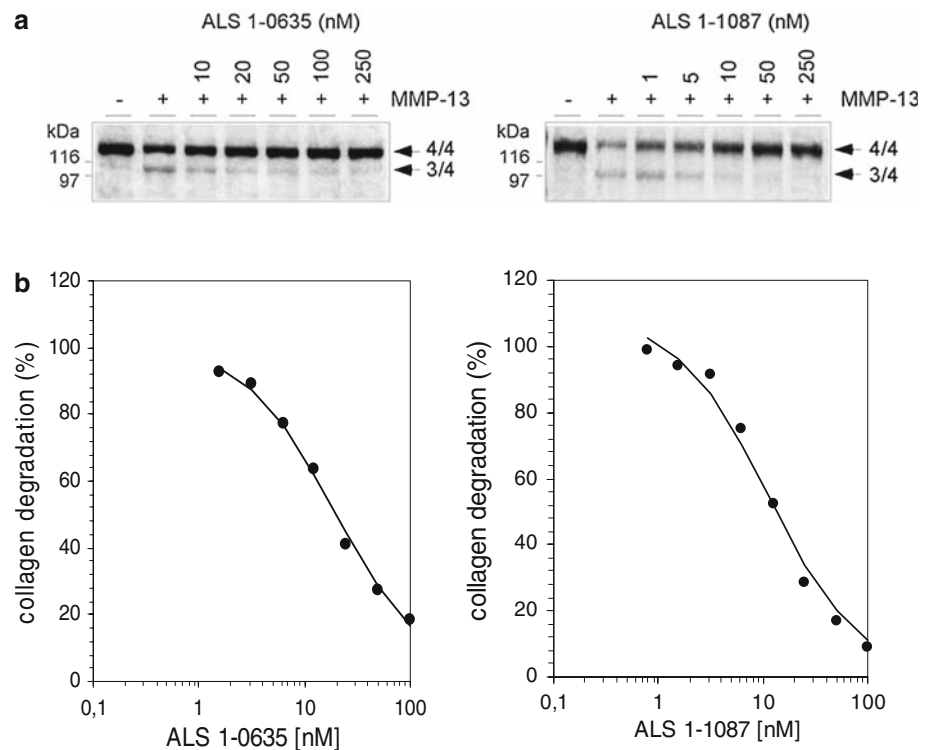
To address the question of whether ALS 1-0635 binds to the catalytic zinc ion of MMP-13, multiple inhibition analysis was performed. The IC_{50} value determination of ALS 1-0635 with MMP-13 was performed in the presence and absence of a fixed concentration of the zinc-binding agent acetohydroxamic acid (AcNHOH). A dual inhibition analysis characterizes the binding sites of the two studied inhibitors. The two inhibitors can either bind exclusively (competitive with respect to each other) to the enzyme or nonexclusively. The IC_{50} determination of ALS 1-0635 alone and in the presence of a fixed concentration of AcNHOH was performed using fluorescent peptide substrate and the catalytic domain of MMP-13. The IC_{50} of ALS 1-0635 was calculated to be 1.44 nM. In the presence of AcNHOH, the IC_{50} value was lowered (1.39 nM). The ratio of IC_{50} (ALS 1-0635 + AcNHOH)/ IC_{50} (ALS 1-0635) = 1.39/1.44 = 0.96 is smaller than 1.

For the Dixon plot analysis, the reciprocal velocity was calculated. Linear fitting of the experimental data was performed (Fig. 3a). Dixon plot for ALS 1-0635 alone and ALS 1-0635 in the presence of AcNHOH resulted in intersecting lines, typical for nonexclusive inhibitors.

Table 2 Comparison of ALS 1-0635 and ALS 1-1087 activity against human and bovine catalytic domain of MMP-13 and in MMP-13-catalyzed hydrolysis of type I and type II collagen

Inhibitor	IC ₅₀ (nM)			
	hMMP-13cat + synth. peptide	bMMP-13cat + synth. peptide	hMMP-13fl + bov. DQ-type I collagen	hMMP-13fl + bov. type II collagen
ALS 1-0635	2.1	1.2	20	27
ALS 1-1087	1.3	3.5	10	4.4

