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Research Paper

Hexosaminidase inhibitors as new drug candidates for the therapy of osteoarthritis

Junjie Liu [a], Alexander R. Shikhman [b,1], Martin K. Lotz [b], Chi-Huey Wong [a,*]

[a] *Department of Chemistry, The Scripps Research Institute, La Jolla, CA 92037, USA*
[b] *Division of Arthritis Research, The Scripps Research Institute, La Jolla, CA 92037, USA*

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Abstract

Background: Articular cartilage from patients with osteoarthritis is characterized by a decreased concentration and reduced size of glycosaminoglycans. Degeneration of the cartilage matrix is a multifactorial process, which is due in part to accelerated glycosaminoglycan catabolism. Recently, we have demonstrated that hexosaminidase represents the dominant glycosaminoglycan-degrading glycosidase released by chondrocytes into the extracellular compartment and is the dominant glycosidase in synovial fluid from patients with osteoarthritis. Inhibition of hexosaminidase activity may represent a novel approach to the prevention of cartilage matrix glycosaminoglycan degradation and a potentially new strategy to treat osteoarthritis.

Results: We have synthesized and investigated a series of iminocyclitols designed as transition-state analog inhibitors of human hexosaminidase, and demonstrated that the five-membered iminocyclitol **4** expresses the strongest inhibitory activity with $K_i = 24$ nM. Inhibition of hexosaminidase activity in human cultured articular chondrocytes and human chondrosarcoma cells with iminocyclitol **4** resulted in accumulation of hyaluronic acid and sulfated glycosaminoglycans in the cell-associated fraction. Similarly, incubation of human cartilage tissue with iminocyclitol **4** resulted in an accumulation of glycosaminoglycans in the pericellular compartment.

Conclusions: Inhibition of hexosaminidase activity represents a new strategy for preventing or even reversing cartilage degradation in patients with osteoarthritis.

Keywords: Cartilage; Glycosaminoglycan; Hexosaminidase; Iminocyclitol; Inhibitor; Osteoarthritis

1. Introduction

Osteoarthritis is the most common joint disorder. It had been estimated that in 1990, 12.1% of Americans aged 25 and older (nearly 21 million) had clinical signs and symptoms of osteoarthritis [1]. The cost of the illness has risen over recent decades accounting for up to 1–2.5% of the gross national product of many industrialized countries [2,3]. Despite its immense public health impact, the conservative treatment of osteoarthritis is limited to a few classes of medications, such as paracetamol, non-steroidal anti-inflammatory drugs, injectable intra-articular corticosteroids and hyaluronic acid, which provide primarily symptomatic relief and have not yet been demonstrated to interfere with the progression of the disease [4–6].

It is well recognized that articular cartilage from patients with osteoarthritis and rheumatoid arthritis is characterized by decreased concentrations of proteoglycans and glycosaminoglycans (GAGs), as well as by a decreased size of GAG molecules [7]. Loss of articular proteoglycans in established joint disease could be more significant than the collagen loss [8]. In addition to quantitative changes, affected cartilage also undergoes certain qualitative changes. Among these changes are a disproportionately increased ratio of chondroitin 4-sulfate to chondroitin 6-sulfate [9], a decreased ratio of keratan sulfate to chondroitin sulfate [9], and decreased sulfation of the terminal residues in chondroitin and dermatan sulfate chains [10]. GAGs are important sources of hydraulic resistance of synovial cavities. An acute depletion of hyaluronic acid and chondroitin sulfate induced by intra-articular injection

[1] Also corresponding author. E-mail: ashikman@scrippsclinic.com

* Correspondence: Chi-Huey Wong;
E-mail: wong@scripps.edu

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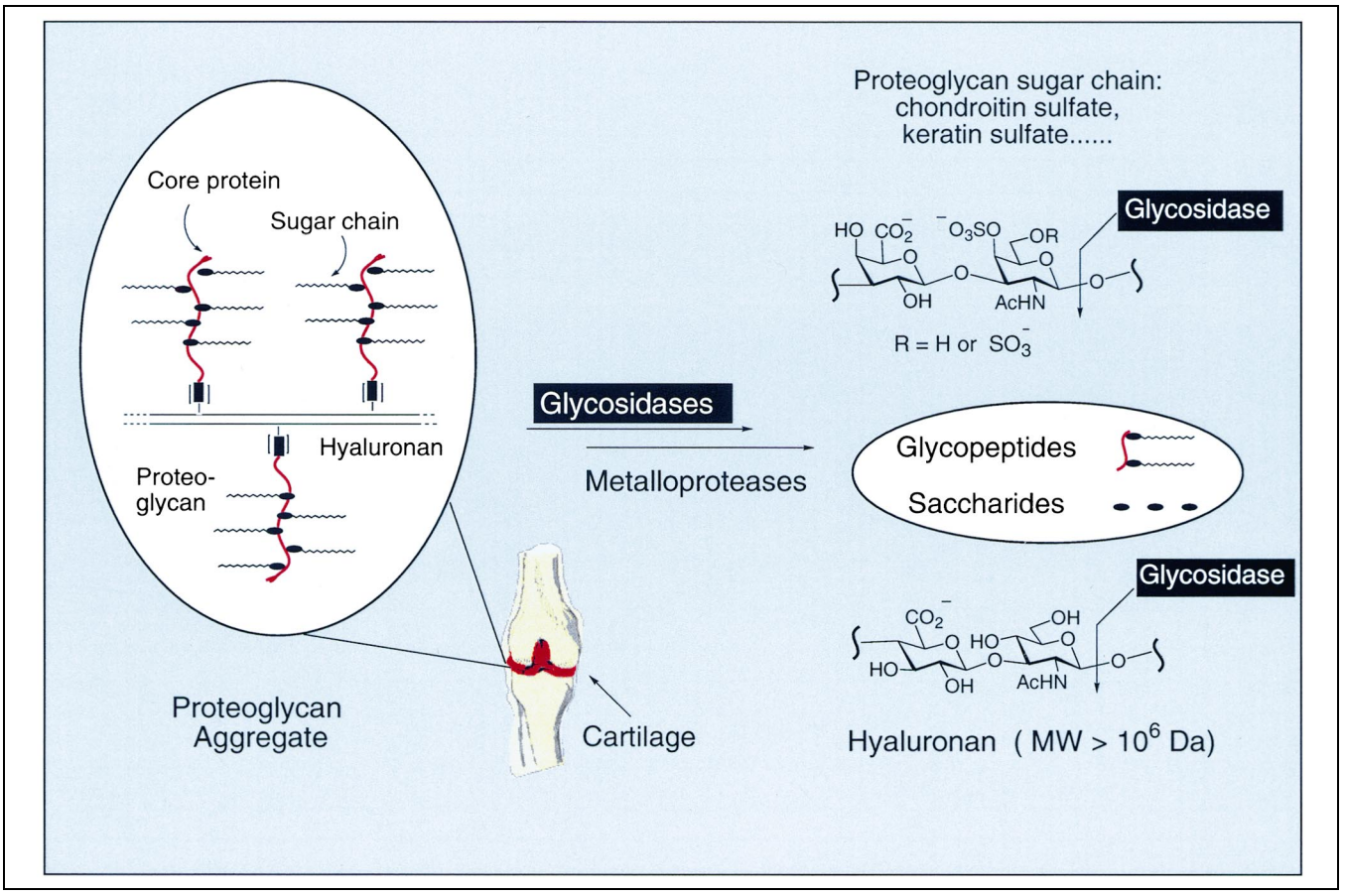


