# Discovery and Evaluation of a Non-Zn Chelating, Selective Matrix Metalloproteinase 13 (MMP-13) Inhibitor for Potential Intra-articular Treatment of Osteoarthritis

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ABSTRACT: Osteoarthritis (OA) is a nonsystemic disease for which no oral or parenteral disease-modifying osteoarthritic drug (DMOAD) is currently available. Matrix metalloproteinase 13 (MMP-13) has attracted attention as a target with disease-modifying potential because of its major role in tissue destruction associated with OA. Being localized to one or a few joints, OA is amenable to intra-articular (IA) therapy, which has distinct advantages over oral therapies in terms of increasing therapeutic index, by maximizing drug delivery to cartilage and minimizing systemic exposure. Here we report on the synthesis and biological evaluation of a non-zinc binding MMP-13 selective inhibitor, 4-methyl-1-(S)-({5-[(3-oxo-3,4-dihydro-2H-benzo[1,4]oxazin-6-ylmethyl)carbamoyl]pyrazolo[1,5-a]pyrimidine-7-carbonyl}amino)indan-5-carboxylic acid (**1**), that is uniquely suited as a potential IA-DMOAD: it has long durability in the joint, penetrates cartilage effectively, exhibits nearly no detectable systemic exposure, and has remarkable efficacy.
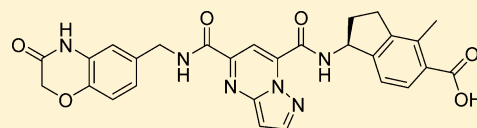
INTRODUCTION

Osteoarthritis (OA) is characterized by progressive loss of articular cartilage resulting in chronic pain and disability. The disease is nonsystemic by nature and is commonly restricted to few joints. In addition to the loss of cartilage, there is an observed reduction in the viscous properties of the synovial fluid.

There is no cure for OA; disease management is limited to treatments that are palliative at best and do little to address the underlying cause of disease progression. Therapeutic interventions in OA have been hindered by the difficulty of targeting drugs to articular cartilage. Because articular cartilage is an avascular and alymphatic tissue, traditional routes of drug delivery (oral, intravenous, intramuscular) ultimately rely on transsynovial transfer of drugs from the synovial capillaries to cartilage by passive diffusion. Thus, in the absence of a mechanism for selectively targeting drug to the cartilage, traditional routes of delivery have found it necessary to expose the body systemically to high concentrations of drugs to achieve a sustained, intra-articular (IA) therapeutic dose. As a consequence of high systemic exposures, most of the traditional therapies for OA have been plagued by serious toxicities.

A variety of IA agents, including corticosteroids and several hyaluronate products, are currently available for symptomatic relief of OA.[1] Corticosteroid clinical trials data suggest that for most patients the treatment results in a slightly better improvement in symptoms than placebo and that the effects do not last more than 4 weeks.[2,3] The precise mechanism by which corticosteroids exert their beneficial effects in an OA joint is not known. They are believed to act as broad-spectrum anti-inflammatory agents. The FDA has designated several preparations of IA viscosupplements based on hyaluronate as new devices for the treatment of pain in OA of the knee. It is reported that pain relief is usually obtained by 8−12 weeks after the first injection and can last up to six months. The IA hyaluronate products are believed to work through viscosupplementation of the compromised synovial fluid in the OA diseased joint.[4]

There is now new evidence for development of potential disease-modifying osteoarthritis drugs (DMOADs), arising from a fuller understanding of joint metabolism. There is overwhelming evidence to demonstrate that matrix metalloproteinases (MMPs), specifically MMP-13, have a major role in tissue destruction associated with OA. MMPs are a family of zinc-dependent endopeptidases involved in the degradation of extracellular matrix and tissue remodeling.[5] MMPs have long been considered as attractive therapeutic targets for treatment of OA;[6] however, broad-spectrum MMP inhibitors developed for treatment of arthritis have failed in clinical trials due to painful, joint-stiffening side effects termed musculoskeletal syndrome (MSS).[7,8] It is believed that MSS is caused by nonselective inhibition of multiple MMPs other than MMP-13.[8−10] MMP-13 is the major collagenase in OA cartilage and the most efficient type II collagen-degrading MMP.[11] Therefore, current drug development strategies for treatment of OA are focused on inhibition of MMP-13 with high isoform selectivity.[12,13]

MMPs have an extended zinc-binding motif, which contains three histidines bound to zinc and a glutamate that acts as the

catalytic site for substrate hydrolysis.[12] Most small molecule inhibitors described to date gain activity by interacting with this catalytic site via a strong zinc-chelating moiety (e.g., hydroxamate) and by tight binding within the largely hydrophobic S1′ pocket.[13] The S1′ pocket is in part formed by the selectivity loop, which varies in length and amino acid sequence for different MMP isoforms. These S1′ differences between MMP family members have been utilized to design MMP inhibitors with different selectivity profiles.[12,13] After the discovery of highly selective nonzinc-chelating MMP-13 inhibitors by Engel et al. (e.g., **A** in Figure 1),[14] which bind
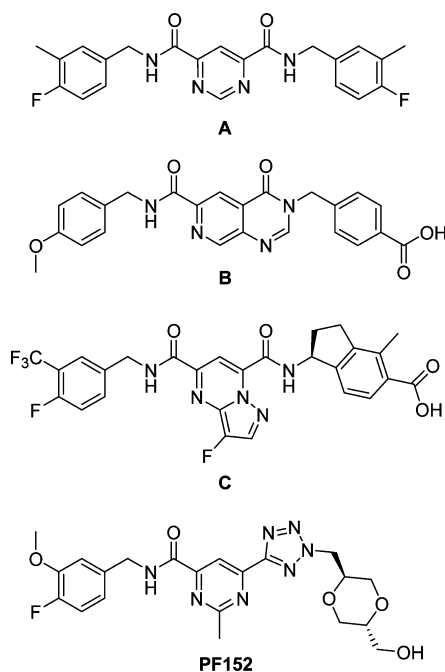
Figure 1. Reported nonzinc binding, highly selective MMP-13 inhibitors (**A**,[14] **B**,[16e] **C**,[10,22] and **PF152**[18]).