Fig. 1 Inhibition of MMP-13-catalyzed cleavage of bovine type II (a) and type I (b) collagen by ALS 1-0635 and ALS 1-1087. **a** SDS-PAGE-gels were stained with Coomassie brilliant blue. The arrows indicate the relative positions of the uncleaved collagen type II α chains (4/4) and the collagenase-generated $\frac{3}{4}$ -fragment. Collagen was incubated with activated MMP-13 for 4 h at 27°C in the presence of increasing concentrations of inhibitors. The density of the $\frac{3}{4}$ -length product was quantified (Table 2). **b** Fluorogenic assay was used to determine the inhibition of the cleavage of bovine type I collagen (FITC-labeled). Percent (%) collagen degradation was plotted versus the concentration of the inhibitor and the IC₅₀ was calculated



Inhibition of collagen degradation in human OA cartilage explants

Osteoarthritic articular cartilage is characterized by enhanced cleavage of type II collagen, which is associated with the upregulation of synthesis and activities of collagenases, in particular MMP-13. In order to evaluate the potential of selective MMP-13 inhibitors to block excessive cartilage degradation in OA cartilage, human cartilages were cultured in the presence of 5 μ M ALS 1-0635, ALS 1-1087, and 500 nM GM6001, while the release of collagen fragments was measured and compared to untreated cartilage. To account for the high variability among the OA cartilage specimens within the joint and among different patients, OA cartilage of seven patients was tested in this study.

Supernatants of the human OA cartilage explants were collected at days 3, 7, 10, 14, and 17 and analyzed individually for collagen degradation products. For every OA cartilage specimen, cumulative collagen degradation over

the period of 17 days was calculated (Fig. 4). The amount of released collagen fragments (C1,2C epitope) from the control cartilage in the absence of any inhibitor was set to 100% and the inhibition in the presence of the inhibitors was calculated for each single experiment (percent of control). The total amounts of collagen degradation (C1,2C epitope) in the control cartilage explants range from 0.075 to 0.298 μ g/mL. In the presence of the broad spectrum MMP inhibitor GM6001, the collagen degradation was reduced to 6–34% of the control, whereas in the presence of ALS 1-0635 or ALS 1-1087 between 21 and 96 or 21 and 76%, respectively, of the control amount was detected. The mean inhibition of collagen degradation was 41% for ALS 1-0635, 49% for ALS 1-1087, and 81% for GM6001.

Inhibition of II-1/OSM-stimulated collagen degradation

Supernatants of bovine cartilage explants after 10 days of culture in the presence and absence of the inhibitors were

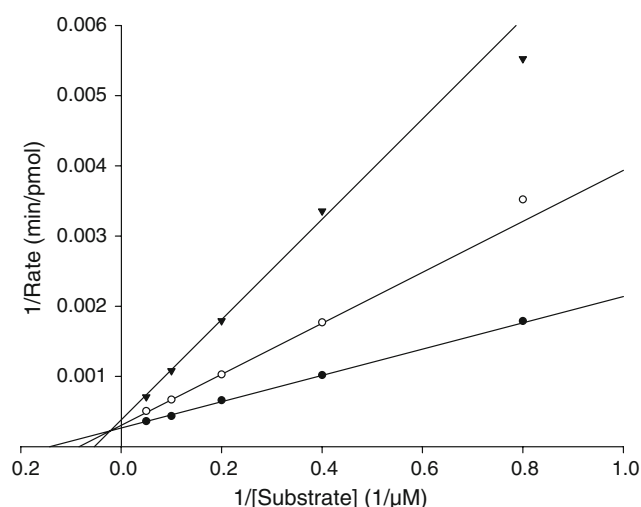


Fig. 2 Kinetic analysis of MMP-13 inhibition by ALS 1-0635. Lineweaver-Burk linear transformation is plotted for 0 (filled circles), 1 (open circles), and 3 nM (triangles) ALS 1-0635. The best fit was achieved for a mixed-type inhibitor