Fig. 1. Structure of the cartilage matrix extracellular proteoglycan (PG)–hyaluronate complexes and mechanisms of their degradation in arthritic cartilage. PG–hyaluronate complexes represent one of the main structural components of the cartilage extracellular matrix. The PG monomers consist of a protein core with covalently attached side chains of sulfated GAGs (chondroitin sulfate, dermatan sulfate and keratan sulfate). The PG monomers are non-covalently bound to hyaluronic acid and form PG–hyaluronate complexes. Degradation of the cartilage matrix PGs is a multifactorial process, which is due in part to the action of glycosidases (including hexosaminidase) and metalloproteinases, such as collagenases, stromelysins, aggrecanases, and cysteine proteases such as cathepsins.

of hyaluronidase resulted in a drastic (5–7-fold) increase in synovial permeability [11].

Degradation of the cartilage matrix (Fig. 1) is a multifactorial process, which is due in part to the action of metalloproteinases, such as collagenases, stromelysins, aggrecanases [12–18], and cysteine proteases such as cathepsins [19–21]. Glycosidases represent another important group of enzymes involved in GAG degradation [22,23]. Recently, we demonstrated that hexosaminidase is the dominant GAG-degrading glycosidase released by chondrocytes into the extracellular compartment, and it is the dominant glycosidase in synovial fluid of patients with osteoarthritis [24]. Stimulation of chondrocytes with the pro-inflammatory cytokine interleukin-1β results in a selective secretion of hexosaminidase [24].

Hexosaminidase (EC 3.2.1.52) belongs to the group of lysosomal hydrolases and catalyzes the hydrolysis of terminal, non-reducing *N*-acetyl-β-D-glucosamine and *N*-acetyl-β-D-galactosamine residues in glycoproteins, G_{M2}-gangliosides, and GAGs, including chondroitin 4-sulfate, chondroitin 6-sulfate, hyaluronic acid, keratan sulfate

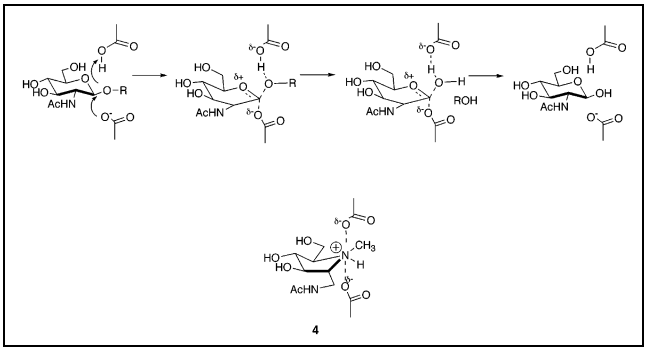
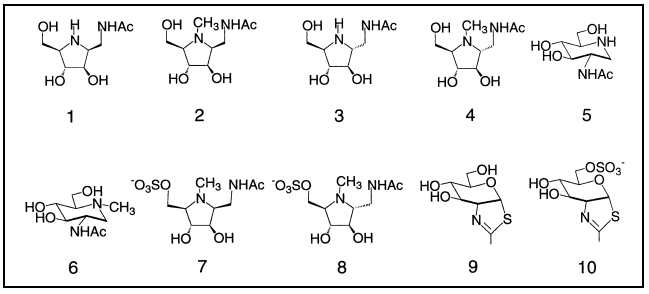


Fig. 2. The postulated mechanism of hexosaminidase catalysis and a designed transition state analog inhibitor. Hexosaminidase hydrolyzes the β-glycosidic linkage of β-*N*-acetylglucosamine or β-*N*-acetylgalactosamine. An oxonium ion transition state is considered to be developed in the catalytic process. The partial positive charge on the ring oxygen atom is stabilized by the deprotonated carboxyl group from the enzyme. Iminocyclitol **4** was designed as a transition state analog inhibitor of the hexosaminidase.