to an additional S1′ side pocket, the structural basis for the high selectivity was apparent.[14,15] Several groups with various structural classes exploited these findings (e.g., **B** in Figure 1),[16,17] and the preclinical experiments in various animal models demonstrated that these highly selective MMP-13 inhibitors are efficacious and at the same time can overcome safety problems associated with MMP inhibitors developed so far for the potential treatment of OA.[9,10,18] Thus, there is compelling data demonstrating the potential of MMP-13 inhibitors as disease-modifying osteoarthritis drugs.

Although corticosteroids as well as nonsteroidal agents including hyaluronate products are being widely used to treat signs and symptoms of OA,[19] to date, there is no agent—oral or IA—on the market that has proven to be disease/structure modifying. While there have been efforts to evaluate potential DMOADs including MMP-13 inhibitors via an oral route of administration, very little information is available on potential IA DMOADs. The IA route of administration has a distinct advantage over oral therapies in terms of increasing therapeutic index, by maximizing drug delivery to target cartilage and minimizing systemic exposure.

We hypothesized that a small molecule MMP-13 inhibitor with low solubility could be used to clarify the role of MMP-13 in pathological OA, when applied via IA injection. Reported herein is the synthesis of a low soluble, highly selective

(>20,000-fold over tested MMPs), and highly potent MMP-13 inhibitor and its biological evaluation following IA administration to rats.

■ RESULTS

Chemistry. Target compound **1** was prepared according to the synthetic route outlined in Scheme 1. Thus, 1*H*-pyrazol-3-

Scheme 1. Synthesis of Compound 1[a]
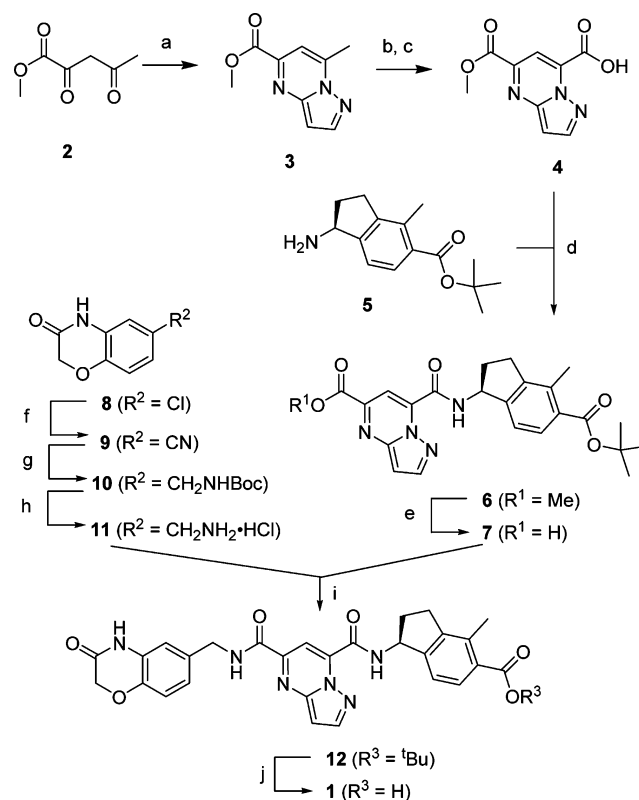
[a]Reagents and conditions: (a) 1*H*-pyrazol-3-amine, MeOH, reflux, 66%; (b) SeO$_2$, TBHP, 50 °C; (c) oxone, rt, 63% (two steps); (d) **5**, HATU, HOAt, NMM, 86%; (e) aqueous LiOH, quant; (f) CuCN, NMP, 250 °C, 24%; (g) NaBH$_4$, Boc$_2$O, cat. NiCl$_2$, 41%; (h) 4 N HCl in dioxane, quant; (i) EDCI, NMM, 94%; (j) HCOOH, 95%.

amine was reacted with methyl acetopyruvate **2** in refluxing methanol, giving compound **3** in 66% yield as the major isomer, which crystallized from MeOH upon cooling of the reaction mixture. Oxidation of the methyl group was carried out in two steps using selene dioxide and *tert*-butyl hydroperoxide (TBHP) to afford the aldehyde intermediate, which was subsequently oxidized with oxone to acid **4** in 63% overall yield. No migration of the methyl ester moiety was observed under these reaction conditions. Reaction of amine **5**[20] with acid **4** using O-(7-azabenzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium-hexafluorophosphate (HATU) and 1-hydroxy-7-azabenzotriazole (HOAt) as powerful coupling reagents afforded methyl ester **6** in 86% yield. Saponification with aqueous lithium hydroxide afforded acid **7** in quantitative yield. The benzoxazinone building block was prepared by reaction of chloride **8** with copper(I) cyanide in *N*-methyl-pyrrolidin-2-one (NMP) at 250 °C overnight. After aqueous workup, crystallization from EtOAc/toluene afforded nitrile **9**. Reduction of the nitrile moiety with sodium borohydride and nickel(II) chloride as catalyst in the presence of di-*tert*-butyl