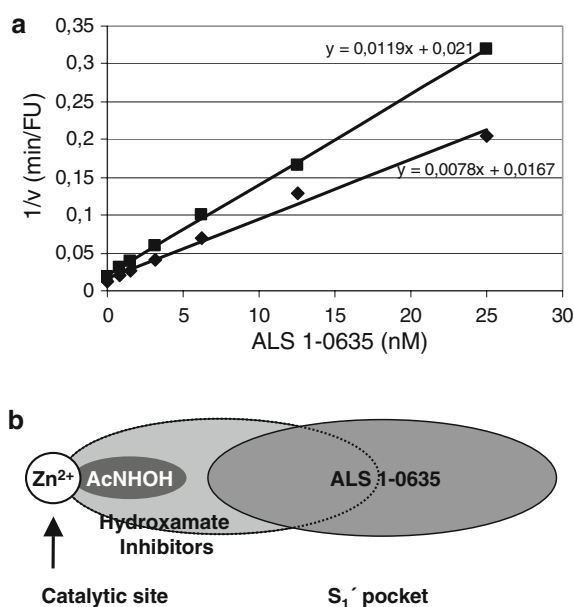


Fig. 3 **a** Dixon plot analysis of ALS 1-0635 alone (diamonds) and in the presence of acetohydroxamic acid AcNHOH (squares). Reciprocal velocity ($1/v$) was plotted versus inhibitor concentration (I). Linear fitting of the experimental data is shown in the diagram. **b** Schematic representation of the binding site of ALS 1-0635

analyzed for the collagen degradation fragments using the C1,2C ELISA. Collagen degradation from explants cultured with IL-1/OSM was increased approximately ninefold in comparison to the control cartilage (Fig. 5). The amount of the released collagen fragments in the control cartilage in the presence of cytokines and in the absence of inhibitors was set to 100%. The collagen degradation in the presence of inhibitors was calculated as a

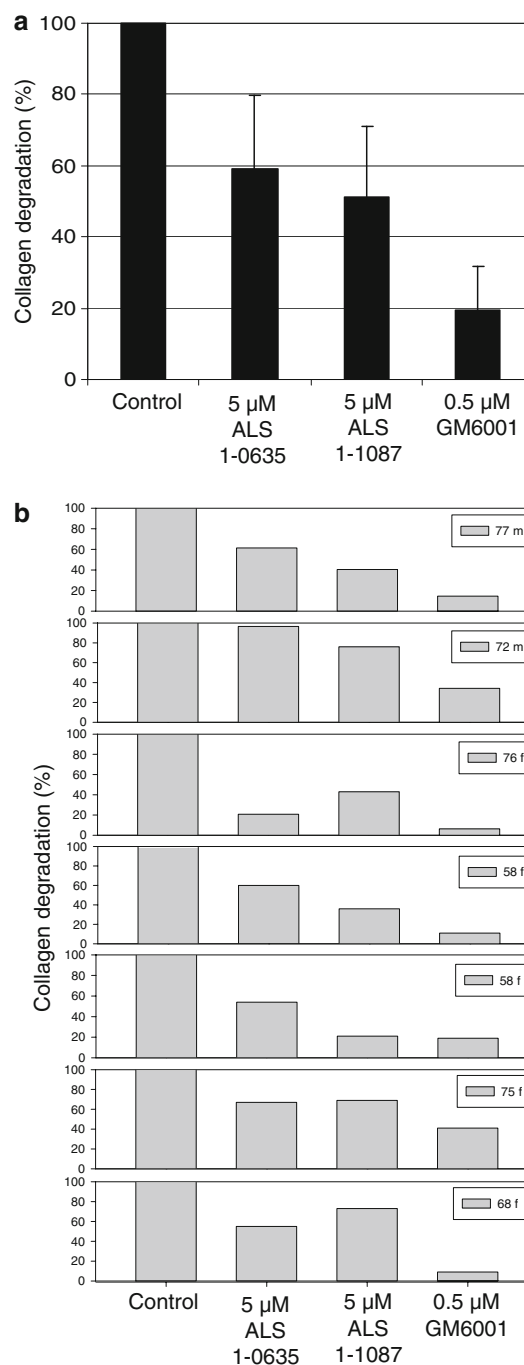


Fig. 4 Inhibition of collagen degradation in human OA cartilage explants. Cumulative collagen degradation using C1,2C ELISA over a period of 17 days in the absence or presence of ALS 1-0635, ALS 1-1087, or GM6001. **a** The mean inhibition from seven different OA tissues and **b** results from each individual experiment. For each experiment, the amount of released collagen from the control cartilage in the absence of inhibitors was set to 100%. Age and gender of patient are indicated (*f* female, *m* male)

percent of control. The broad spectrum MMP inhibitor GM6001 (control inhibitor) fully blocked the collagen degradation. ALS 1-0635 at levels of 500 and 5,000 nM

reduced the collagen degradation by 56 and 82%, respectively. In contrast, ALS 1-1087 reduced collagen degradation by 57% at 5 nM concentration (Fig. 5c) indicating a 100-fold greater activity than ALS 1-0635 in