Scheme 1.

and dermatan sulfate [22]. Hexosaminidase (Fig. 2) catalyzes the glycosidic hydrolysis of β-*N*-acetylglucosamine or β-*N*-acetylgalactosamine. During the course of catalysis, an oxonium ion-like transition state is thought to be generated, which is stabilized by a deprotonated carboxyl group from the enzyme [25,37]. Human hexosaminidase has two major isoenzymes, A and B. Hexosaminidase A (HexA) is a heterodimer composed of α and β subunits, whereas hexosaminidase B (HexB) is a homodimer composed of β subunits only. Both isoenzymes recognize terminal *N*-acetylglucosamine and *N*-acetylgalactosamine, but only isoenzyme A recognizes 6-sulfated residues of these sugars [22].

Although hexosaminidase was shown to be produced by chondrocytes and to possess an enzymatic activity toward several GAGs, its direct involvement in cartilage matrix degradation has never been demonstrated. To test the hypothesis that hexosaminidase serves as one of the key enzymes participating in cartilage matrix GAG degradation, we have synthesized iminocyclitol inhibitors of hexosaminidase and demonstrated that these agents are capable of enhancing GAG accumulation in cultured human articular chondrocytes, cartilage tissue and chondrosarcoma cells. We have shown that compound **4** is a good inhibitor of the hexosaminidase in vitro [29], but no further studies were conducted. Thiazoline inhibitors of other types of hexosaminidase have also been reported to have good inhibition activity in vitro [30]. The iminocyclitols, in which the ring nitrogen atom carries a positive charge under the physiological environment [26,27], are believed to be the transition state analog inhibitors of the enzyme, and many such structures have been shown to be inhibitors of glycosidases [26–29]. We describe here the application of iminocyclitols as potential chondroprotective agents and suggest a new avenue to the development of drugs to treat osteoarthritis.

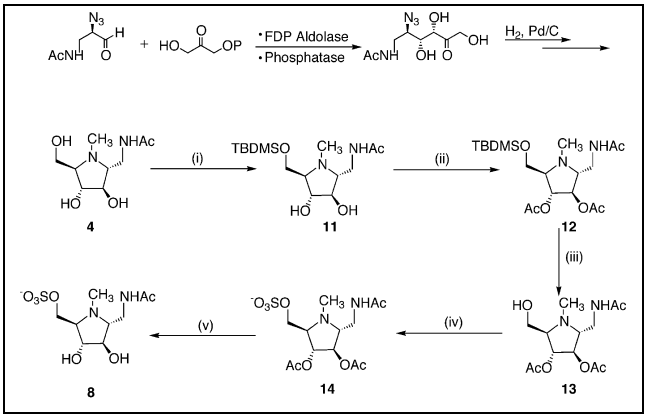


Fig. 3. Synthesis of α-iminocyclitol sulfate **8**. Reagents and conditions: (i) TBDMSCl, TEA, DMF, 0°C, 88%; (ii) Ac_2O, Pyr. 90%; (iii) AcOH/H_2O/THF (5:1:3), 50°C, 80%; (iv) SO_3·Pyr, pyridine, rt, 82%; (v) NaOMe (cat.), MeOH.

2. Results and discussion

2.1. Synthesis of the iminocyclitol inhibitors

Iminocyclitols (**1**–**8**, Scheme 1) were prepared chemoenzymatically following the procedure previously reported from our laboratory [26,28]. The key synthetic step is the aldol addition reaction of an aldehyde with dihydroxyacetone phosphate catalyzed by fructose-1,6-diphosphate aldolase.

As mentioned earlier, human hexosaminidase has two major isoenzymes [22]: HexA and HexB. HexB is a homodimer, which consists of two subunits β, while HexA is a heterodimer made of subunits α and β. Due to this difference, HexA can hydrolyze both neutral and negative-charge substrates, mainly sulfated substrates, while HexB can only hydrolyze the neutral substrates. Therefore, selective inhibition of HexA activity may have more pronounced effects on protection of sulfated GAGs from enzymatic degradation and spare non-sulfated GAGs such as hyaluronic acid.

Based on this hypothesis, the 6-hydroxyls of iminocyclitols **2** and **4** were sulfated. Following the synthetic route

Table 1
Inhibition of **1**–**10** against hexosaminidases in K_i (nM)

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**[a]	**9**	**10**[a]
K_i	240	65	40	24	1.2×10^3	8.6×10^2	–[b]	5.0×10^3 1.0×10^2 [c]	1.1×10^4	1.0×10^5 1.0×10^4 [c]

[a]Concentration at which 50% of the enzyme activity is inhibited.
[b]No inhibition at 100 μM.
[c]Inhibition of HexA.

shown in Fig. 3, the 6-sulfated α-iminocyclitol **8** was prepared from **4**.

The hydroxyl groups in β-iminocyclitol **2** seemed much less reactive than those in α-iminocyclitol **4** (Fig. 4). The 6-hydroxyl did not react with *tert*-butyldimethylsilyl chloride even at elevated temperature (130°C). However, the primary hydroxyl group of **2** reacted exclusively with *tert*-butyldimethylsilyl triflate at 0°C to afford the 6-silyl ether-protected iminocyclitol **9** in 90% yield. The different reactivity between **2** and **4** appears to be caused by the difference in orientation of the *N*-acetyl group.