Table 1. Selectivity and Potency of Compound 1 and Compound C[a]

compd no.	IC$_{50}$ (nM) vs catalytic domain										
	MMP-1	MMP-2	MMP-3	MMP-7	MMP-8	MMP-9	MMP-12	**MMP-13**	MMP-14	TACE	Agg 1
1	>20000	>20000	8200	>20000	3200	>20000	655	**0.03**	>20000	>20000	2400
C	>20000	>20000	15000	>20000	>20000	>20000	>20000	**1.3**	>20000	>20000	7000

[a]Errors are in the range 5−10% of the reported value.

dicarbonate (Boc$_2$O)[21] furnished the Boc-protected derivative **10**, which was finally deprotected with hydrochloride in dioxane to give benzoxazinone building block **11**. Final coupling of amine **11** and acid **7** using 1-(3-dimethylamino-propyl)-3-ethylcarbodiimide hydrochloride (EDCI) and N-methylmorpholine (NMM) as base furnished intermediate **12**, which was deprotected in formic acid at 45 °C to give target compound **1** in high overall yield.

PHARMACOKINETICS

Systemic Exposure. Little or no systemic exposure in rats was observed up to 100 mg/kg oral or intraperitonial doses, or 0.5 mg/kg IA dose. Only a minimal exposure with a rapid clearance of compound **1** from the systemic circulation was seen in the 1.0 mg/kg intravascular dose (data not shown). However, the studies on the microsomal stability of compound **1** indicated 95% and 96% of the unmetabolized compound in the rat and human microsomes, respectively, after 60 min of incubation.

Safety, Tolerability, and Pharmacokinetics Following Intra-articular Administration. Following IA injection of the right knee joint of rats with the test compound **1**, synovial lavage, cartilage, and plasma samples were collected at various time points for a period of 8 weeks and analyzed for compound concentrations using LC/MS/MS methods. Compound concentrations ranging from 0.30 ± 0.02 to 3.40 ± 1.28 μM (mean ± SE) were measured in the synovial lavage and from 4.09 ± 0.57 to 7.10 ± 0.66 μM in the cartilage for a period of about 21 days post a single injection of 0.5 mg of compound/joint. However, by day 56, the concentration of compound **1** in the synovial lavage was below the level of detection, whereas concentrations of ≥2.0 μM were maintained in the cartilage. In comparison, plasma concentrations remained near or below the level of detection for the entire duration of the study. On day 56, the joints were scored for behavioral signs of potential musculoskeletal side effects. None of the six animals in the compound-treated group displayed any signs of impaired use of their limbs. There was no difference between the control and drug treated groups in terms of the total score received (total score = 0) for behavioral signs of toxicity, as assessed by the ability to use their hind legs.

PHARMACOLOGY

Potency and Selectivity. The potency (IC$_{50}$) of compound **1** against the catalytic domain of recombinant human MMP-13 was determined[22] to be 0.03 nM (Table 1). Compared to other derivatives of the same compound class,[10,23] the introduction of the 4H-benzo[1,4]oxazin-3-one-6-ylmethyl moiety improved potency by at least ten times that seen with structurally simpler moieties (e.g., 3-trifluoromethyl-4-fluorobenzyl as in compound **C** of Table 1). This was also observed in the compound classes with different core structures.[24,25] Compound **1** is >20000-fold selective over all the MMPs, TACE and Aggrecanase 1, tested (Table 1).

Although still remarkably selective toward MMP-13, MMPs with similar size and shape of the S1′ pocket—namely MMP-3, -8, and -12[17]—are targeted to some extent by compounds in this class. Comparison with other compounds of the same class (e.g., **C** in Figure 1) indicated that to a major extent the benzoxazinone moiety is responsible for this effect.[10] Remarkably, the fragment 6-methyl-4H-benzo[1,4]oxazin-3-one alone is reported to inhibit MMP-12 with an IC$_{50}$ of 355 nM.[26]

Inhibition of In Vitro Cartilage Degradation. To characterize the functional activity of compound **1**, its ability to block collagen degradation was studied in vitro in bovine articular cartilage explants by measuring the interleukin-1α (IL-1α)/oncostatin-M (OSM)-induced release of C1,2C, the product of collagen degradation.[22] Compound **1** was effective in blocking collagen degaradation in a dose-dependent manner with an IC$_{50}$ of approximately 20 nM (Table 2).

Table 2. Results from in Vitro Cartilage Degradation Assay and Ex Vivo Model of Cartilage Degradation[a]

compd no.	cat. MMP-13 IC$_{50}$ (nM)	bovine cartilage explant (nM)	ex vivo model of cartilage degradation (% inhibition)			
			24 h	7 d	14 d	21 d
1	0.03	∼20	100	100	100	100
C	1.3	∼5	95	60	0	n.t.

[a]n.t. = not tested.

Activity in the Ex Vivo Model of Rat Articular Cartilage Degradation. To gain insight into the functional activity of the compound in vivo, the extent and duration of chondroprotective effects of the compound in the rat knee joint was assessed. Following a single injection (0.5 mg/joint) of compound **1**, rats were sacrificed at various times and cartilage obtained from knee joints was challenged with human MMP-13 ex vivo and the resulting collagen loss was measured using C1,2C ELISA. Compound **1** was effective in blocking 100% of MMP-13-induced release of C1,2C at 1, 7, 14, and 21 days post-IA injection (Table 2).