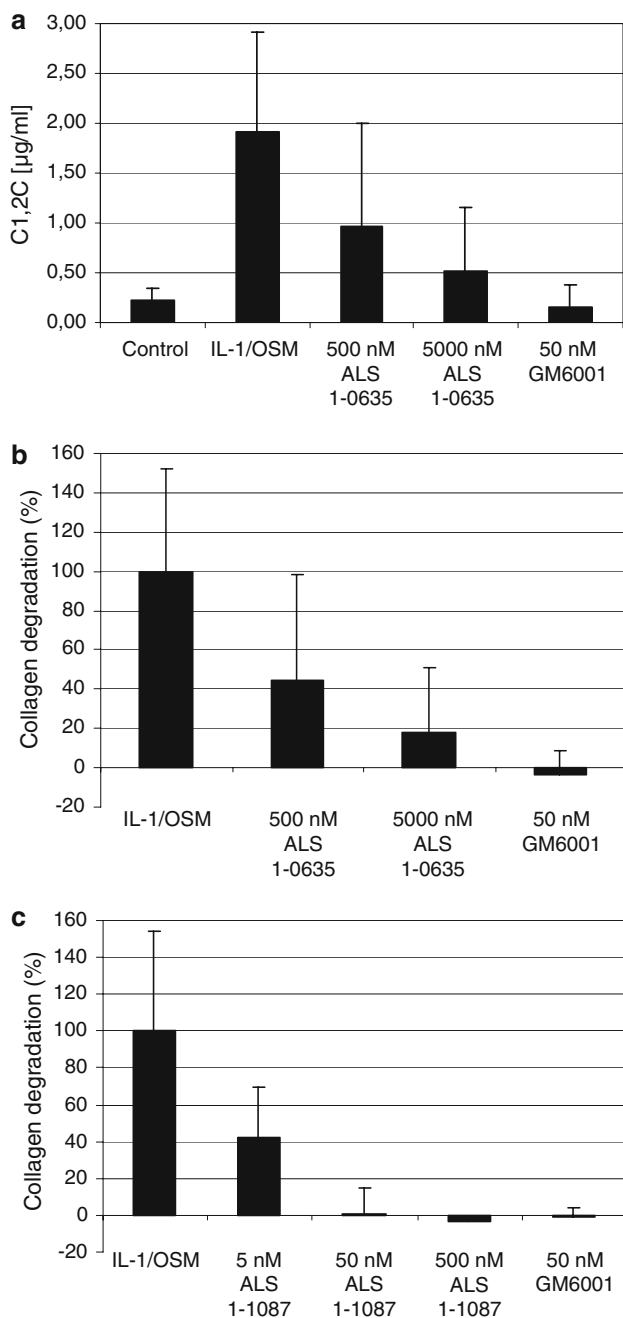


Fig. 5 Inhibition of IL-1/OSM-stimulated collagen degradation in bovine articular cartilage explants. Collagen degradation products in the supernatants of cartilage explants after 10 days of stimulation with cytokines were analyzed using C1,2C ELISA. **a** Collagen degradation in the control cartilage (stimulated with IL-1/OSM but without inhibitor) was set to 100%. **b** Inhibition of collagen degradation by ALS 1-0635. **c** Inhibition of collagen degradation by ALS 1-1087

bovine explants. ALS 1-1087 at levels of 50 and 500 nM fully blocked the collagen degradation.

The capacity of ALS 1-0635 and ALS 1-1087 to inhibit collagen degradation was studied in several human OA cartilage explants. Cartilage was isolated and cultured in the presence of IL-1 α , OSM, and the inhibitors. After 21 days of culture in the presence of the proinflammatory cytokines, the collagen degradation was increased in comparison to the noninduced control cartilage in all tissues. However, the degree of collagen degradation was very different, ranging from 3- to 72-fold increase. The collagen degradation in the presence of the inhibitors was calculated as a percent of control. Both inhibitors were analyzed in five different tissues (Fig. 6). ALS 1-0635 at levels of 500 and 5,000 nM reduced the collagen degradation by 0–75 and 54–100%, respectively. ALS 1-1087 at levels of 500 and 5,000 nM reduced the collagen degradation by 45–82 and 55–84%, respectively. A 500 nM dose of GM6001 reduced the collagen degradation by 73–100% in the different OA biopsies.