The remaining two hydroxyls were then protected as benzyl ethers. The activation of 6-hydroxyl by the benzyl group was critical for the sulfation reaction. We found that the diacetyl-protected iminocyclitol **15** did not react with pyridine sulfur trioxide even under forcing reaction conditions. Compound **9** was prepared according to the procedure reported previously by Knapp et al. [30]. Compound **10** was prepared from **9** by reaction with pyridine sulfur trioxide. Both **9** and **10** were used to see if the participation of the neighboring C-2 acetamido group was involved during the degradation of GAGs by the enzyme.

2.2. Inhibition activity

All the iminocyclitols were assayed in vitro under the condition similar to the one described previously [28,29]. The substrate was used in the micromolar range and inhibitors in the micro- to nanomolar range. All inhibitors were found to be potent competitive inhibitors of β-*N*-acetylhexoaminidases from human placenta (Table 1). Especially, the five-membered iminocyclitol **4** exhibits a K_i of 24 nM. The potent inhibition activity may be attributed to the protonated iminocyclitol at the physiological pH to mimic the positive charge of the oxonium ion [26,27]. Since there is less torsion force and more half-chair-like characteristic in the five-membered ring [29], the transition state conformation is mimicked better by the five-membered ring than by the six-membered ring system. The participation of the C-2 acetamido group seems not so critical here.

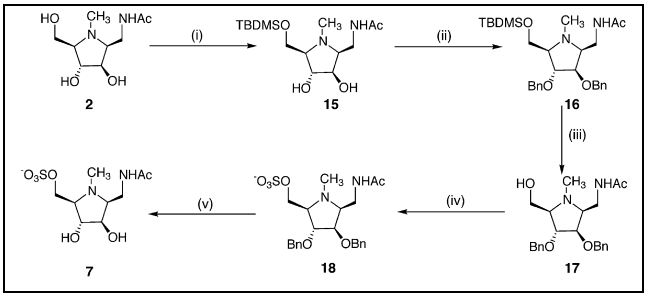


Fig. 4. Synthesis of β-iminocyclitol sulfate **7**. Reagents and conditions: (i) TBDMSOTf, TEA, 0°C, DMF 90%; (ii) BnBr, NaH, 0°C–rt, 90%; (iii) TBAF, THF, 4 h, 80%; (iv) SO_3·Pyr, pyr, 80%; (v) $Pd(OH)_2$-C, H_2, 75%.

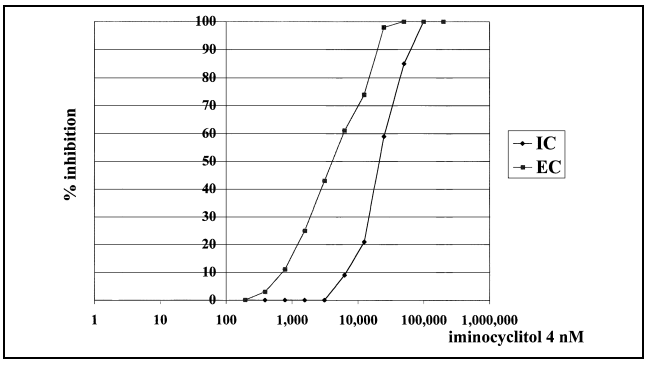


Fig. 5. Effect of the iminocyclitol **4** on extracellular (EC) and intracellular (IC) activities of hexosaminidase in cultured human articular chondrocytes. Human articular chondrocytes were incubated with various concentrations of the iminocyclitol **4** for 24 h at 37°C. EC hexosaminidase activities were measured in culture supernatants. IC hexosaminidase activities were measured in cell lysates, which were produced by solubilization of chondrocytes in equal volumes of Cell Death ELISA^{PLUS} buffer (Boehringer Mannheim, Indianapolis, IN, USA). EC and IC hexosaminidase activities in chondrocyte culture without the inhibitor were used as controls. Vertical axis represents percent hexosaminidase inhibition, which was calculated as ($E_{460/360}$ experimental/$E_{460/360}$ control) ×100%. Horizontal axis represents nM concentrations of the iminocyclitol **4** added to cell cultures at time 0 of incubation.

2.3. Effect of iminocyclitol **4** on extracellular and intracellular hexosaminidase activity in cultured human articular chondrocytes

Incubation of human articular chondrocytes with iminocyclitol **4** resulted in a strong, dose-dependent inhibition of extracellular and intracellular hexosaminidase activities (Fig. 5). The inhibitor did not affect cell viability measured by a standard MTT (thiazolyl blue) assay. In addition, the inhibitor did not affect the extracellular and intracellular enzymatic activities of α-*N*-acetylglucosaminidase, β-galactosidase and hyaluronidase in chondrocyte cultures (data not shown). Therefore, results of the experiments demonstrated that compound **4** expresses specific inhibitory activity toward human hexosaminidase in vitro.

2.4. Effect of hexosaminidase inhibition on sulfated GAG and hyaluronic acid accumulation in cultured human articular chondrocytes and chondrosarcoma cells

Incubation of human chondrosarcoma cells with the inhibitor **4** resulted in a statistically significant increase in the accumulation of cell-associated hyaluronic acid and sulfated GAGs in the cell-associated fraction, and in a statistically significant decrease in secretion of hyaluronic and sulfated GAGs into the supernatant as compared with untreated cells (Fig. 6). Similar results were obtained with cultured human articular chondrocytes (Fig. 7). However, the response of the chondrocytes was more heterogeneous than that of chondrosarcoma cells, which most likely reflects the heterogeneity in the amplitude of hexosaminidase activities in the chondrocytes. Overall, the ex-

perimental data have confirmed the assumption that hexosaminidase participates in degradation of the extracellular matrix and that the inhibition of hexosaminidase activity can decelerate this process.