DISCUSSION AND CONCLUSION

Therapeutic interventions in OA have been hindered by the difficulty of targeting drugs to articular cartilage. Because articular cartilage is an avascular and alymphatic tissue, traditional routes of drug delivery (oral, intravenous, intramuscular) ultimately rely on trans-synovial transfer of drugs from the synovial capillaries to cartilage by passive diffusion. Thus, in the absence of a mechanism for selectively targeting drug to the cartilage, traditional routes of delivery have found it necessary to expose the body systemically to high concentrations of drugs to attain therapeutic concentrations in the joint. As a consequence of high systemic exposures, most of the traditional therapies for OA have been plagued by serious toxicities. Thus, given the side-effect profiles of current oral

therapies for OA, a chronic but nonlethal disease, there exists an opportunity for alternative OA therapies. OA being localized to a few large joints, there is a competitive advantage to deliver a small molecule locally to the affected joint. IA delivery would ideally minimize structural damage and inflammation and provide symptomatic relief of OA, while minimizing systemic exposure. In addition, the agent should minimize toxicity and provide cost-of-goods savings and convenience of dosing at 4 to 6 injections/year. Clearly, the IA route of administration, by maximizing drug delivery to cartilage, the site where it is most needed, and minimizing systemic exposure, potentially offers the distinct advantage over traditional therapies in terms of increasing therapeutic index.

A variety of IA agents, including corticosteroids and several hyaluronate products, are currently available for short-term symptomatic relief of OA. IA corticosteroid injections are effective in providing symptomatic relief for weeks and sometimes months. However, because of concerns of damage to the cartilage, corticosteroids are not used for long-term treatment of OA. There are five preparations of IA hyaluronate marketed in the US for treatment OA knee pain. They are generally reported to provide short-term positive effects on pain and function after a series of 3−5 injections. However, the mechanisms of their effect are not known, and most have a synovial half-life of only 2−8 days. There are currently no FDA approved oral or IA therapies that alter joint structure in OA.

Compound **1** is uniquely suited as a potential IA-DMOAD candidate. It is the most potent and selective MMP-13 inhibitor reported so far (IC_{50}: 0.03 nM). As we have previously reported, MMP-13 specific inhibitors are without the musculoskeletal liability that has been associated with broad-spectrum MMP inhibitors.[10] Compound **1** is distinguished for its remarkable durability in the joint. A single IA injection of compound **1** was sufficient to sustain high-levels of the compound in cartilage (2 μM) for up to 8 weeks. Combined with this outstanding durability in the joint, compound **1** has a PK profile that minimizes systemic exposure. Negligible or no levels of the compound were detected in the plasma following 100 mg/kg oral and intraperitoneal routes, the 1 mg/kg IV route, or the 0.5 mg/kg IA route of administration of the compound. Consistent with this joint durability and systemic PK profile, compound **1** showed excellent *in vitro* and *ex vivo* efficacy while demonstrating no clinical signs of MSS in the animals or gross changes in the joint in an 8 week IA durability study. *In vitro*, compound **1** inhibited IL-1 and oncostatin M-stimulated collagen loss from bovine articular cartilage with an $IC_{50} \sim 20$ nM. IA administration of compound **1** demonstrated dose-dependent chondroprotection in rats with *ex vivo* challenge of cartilage with hMMP-13 (IC_{50} = 0.09 mg/joint, data not shown). Taken together, these findings demonstrate that the compound represents a promising new class of potential IA-DMOADs. To date, there is no agent—oral nor IA—on the market that has proven to be disease/structure modifying.[27]

In summary, compound **1** is the most potent MMP-13 selective inhibitor reported so far. Mechanistically, given the compelling role of MMP-13 in cartilage degradation associated with OA, compound **1** has the disease-modifying potential. It is uniquely suited for IA delivery: It is able to penetrate and attain high, sustained concentrations (≥ 2 μM for 8 weeks) in cartilage. Correlating with its long durability in the joint, the compound demonstrated sustained efficacy (100% inhibition for 3 weeks). Despite outstanding efficacy, the compound

exhibited no significant systemic exposure. The compound showed no risk of joint damage: no joint irritation or adverse effects in both acute (48 h) and chronic (3 weeks) studies in rats (data not shown). It also showed no gross changes in the joint or signs of MSS in an 8 week durability study (0.5 mg/joint for 8 weeks). The compound is amenable to IA delivery and has sufficient durability to allow for a minimal number of injections annually. Taken together, these findings demonstrate that compound **1** represents a promising first-in-class IA-DMOAD candidate.

■ EXPERIMENTAL SECTION

Chemistry. ^{1}H NMR spectra were recorded on a Bruker Avance (250 MHz) instrument at 300 K in CDCl₃ or DMSO-d_6. Chemical shifts are reported in δ values (ppm); the hydrogenated residues of deuterated solvent were used as internal standard (CDCl₃: δ 7.26 and DMSO: δ 2.50). Signals are described as s, d, t, dd, m, and b for singlet, doublet, triplet, double−doublet, multiplet, and broad, respectively. Mass spectra (LC-MS) were measured on a Ion Trap Esquire 3000+ instrument. Chemical names follow IUPAC nomenclature. Starting materials were used without purification. Column chromatography was performed using silica gel (40−63 μm), and the reaction progress was determined by thin layer chromatography analyses on Merck silica gel plastic plates 60F₂₅₄. The purity of tested compounds was established by LC/MS and combustion analysis to be >95%.