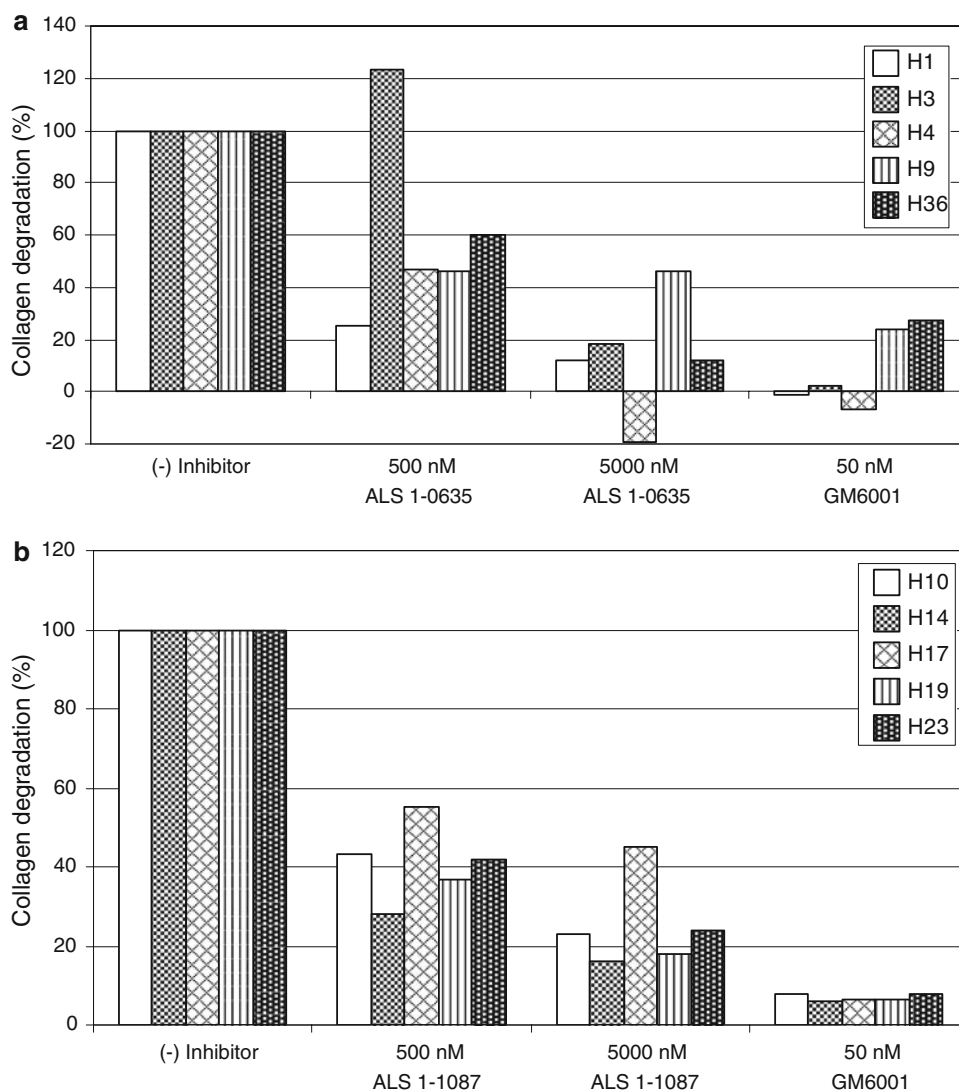
To validate the inhibition of collagen degradation by a selective MMP-13 inhibitor, we compared the results generated by C1,2C ELISA with C2C ELISA and hydroxyproline determination of the same supernatants. The effects of the inhibitors were comparable in all three assays (Fig. 7). Whereas C1,2C and C2C antibodies recognize a similar neopeptide generated by collagenases, the hydroxyproline determination quantifies the total amount of degraded collagen released from the cartilage. Similar results generated with the collagenase-specific ELISA and using hydroxyproline determination confirm the primary role of MMP-13 in cartilage collagen degradation.