2.5. *Effect of hexosaminidase inhibition on morphology of cultured human cartilage tissue*

In order to evaluate the effect of hexosaminidase inhibition on cartilage morphology, human articular cartilage tissue was incubated with iminocyclitol **4** and subsequently stained with safranin O to detect sulfated GAGs. Results of the experiments demonstrated that incubation of cartilage tissue with the inhibitor resulted in a remarkable accumulation of safranin O-positive material in the pericellular cartilage compartment (Fig. 8). Therefore, the generated histomorphological data also confirmed the notion regarding the positive effect of hexosaminidase inhibition on GAG accumulation.

2.6. *Sulfated iminocyclitol **8** as a selective inhibitor of human HexA*

The activities of sulfated inhibitors were also investigated, and no inhibition was observed for sulfated β-iminocyclitol **7** at 100 μM range. Incubation of human articular chondrocytes with iminocyclitol **8** demonstrated a selective inhibition of HexA in the culture supernatants (Fig. 9A). In addition, inhibitor **8** showed a better inhibition activity against human placental HexA (IC_{50} = 0.1 μM) than the total placental hexosaminidases (IC_{50} = 5 μM) (Fig. 9B). This result verified our initial hypothesis.

Although the negative-charge carrying inhibitor is difficult to transport through the circulating system [31], prodrugs of the sulfated inhibitor may be designed based on this result. As mentioned earlier, as important components for the construction of different tissues and for the display of secretions of many important functions, GAGs are distributed with different ratios of hyaluronic acid (non-sulfated) to other sulfated GAGs in different human tissues based on their functions. Selective inhibition of the degradation of sulfated GAGs would have fewer side effects on the metabolism of hyaluronic acid. One example of this advantage was shown in the inhibition of tumor angiogenesis and metastasis [32], where sulfated inhibitors were used to inhibit the heparanase-catalyzed cleavage of heparan, thus blocking heparan sulfate recognition by growth factors.

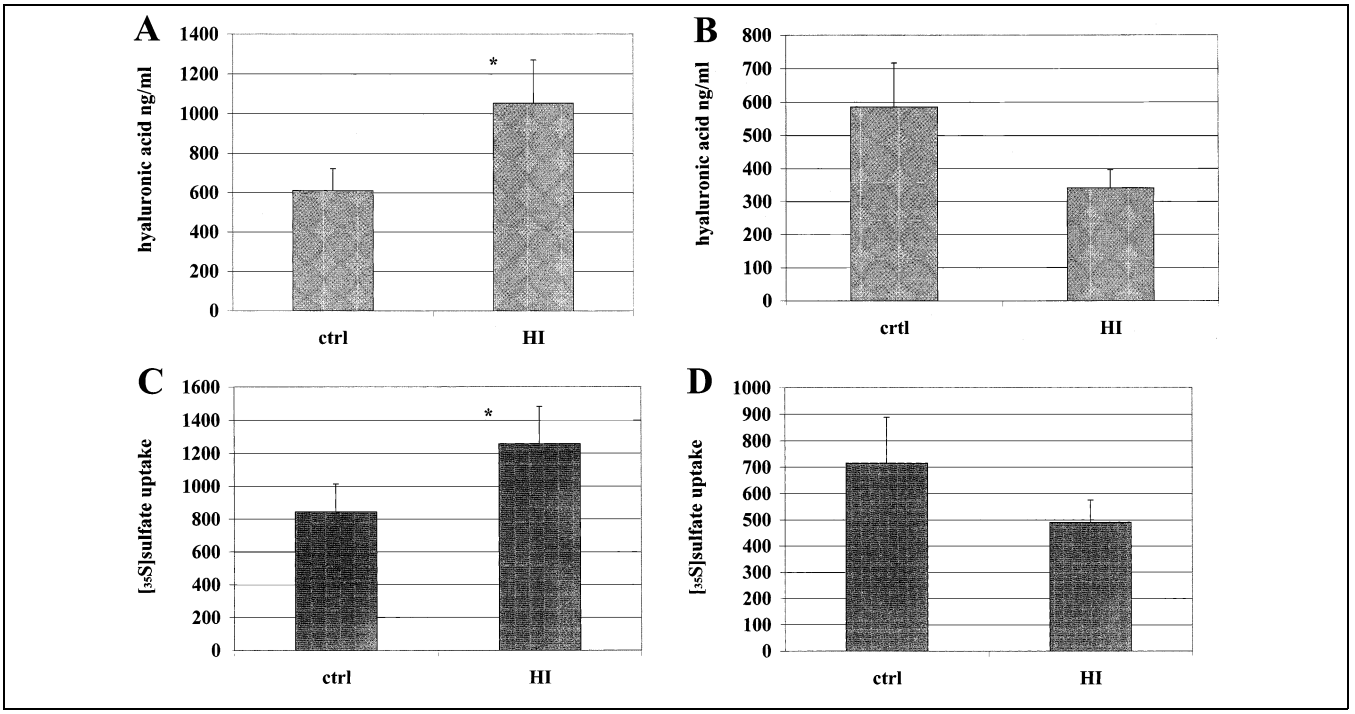


Fig. 6. Effect of hexosaminidase inhibition on hyaluronic acid and sulfated GAG accumulation in cultured human chondrosarcoma cell line SW 1353. Chondrosarcoma cells were incubated with the iminocyclitol **4** (50 μM) for 24 h at 37°C. Chondrosarcoma cells incubated in the absence of the inhibitor were used as a control. Measurement of hyaluronic acid and metabolic labeling of sulfated GAGs with [^{35}S]sulfate were performed according to the technique described in Section 4. Bars labeled with letters 'HI' represent cells treated with the iminocyclitol **4**. Bars labeled with letters 'ctrl' represent untreated chondrosarcoma cells. (A) Concentration of hyaluronic acid (ng/ml) in cell-associated fractions. (B) Concentration of hyaluronic acid (ng/ml) in supernatants. (C) Metabolic labeling of sulfated GAGs in cell-associated fractions with [^{35}S]sulfate. (D) Metabolic labeling of sulfated GAGs in supernatants with [^{35}S]sulfate. Vertical axes in C and D represent [^{35}S]sulfate uptake in relative units of radioactivity. Asterisks indicate statistically significant differences.