7-Methyl-pyrazolo[1,5-*a*]pyrimidine-5-carboxylic Acid Methyl Ester (3). To a solution of 1*H*-pyrazol-3-amine (2.50 g, 30.1 mmol) in MeOH (90 mL) was added methyl acetopyruvate (4.42 g, 30.7 mmol). The mixture was heated to reflux for 5 h and then cooled to room temperature overnight. The precipitated yellow needles were collected by filtration and recrystallized from MeOH to afford the major isomer **3** (3.80 g, 66%) as off-white needles (TLC (cyclohexane/ethyl acetate =2:1): R_f = 0.21 for **3** in comparison to R_f = 0.13 for the regioisomer). ^{1}H NMR (DMSO-d_6): δ 8.40 (d, *J* = 2.4 Hz, 1H, CH), 7.56 (d, *J* = 0.9 Hz, 1H, CH), 6.99 (d, *J* = 2.4 Hz, 1H, CH), 3.93 (s, 3H, CH₃), 3.32 (s, 3H, CH₃). C₉H₉N₃O₂; MW: 191; MS 192 [M + H]⁺.

Pyrazolo[1,5-*a*]pyrimidine-5,7-dicarboxylic Acid 5-Methyl Ester (4). To a suspension of SeO₂ (44.4 g, 400 mmol) in dry 1,4-dioxane (200 mL) was added *tert*-butyl hydroperoxide (200 mL of a 5−6 M solution in decane), and this solution was placed in a preheated oil bath (50 °C) and vigorously stirred at this temperature until a clear colorless solution was obtained (approximately 30 min). Then **3** (38.2 g, 200 mmol) was added under vigorous stirring until complete consumption (approximately 19 h). The clear yellow to orange solution was cooled to room temperature and diluted with 1,4-dioxane (800 mL) and water (120 mL), and oxone (123 g, 200 mmol) was added successively. The resulting orange suspension was stirred at room temperature until the intermediate aldehyde was consumed (approximately 68 h). The yellow suspension was filtered through a frit (P2), and the filter cake was washed with 1,4-dioxane (100 mL) and air-dried. The remaining gluey yellow solid residue was washed with CHCl₃/methanol (95:5, 2 × 400 mL) and air-dried. The obtained yellow powdery solid was transferred into an extraction thimble and placed in a Soxhlet extractor (500 mL) and continuously extracted with refluxing CHCl₃ (1.6 L) until a beige solid remained in the extraction thimble (approximately 48 h). The dark yellow CHCl₃ extract was concentrated to dryness under reduced pressure, ground, and suspended in methanol (200 mL), filtered through a frit (P3), washed with methanol (100 mL), and dried under reduced pressure for 15 h to obtain the product (27.8 g, 63%) as a yellow powder. ^{1}H NMR (DMSO-d_6): δ 8.44 (s, 1H, CH), 7.47 (s, 1H, CH), 6.98 (s, 1H, CH), 3.93 (s, 3H, CH₃). C₉H₇N₃O₄; MW: 221; MS 222 [M + H]⁺.

7-(5-*tert*-Butoxycarbonyl-4-methyl-indan-1-(*S*)-ylcarbamoyl)-pyrazolo[1,5-*a*]pyrimidine-5-carboxylic Acid Methyl Ester (6). Acid **4** (43.7 g, 198 mmol) and 1-(*S*)-amino-4-methyl-indan-5-carboxylic acid *tert*-butyl ester **5** (44.5 g, 180 mmol) and 1-hydroxy-7-azabenzotriazole (26.8 g, 197 mmol) were dissolved in dry DMF

(1 L), and then subsequently *N*-methylmorpholine (22 mL) and *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium-hexafluorophosphate (90 g, 237 mmol) were added at room temperature. After 1 h, precipitation occurred and the black suspension was allowed to stand overnight and was evaporated under reduced pressure. To the residue was added 10% citric acid, and the precipitate was filtered, washed with water, and absorbed on silica. Purification by flash chromatography using cyclohexane/ethyl acetate 7/3 to 3/7 as eluent afforded the product (70.0 g, 86%) as yellow solid. ^{1}H NMR (DMSO-d_6): δ 10.36 (d, J = 7.9 Hz, 1H, NH), 8.57 (d, J = 2.5 Hz, 1H, CH), 8.10 (s, 1H, CH), 7.61 (d, J = 7.9 Hz, 1H, CH), 7.33 (d, J = 7.9 Hz, 1H, CH), 7.26 (d, J = 2.5 Hz, 1H, CH), 5.68 (q, 1H, CH), 4.01 (s, 3H, CH$_3$), 3.15–2.61 (m, 3H, CH$_2$, C*HH*), 2.44 (s, 3H, CH$_3$), 2.11–1.98 (m, 1H, CH*H*), 1.58 (s, 9H, 3 CH$_3$). C$_{24}$H$_{26}$N$_4$O$_5$; MW: 450; MS 451 [M + H]$^+$.