The selective MMP-13 inhibitors had no effect on proteoglycan degradation in bovine and human cartilage explants. The proteoglycan degradation was measured on days 3 and 7 of the cytokine induction by colorimetric assay using dimethylmethylene blue (DMMB, data not shown). The chondrocyte viability in cartilage explants was also not affected by the selective MMP-13 inhibitors (data not shown). In the absence of proinflammatory stimulus, the chondrocytes remained viable over the first 3 weeks, and the viability was only reduced by ~20% after 4 weeks of culture, as measured by Alamar Blue staining (data not shown).

Cartilage penetration assay

To determine whether the inhibitors can penetrate the cartilage tissue, cartilage slices were incubated with a 1 μM solution of ALS 1-0635 or ALS 1-1087. After 48 h, the cartilage was digested with papain and the concentration of the inhibitor in the cartilage was determined by HPLC-MS. In parallel, the experiments were performed in

Fig. 6 Inhibition of IL-1/OSM-stimulated collagen degradation in human OA cartilage explants. Collagen degradation products were analyzed in the supernatants of explants after 21 days of cytokine stimulation using C1,2C ELISA. Inhibition of collagen degradation by ALS 1-0635 (a) and ALS 1-1087 (b) was tested in five different OA cartilage tissues (different patients, H1–H36). Collagen degradation in the control cartilage (stimulated with IL-1/OSM but without inhibitor) was set to 100%



the presence of 1% HSA to estimate the potential role of protein binding on cartilage penetration. After a 48 h incubation with the inhibitor, the cartilage contained high concentrations of ALS 1-0635 and ALS 1-1087, respectively (Table 3). In the presence of HSA, the cartilage penetration was reduced in correlation to the high protein binding of the inhibitors (98.6 and 98.8%, respectively, data not shown).

Discussion

Alantos Pharmaceuticals has identified extremely selective inhibitors of MMP-13 that are structurally unique and do not contain a zinc binding group [23]. These inhibitors inhibit the catalytic domain of MMP-13 in the single digit nanomolar range, showing no effect on other closely related MMPs, TACE, or aggrecanases at micromolar

concentration (selectivity of at least four orders of magnitude).

The analysis of the kinetic mechanism of the inhibition of ALS 1-0635 has shown that the compound is a mixed-type inhibitor. The mixed-type inhibition is a form of noncompetitive inhibition, where the inhibitor and substrate bind independently of each other to the enzyme. Recently described MMP-13 inhibitors that bind deeply into the S1'-pocket [12] showed a noncompetitive kinetic mechanism suggesting that these inhibitors may block the enzymatic reaction by inducing rigidity to the S1-specificity loop. The noncompetitive mechanism of inhibition is a major difference from the previous nonselective MMP inhibitors that occupy the substrate binding cleft and bind to the catalytic zinc.

Dixon plot analysis shows that ALS 1-0635 and aceto-hydroxamic acid bind to the enzyme or the enzyme/substrate complex simultaneously, implicating different