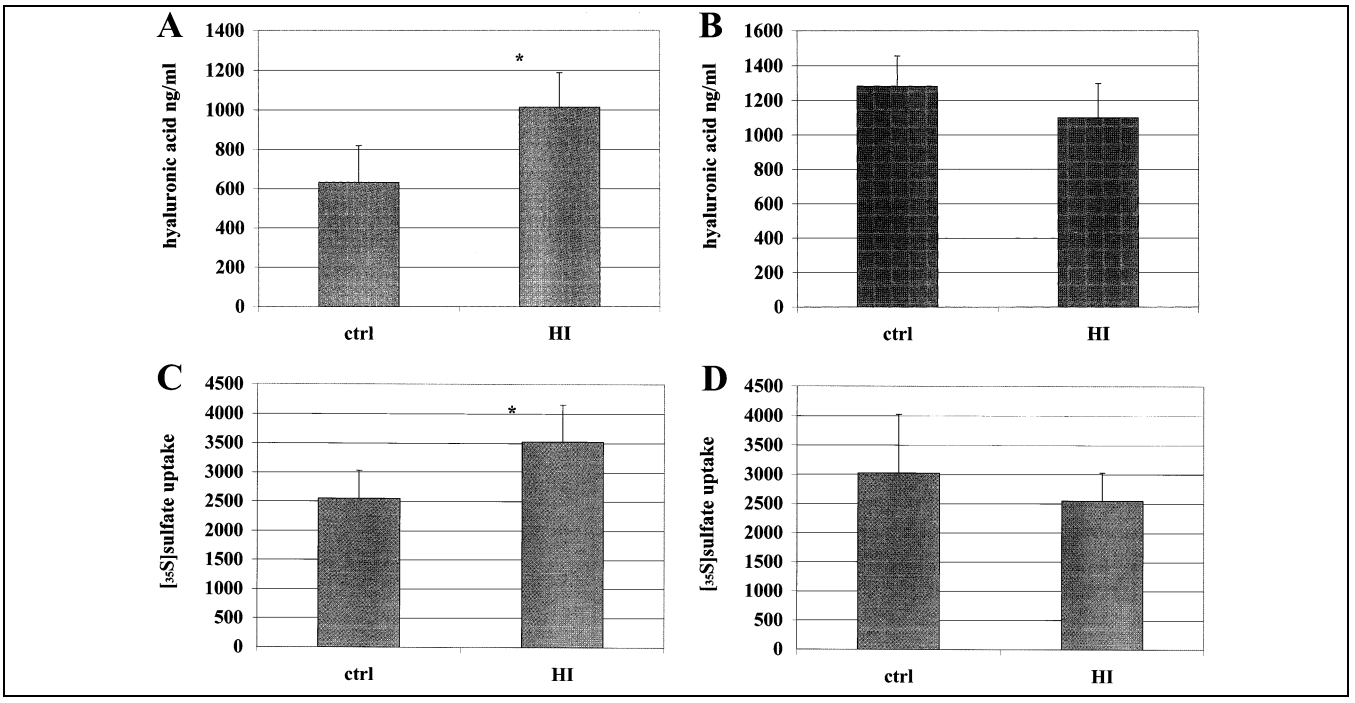


Fig. 7. Effect of hexosaminidase inhibition on hyaluronic acid (A and B) and sulfated GAG accumulation (C and D) in cultured human articular chondrocytes. Cultured human articular chondrocytes were incubated with the iminocyclitol **4** (50 μM) for 24 h at 37°C. Chondrocytes incubated in the absence of the inhibitor were used as a control. Measurement of hyaluronic acid and metabolic labeling of sulfated GAGs with [^{35}S]sulfate were performed according to the technique described in Section 4. Bars labeled with letters 'HI' represent cells treated with the iminocyclitol **4**. Bars labeled with letters 'ctrl' represent untreated chondrosarcoma cells. (A) Concentration of hyaluronic acid (ng/ml) in cell-associated fractions. (B) Concentration of hyaluronic acid (ng/ml) in supernatants. (C) Metabolic labeling of sulfated GAGs in cell-associated fractions with [^{35}S]sulfate. (D) Metabolic labeling of sulfated GAGs in supernatants with [^{35}S]sulfate. Vertical axes in C and D represent [^{35}S]sulfate uptake in relative units of radioactivity. Asterisks indicate statistically significant differences.

3. Significance

The results of our earlier reported experiments demonstrated that hexosaminidase is one of the dominant glycosidases secreted by human articular chondrocytes in vitro and it is also one of the dominant glycosidases found in human synovial fluid [24]. In the present article we describe data showing that inhibition of hexosaminidase activity results in GAG accumulation in cultured human articular chondrocytes and cartilage.