7-(5-*tert*-Butoxycarbonyl-4-methyl-indan-1-(*S*)-ylcarbamoyl)-pyrazolo[1,5-*a*]pyrimidine-5-carboxylic Acid (7). Compound **6** (69.5 g, 154 mmol) was dissolved in THF (1.2 L) at 40 °C and then a solution of lithium hydroxide (4.8 g, 200 mmol) in water (200 mL) was added. The yellow solution turned green and was stirred for 2 h at room temperature. The solution was adjusted to pH 4 by addition of 1N HCl (180 mL), and then the THF was removed under reduced pressure. To the remaining suspension was added additional 1N HCl (30 mL), and then the suspension was cooled to ~10 °C, filtered, and extensively washed with water (~1 L) and dried at 40 °C to afford the product (68.8 g, quant.) as a yellow solid. ^{1}H NMR (DMSO-d_6): δ 10.38 (d, J = 8.1 Hz, 1H, NH), 8.53 (d, J = 2.5 Hz, 1H, CH), 8.09 (s, 1H, CH), 7.61 (d, J = 7.9 Hz, 1H, CH), 7.33 (d, J = 7.9 Hz, 1H, CH), 7.20 (d, J = 2.7 Hz, 1H, CH), 5.68 (q, 1H, CH), 3.40 (bs, 1H, OH), 3.13–2.64 (m, 3H, CH$_2$, C*HH*), 2.44 (s, 3H, CH$_3$), 2.12–1.97 (m, 1H, CH*H*), 1.58 (s, 9H, 3 CH$_3$). C$_{23}$H$_{24}$N$_4$O$_5$; MW: 436; MS 437 [M + H]$^+$.

3-Oxo-3,4-dihydro-2*H*-benzo[1,4]oxazine-6-carbonitrile (9). A suspension of 6-chloro-3-oxo-3,4-dihydro-2*H*-benzo[1,4]oxazin-3-one (3.2 g, 17.4 mmol) and CuCN (2.9 g) in dry *N*-methyl-pyrrolidin-2-one (15 mL) was placed in a preheated oil bath (~250 °C) under argon. After stirring at this temperature overnight, the mixture was concentrated, diluted with water (200 mL), and extracted with EtOAc (3 × 200 mL). The combined organic layers were washed with water (2 × 200 mL) and brine (200 mL), dried over MgSO$_4$, filtered, and concentrated. The remaining residue crystallized from EtOAc/toluene to afford the product (720 mg, 24%) as a tan solid. ^{1}H NMR (DMSO-d_6): δ 10.99 (s, 1H, NH), 7.41 (dd, J = 8.4 Hz, J = 1.8 Hz, 1H, CH), 7.21 (d, J = 1.8 Hz, 1H, CH), 7.11 (d, J = 8.4 Hz, 1H, CH), 4.71 (s, 3H, CH$_3$). C$_9$H$_6$N$_2$O$_2$; MW: 174; MS 175 [M + H]$^+$.

(3-Oxo-3,4-dihydro-2*H*-benzo[1,4]oxazin-6-ylmethyl)-carbamic Acid *tert*-Butyl Ester (10). To an ice cooled solution of nitrile **9** (700 mg, 4.02 mmol) in dry MeOH (20 mL) were added di-*tert*-butyl dicarbonate (1.8 g, 8.25 mmol) and NiCl$_2$·6H$_2$O (40 mg), followed by the careful portionwise addition of NaBH$_4$ (900 mg, 23.8 mmol). The resulting black mixture was stirred for 20 min at 0–5 °C and then the ice bath was removed and stirring at room temperature was continued overnight. Diethylenetriamine (0.5 mL) was added, and the mixture was concentrated to dryness. The remaining residue was dissolved in EtOAc and washed subsequently with 10% aqueous citric acid, saturated aqueous NaHCO$_3$, and brine, dried over MgSO$_4$, filtered, concentrated, and purified by flash chromatography using cyclohexane/EtOAc 3/2 to 1/1 as eluent to afford the product (459 mg, 41%) as white needles. ^{1}H NMR (DMSO-d_6): δ 10.70 (s, 1H, NH), 7.33 (t, J = 6.2 Hz, 1H, NH), 6.89–6.75 (m, 3H, 3 CH), 4.52 (s, 2H, CH$_2$), 4.01 (d, J = 6.3 Hz, 2H, CH$_2$), 1.39 (s, 9H, 3 CH$_3$). C$_{14}$H$_{18}$N$_2$O$_4$; MW: 278; MS 301 [M + Na]$^+$.

6-Aminomethyl-4*H*-benzo[1,4]oxazin-3-one Hydrochloride (11). To compound **10** (450 mg, 1.62 mmol) was added a 4 M solution of HCl in 1,4-dioxane (3 mL), and the reaction mixture was stirred at room temperature for 3 h and then concentrated and dried under high vacuum to afford the product (350 mg, quant.) as an off-white solid. ^{1}H NMR (DMSO-d_6): δ 10.94 (s, 1H, NH), 8.46 (bs, 3H, NH$_3$), 7.09–6.95 (m, 3H, 3 CH), 4.57 (d, J = 4.2 Hz, 2H, CH$_2$), 3.89 (s, 2H, CH$_2$). C$_9$H$_{11}$ClN$_2$O$_2$; MW: 214; MS 162 [M − NH$_3$Cl]$^+$.