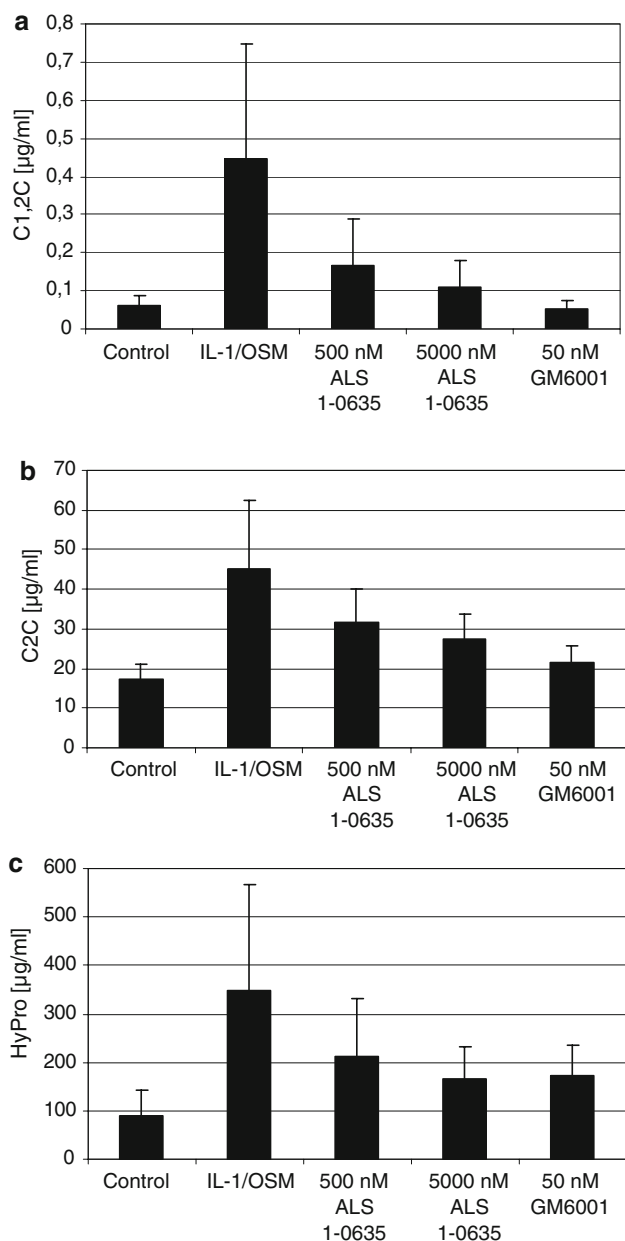


Fig. 7 Inhibition of collagen degradation by ALS 1-0635 in human OA cartilage using different biomarkers. Supernatant of human OA cartilage explants stimulated with IL-1/OSM for 24 days was analyzed in parallel for collagen degradation by determination of C1,2C (a), C2C (b), and hydroxyproline (c)

Table 3 Cartilage penetration of ALS 1-0635 and ALS 1-1087 in presence and absence of HSA

1 µM Inhibitor in PBS	Inhibitor concentration in cartilage (µM)	Inhibitor concentration in cartilage in presence of 1% HSA (µM)
ALS 1-0635	2.0	0.26
ALS 1-1087	4.2	0.36

binding sites at MMP-13 for these two inhibitors. As aceto-hydroxamic acid inhibits Zn-metalloproteases by complexing the catalytic zinc ion, ALS 1-0635 cannot bind to the catalytic zinc (Fig. 3b). The noncompetitive mechanism of inhibition and the lack of binding to the catalytic zinc differentiate the ALS 1-0635 and ALS 1-1087 compounds from the previous nonselective MMP inhibitors and likely contribute to their extremely high selectivity over other MMPs.

MMP-13 is the major type II collagenase *in vitro*, and cleavage of the triple helical collagen [24, 25], which initiates the collagen degradation, is probably the most important function of MMP-13 in cartilage. To evaluate the potential of the selective MMP-13 inhibitors to inhibit the collagenase activity of MMP-13, we have measured the inhibition of cleavage of type I and II collagen and also compared the inhibition of human and bovine MMP-13. The inhibitors efficiently blocked the collagenase activity of MMP-13 (IC₅₀ between 4 and 27 nM), and the inhibition was independent of the type of collagen and species.

Osteoarthritic articular cartilage is characterized by enhanced cleavage of type II collagen [11], which is associated with the upregulation of synthesis and activities of collagenases, in particular MMP-13 [26]. The three known human collagenases (MMP-1, MMP-8, and MMP-13) have been shown to cleave triple-helical type II collagen. Synthetic broad-spectrum or partially selective metalloproteinase inhibitors have been shown previously to inhibit degradation of type II collagen in OA cartilage [20]. The results of this study demonstrate that fully selective MMP-13 inhibitors reduce the collagen degradation in human OA cartilage explants. The total amount of collagen degradation and the inhibition values differ with the individual cartilage. This may be a result of different degrees of OA cartilage damage of the samples used in the experiment, patient variability, or differences in the disease etiology. While the broad spectrum MMP inhibitor GM6001 blocked the collagen degradation by 81% (mean inhibition), a reduction of approximately 41–49% (mean) of the collagen degradation was achieved by selective MMP-13 inhibitors. Therefore it appears likely that MMP-13 is responsible for at least 50–60% of the enhanced collagenase activity in OA cartilage. GM6001 reduced collagen degradation more effectively, indicating involvement of other MMPs or proteases nonspecifically blocked by this inhibitor. However nonselective inhibition is probably the reason for joint toxicities observed *in vivo*, while selective MMP-13 inhibitors overcome the MSS issue [12, 14]. To reduce collagen degradation in OA cartilage significantly, high nanomolar concentrations of ALS 1-0635 and ALS 1-1087 are necessary. Therefore a crucial precondition for efficacy of these selective MMP-13 inhibitors *in vivo* is high bioavailability and high