The idea regarding possible therapeutic benefits from hexosaminidase inhibition is based on the following observations. First, Cantz and Kresse [33] established that fibroblasts isolated from patients with Sandhoff disease accumulate excessive amounts of GAGs. The impaired catabolism of GAGs was reversed by addition of hexosaminidases [35]. Second, Suzuki et al. [34] demonstrated that mice deficient in both hexosaminidase isoenzymes developed mucopolysaccharidosis-like pathology. Articular chondrocytes from hexosaminidase-deficient mice were found to contain increased quantities of GAGs [36]. These findings confirmed the notion that hexosaminidases play a key role in the degradation of GAGs. The process of GAG accumulation in hexosaminidase-deficient individuals is qualitatively opposite to the trend which takes place in articular cartilage affected by arthritis. Accordingly, inhibition of hexosaminidase activity may be considered a desirable intervention directed toward the prevention of GAG loss or even restoration of impaired cartilage GAGs.

In the present study we demonstrate that inhibition of hexosaminidase activity with an iminocyclitol inhibitor resulted in accumulation of GAGs in the cell-associated fraction and decreased release of GAGs into the culture supernatant. The data confirm two assumptions: (1) hexosaminidase participates in the degradation of extracellular matrix in human cartilage; (2) inhibition of hexosaminidase activity with the aid of chemical inhibitors delays the catabolism of cartilage matrix GAGs.

In summary, the described findings open a new avenue in the development of a new class of drugs possessing chondroprotective activity.

4. Materials and methods

4.1. General methods

The reagents used were purchased from Aldrich, Sigma, or TCI America. The solvents were reagent grade and used as supplied. High resolution mass spectra (HRMS) were recorded on a VG ZAB-ZSE instrument with fast atom ion bombardment (FAB). ^{1}H NMR spectra and ^{13}C NMR were carried out on a Bruker

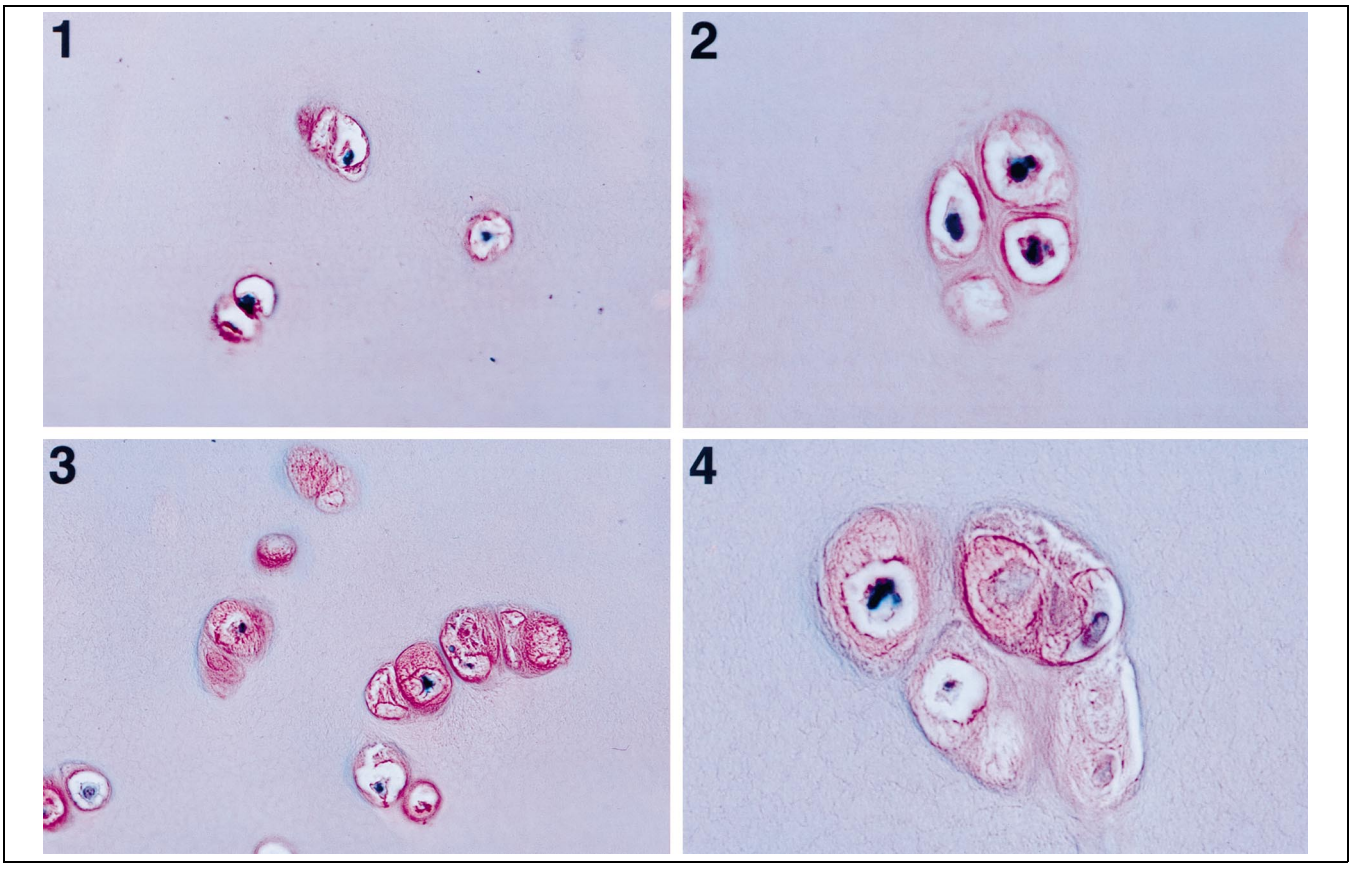


Fig. 8. Effect of hexosaminidase inhibition on histomorphology of cultured human cartilage explants. Human cartilage tissue explants were incubated with the iminocyclitol **4** (50 μM) for 48 h at 37°C. Cartilage explants from the same donor incubated with culture medium only were used as controls. After incubation, the tissue was stained with safranin O. Panels 1 (low magnification) and 2 (high magnification) represent microscopic images of the control, untreated cartilage explants. Panels 3 (low magnification) and 4 (high magnification) represent microscopic images of the cartilage implants treated with iminocyclitol **4**.