4-Methyl-1-(*S*)-({5-[(3-oxo-3,4-dihydro-2*H*-benzo[1,4]oxazin-6-ylmethyl)-carbamoyl]pyrazolo[1,5-*a*]pyrimidine-7-carbonyl}amino)indan-5-carboxylic Acid *tert*-Butyl Ester (12). To a solution of **7** (25.4 g, 58.2 mmol), amine **11** (15.0 g, 70 mmol), 1-hydroxy-7-azabenzotriazole (8.7 g, 64 mmol), and *N*-methylmorpholine (13 mL) in dry DMF (1 L) was added *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride (22.3 g, 116 mmol) at room temperature. After 15 min, precipitation occurred. The suspension was stirred over a weekend, diluted with DMF (1 L), and heated to 40 °C, and then the solution was filtered over Celite and evaporated under reduced pressure. The residue was slurried in 5% aqueous citric acid (~5 L), filtered, and washed with 5% aqueous citric acid and then water. Drying at 40 °C under reduced pressure afforded the product (32.7 g, 94%) as a yellow solid. ^{1}H NMR (DMSO-d_6): δ 10.72 (s, 1H, NH), 10.37 (d, J = 8.1 Hz, 1H, NH), 9.66 (t, J = 6.2 Hz, 1H, NH), 8.54 (d, J = 2.5 Hz, 1H, CH), 8.14 (s, 1H, CH), 7.61 (d, J = 7.7 Hz, 1H, CH), 7.33 (d, J = 7.9 Hz, 1H, CH), 7.13 (d, J = 2.4 Hz, 1H, CH), 6.98–6.91 (m, 3H, 3 CH), 5.69 (q, 1H, CH), 4.56 (s, 2H, CH$_2$), 4.45 (d, J = 6.3 Hz, 2H, CH$_2$), 3.13–2.67 (m, 3H, CH$_2$, C*HH*), 2.44 (s, 3H, CH$_3$), 2.12–1.97 (m, 1H, CH*H*), 1.58 (s, 9H, 3 CH$_3$). C$_{32}$H$_{32}$N$_6$O$_6$; MW: 596; MS 619 [M + Na]$^+$.

4-Methyl-1-(*S*)-({5-[(3-oxo-3,4-dihydro-2*H*-benzo[1,4]oxazin-6-ylmethyl)carbamoyl]pyrazolo[1,5-*a*]pyrimidine-7-carbonyl}amino)indan-5-carboxylic Acid (1). Compound **12** (72.9 g, 122 mmol) was added in portions to a warm (45 °C) solution of formic acid (1.6 L) and stirred for 2 h, cooled to room temperature, then filtered, washed with formic acid and then water, and finally dried on a lyophilization apparatus for 70 h to afford the product (62.7 g, 95%) as a pale yellow solid. ^{1}H NMR (DMSO-d_6): δ 12.84 (bs, 1H, COOH), 10.72 (s, 1H, NH), 10.37 (d, J = 7.9 Hz, 1H, NH), 9.66 (t, J = 6.2 Hz, 1H, NH), 8.55 (d, J = 2.7 Hz, 1H, CH), 8.14 (s, 1H, CH), 7.73 (d, J = 7.9 Hz, 1H, CH), 7.33 (d, J = 7.7 Hz, 1H, CH), 7.13 (d, J = 2.5 Hz, 1H, CH), 6.98–6.91 (m, 3H, 3 CH), 5.68 (q, 1H, CH), 4.56 (s, 2H, CH$_2$), 4.45 (d, J = 6.3 Hz, 2H, CH$_2$), 3.13–2.67 (m, 3H, CH$_2$, C*HH*), 2.50 (s, 3H, CH$_3$), 2.13–2.01 (m, 1H, CH*H*). ^{13}C NMR (DMSO-d_6): δ 168.90, 164.85, 162.18, 158.37, 149.41, 147.91, 145.79, 145.62, 143.54, 142.15, 138.59, 134.93, 133.30, 139.48, 129.06, 127.00, 122.25, 120.96, 115.83, 115.03, 106.35, 98.49, 66.66, 54.96, 42.10, 32.37, 28.86, 16.80. C$_{28}$H$_{24}$N$_6$O$_6$; MW: 540; MS 541 [M + H]$^+$.

Potency and Selectivity Assays. MMP-13 activity and the selectivity assays were performed using the catalytic domains of recombinant human MMPs (MMP-1, -2, -3, -7, -9, -12: Biomol, Hamburg, Germany; MMP-8: Calbiochem, Schwalbach, Germany; MMP-13, -14: Invitek, Berlin, Germany) and the appropriate fluorogenic peptide substrates. MMP-13 activity was tested using the specific MMP-13 substrate MCA-Pro-Cha-Gly-Nva-His-Ala-Dpa-NH$_2$ (Calbiochem). MMP-3 was tested using the NFF-3 substrate MCA-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys(DNP)-NH$_2$ (Calbiochem), and all remaining MMPs were tested using OmniMMP substrate MCA-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH$_2$·AcOH (Biomol).[28] 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-NH$_2$ (Calbiochem).

The MMP-3 assay was performed in 50 mM MES buffer, pH 6.0, 10 mM CaCl$_2$, and 0.05% Brij-35. The aggrecanase-1 assay and all other MMP assays were performed in 50 mM Tris-HCl buffer, pH 7.5, 150 mM NaCl, 5 mM CaCl$_2$, and 0.05% Brij-35. TACE assay was run in 25 mM Tris-HCl buffer, pH 9, 1.25 μM Zn(OAc)$_2$, and 0.005% Brij-35. Concentrations of enzymes and substrates were optimized for each assay and varied between 1 and 10 nM enzyme and 4–10 μM substrate. The enzyme activity was measured after 10 min preincubation of the enzyme with varying concentrations of the inhibitor. Each assay was run at least in duplicate, and the IC$_{50}$ values were calculated using the Life Science Workbench (LSW) Data Analysis Plugin for Microsoft Excel. The data were fitted into the formula $y = V_{max}/(1 + [I]/IC_{50})$.[22]