cartilage penetration. Promising results from in vivo studies in rats with ALS 1-0635 addressing drug exposure have recently been published by Baragi et al. [14].

A number of previous studies demonstrated elevated levels of IL-1 α and OSM in OA/RA joints as well as the synergistic upregulation of aggrecanases and collagenases by these cytokines in cartilage and associated degradation of cartilage [16, 17, 27–29]. The IL-1/OSM-stimulated cartilage explant model can be used to test the effectiveness of proteinase inhibitors. Nonselective broad spectrum MMP inhibitors showed efficacy in blocking collagen and proteoglycan degradation in this system [19, 21, 28, 30]. Bovine articular cartilage is readily available, and this explant culture system provides a rapid, reproducible, and reliable model system to study the mechanisms of cartilage degradation and as such has become a standard for studying the efficacy of novel therapeutics. Our results show that selective MMP-13 inhibitors fully blocked collagen degradation in bovine articular cartilage explants and therefore validate MMP-13 as the primary collagenase in this model.

Selective MMP-13 inhibitors ALS 1-0635 and ALS 1-1087 displayed a remarkable difference in activity in the bovine articular cartilage explants. ALS 1-1087 was approximately 100-fold more potent in inhibition of collagen degradation although both inhibitors showed very similar chemical and biological properties, for instance their similar activity in different in vitro assays or protein binding and metabolic stability. The slightly increased cartilage penetration of ALS 1-1087 cannot be the sole explanation for such an increase in activity in cartilage explant assays. Further studies will be necessary to understand the principle behind this difference in activity and to evaluate the best profile of the selective MMP-13 inhibitors.

In comparison to the bovine system, the IL-1/OSM-induced degradation of collagen in human OA cartilage was significantly lower and slower, simultaneously showing very high sample variability. Differences in response to IL-1/OSM may be due to cell density, age, health, or tissue permeability. Selective inhibitors of MMP-13 reduced the collagen degradation up to 80%. However, complete inhibition was never achieved and usually higher concentrations of selective MMP-13 inhibitor were necessary to reduce collagen degradation in human OA cartilage. The significant degree of inhibition by selective MMP-13 inhibitors in most of the tested tissues emphasizes the role of MMP-13 in the pathology of human cartilage degradation. Further studies are necessary to understand the more complex situation in human OA cartilage, the involvement and role of other MMPs or other proteases, and the predictability of results from bovine in vitro model or other animal disease models for the efficacy of the MMP-13 inhibitors in human OA tissue. The selective MMP-13

inhibitors do not induce joint toxicities in animal studies [12] while showing efficacy in animal disease models. The success of the inhibitors in clinical trials will very much depend on the pharmacological and safety profile of the compounds and their efficacy to block collagen degradation in human OA cartilage. Furthermore, selective MMP-13 inhibitors represent a great tool to analyze the role of MMP-13 in arthritis and other pathologies such as cancer, heart failure, or atherosclerosis [31–33]. Results from in vitro studies and the very recently published in vivo animal models [14] permit us to believe that selective MMP-13 inhibitors can protect cartilage in OA patients, thereby slowing further joint damage and possibly facilitating cartilage recovery through balanced synthesis and degradation.

Acknowledgments We thank Joshua Van Veldhuizen, Timothy Powers, and Arthur G. Taveras from Alantos Pharmaceuticals Inc., Cambridge, MA, and Jürgen Boer and Christoph Steeneck from Alantos Pharmaceuticals AG, Heidelberg, Germany, for the synthesis of the inhibitors; Wiltrud Richter and Helge Bertram from the Orthopaedic University Hospital Heidelberg, Germany, for supplying us with human OA cartilage; and A. Robin Poole, Arthur G. Taveras, and Vijay Baragi for helpful discussions.

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