Aggrecanase-1 activity was determined using the InviLISA Kit (Invitek). Human recombinant aggrecanase-1 (0.75 nM) was incubated with different concentrations of inhibitor 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 neoepitopes (ARGSVIL) were detected and quantified by ELISA.[22]

In Vitro Cartilage Degradation Assay. Freshly isolated bovine articular cartilage ($\sim$3-mm^2 pieces) were incubated with 5 ng/mL IL-1α plus 50 ng/mL OSM in the presence or absence of compounds, for 11 days. 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).[22]

Ex Vivo Model of Rat Articular Cartilage Degradation.[29] Male Sprague−Dawley (Harlan, Indianapolis, Indiana) rats weighing 175−200 g, with three animals/group, were used in this study. Rats in the vehicle group received a single IA injection of 50 μL phosphate buffered saline (PBS) into their right knee joint. Rats in the inhibitor groups received a single IA injection (50 μL) of 10 mg/mL of compound **1** suspended in PBS into their right knee joint. The compound suspension was sonicated in a water bath for 30−60 min and vortexed immediately before IA injection into their right knee joint. The animals were sacrificed at 1, 7, 14, and 21 days after the IA injection via CO$_2$ asphyxiation, and the cartilage from the tibia of the right joints was collected. Two vehicle groups (Veh 0% and Veh 100%) were sacrificed at each time point.

The susceptibility of cartilage to degradation was assessed by incubating cartilage sample from each animal with 10 μg/mL solution of activated MMP-13 (activated with 1 mM 4-aminophenylmercuric acetate) for 24 h at 37 °C in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM CaCl$_2$ buffer. Samples from the vehicle group were incubated for the same duration but with or without MMP-13 to represent 100% (Veh 100%) and 0% inhibition (Veh 0%), respectively. Incubation was stopped by adding the chelating agent, ethylenediaminetetraacetic acid to a final concentration of 10 mM. The supernatants were collected and stored at −40 °C for further analysis. Cartilage degradation was assessed by measuring the C1,2C release into the supernatant, using an ELISA (Ibex). The average C1,2C values for the vehicle groups (Veh 100% and Veh 0%) were used to calculate the % inhibition value for each sample:

$$[1 - (C1,2C_{sample} - C1,2C_{veh100\%,ave})$$
$$/(C1,2C_{veh0\%,ave} - C1,2C_{Veh100\%,ave})] \times 100\%$$

Safety, Tolerability, and Pharmacokinetics Following Intra-articular Administration. The study consisted of 24 male Sprague−Dawley rats (Harlan, Indianapolis, Indiana) weighing 175−200 g, with three male animals for each time point. The right knee joint of each rat was injected once with 50 μL/joint of PBS containing 0.5 mg of compound **1** or 50 μL/joint of PBS alone (control rats). Animals were sacrificed on days 1, 7, 14, 21, 28, 42, and 56 days after the IA injection. Plasma from each animal as well as cartilage and synovial fluid lavage from both the injected and contra-lateral joints were collected. Samples were analyzed for compound concentration by LC/MS/MS. On day 56, animals from PBS and compound **1** were scored for behavioral signs indicative of potential musculoskeletal side effect reported for MMP inhibitors.

The cartilage from the tibial articular surface was shaved, weighed, and digested overnight at 65 °C in 100 μL digestion buffer, containing 6.9 mg/mL NaH$_2$PO$_4$, 74.4 mg/mL EDTA, 0.35 mg/mL cysteine, and 10 U/mL papain (Sigma), pH 6.5. Synovial fluid samples were collected by lavaging the knee joints with 100 μL of saline. Blood (400−500 μL) was collected from all study animals at sacrifice into tubes containing dipotassium ethylenediaminetetraacetic acid and processed into plasma.

Concentrations of compound **1** in plasma, cartilage, and synovial samples were determined using an Applied Biosystems/MDS SCIEX 3200 Q TRAP mass spectrometer with an Agilent 1200 Binary SL pump. Separation was performed on an Agilent ZORBAX 1.8 μ SB-C18 2.1 mm × 30 mm HPLC column using formic acid−acetonitrile

gradient elution. Compound **1** was detected by positive electrospray ionization in the multiple reaction monitoring (MRM) mode.

The compound concentration in cartilage (ng/mg) was converted to compound per volume of cartilage (ng/mL) on the basis of the assumption that the density of the cartilage was the same as that of water, 1 g/mL. The cartilage compound concentration was converted to a molar concentration (μM) for comparison across sample types.

On day 56, the animals from control and drug treated groups were observed for behavioral signs of potential musculoskeletal side effects using a procedure reported previously.[8] Two observers individually scored the animals for three categories of resting posture, gait, and willingness to move when stimulated, and the scores in each category were averaged from both observers. The averages were then added together to obtain a total score ranging from 0 to 7.

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■ ABBREVIATIONS

Boc, *tert*-butyloxycarbonyl; DMF, *N*-dimethylformamide; DMOAD, disease-modifying osteoarthritis drug; EDCI, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; HATU, *O*-(7-azabenzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium-hexafluorophosphate; HOAt, 1-hydroxy-7-azabenzotriazole; IA, intra-articular; IL-1α, interleukin-1α; MMP, matrix metalloproteinase; MSS, musculoskeletal syndrome; NMM, *N*-methylmorpholine; NMP, *N*-methylpyrrolidin-2-one; OA, osteoarthritis; OSM, oncostatin-M; TACE, tumor necrosis factor-α converting enzyme; TBHP, *tert*-butyl hydroperoxide; THF, tetrahydrofuran

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