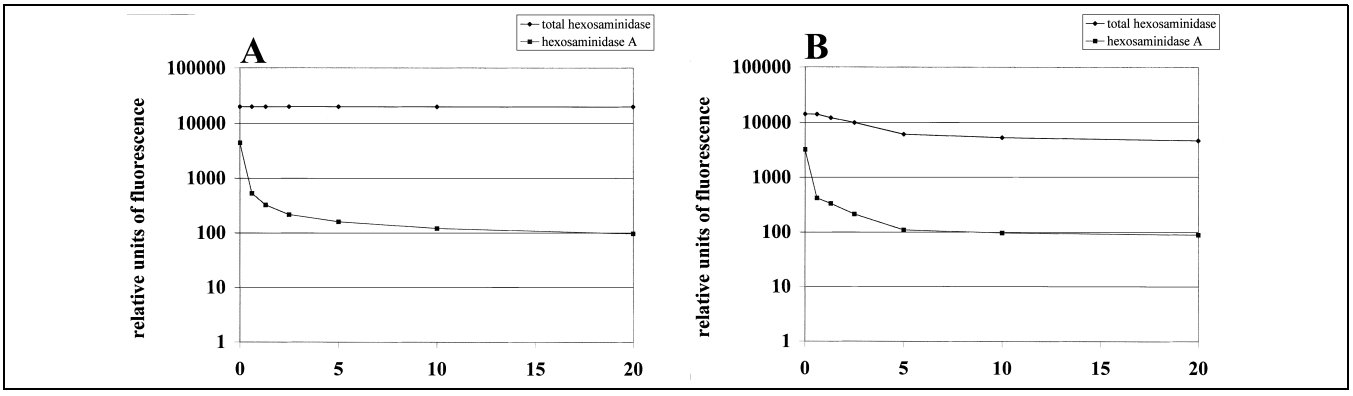


Fig. 9. Effect of iminocyclitol **8** on total hexosaminidase and HexA activities in human cultured chondrocytes (A) and in preparation of human placental hexosaminidase (B). (A) Human articular chondrocytes were cultured with appropriate concentrations of iminocyclitol **8** for 24 h at 38°C. Total hexosaminidase activity was measured in culture supernatants using 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide as a substrate. Activity of HexA was measured in culture supernatants using 4-methylumbelliferyl-6-sulfo-*N*-acetyl-β-D-glucosaminide as a substrate. Vertical axis represents relative fluorescent units of hexosaminidase activity. Horizontal axis represents concentration of iminocyclitol **8** (μM). (B) Human placental hexosaminidase was used as a source of the enzyme. Enzymatic reaction was performed in 0.1 M sodium citrate buffer pH 4.5, containing appropriate concentrations of iminocyclitol **8**, for 2 h at 37°C. Total hexosaminidase activity was measured using 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide as a substrate. Activity of HexA was measured using 4-methylumbelliferyl-6-sulfo-*N*-acetyl-β-D-glucosaminide. Vertical axis represents relative fluorescent units of hexosaminidase activity. Horizontal axis represents concentration of iminocyclitol **8** (μM).

AMC-400, AMX-500, or AMX-600 instruments. Silica gel 60 (230–240 mesh) from Mallinckrodt was used in chromatography.

*4.1.1. Thiazoline 6-sulfate **10***

To a solution of 55 mg of thiazoline **9** in 4 ml of pyridine was added 35 mg of SO_3·NMe_3. The mixture was first stirred under 0°C for 2 h, and then stayed at room temperature for 10 h. After concentration, the residue was purified by silica gel chromatography (methylene chloride/methanol = 4:1) to afford 78 mg (87%) of **10**. ^{1}H NMR (CD_3OD) δ 2.25 (3H, d, J = 1.9 Hz), 3.54 (1H, ddd, J = 2.3 Hz and 9.0 Hz), 3.66 (1H, dd, J = 4.5 Hz and 9.0 Hz), 4.07 (1H, dd, J = 4.3 Hz and 4.5 Hz), 4.10 (1H, dd, J = 5.8 Hz and 11.0 Hz), 4.17 (1H, dd, 2.3 Hz and 11.0 Hz), 4.29 (1H, d, J = 4.5 Hz), 6.32 (1H, d, J = 6.9 Hz); ^{13}C NMR (CD_3OD) δ 20.8, 68.9, 71.1, 74.4, 74.7, 80.9, 90.6, 170.9; HRMS ($M+Na^+$) calcd. 322.0031, found 322.0036.

*4.1.2. Silyl ether **11***

To a 1.2 ml DMF solution containing 70 mg (0.32 mmol) iminocyclitol **4** was added 84 µl (0.48 mmol) triethylamine and then cooled to 0°C, 72 mg of TBDMSCl (0.46 mmol) was added and this mixture was stirred for 10 h at room temperature. After the reaction was completed, the solvent was removed under vacuum and the residue was purified by silica gel chromatography (CH_3Cl/MeOH = 9:1) to afford the silyl ether **11** (93 mg, 88% yield). ^{1}H NMR ($CDCl_3$) δ 0.15 (6H, s), 0.97 (9H, s), 2.00 (3H, s), 2.51 (3H, s), 2.86–2.90 (1H, td, J = 3.3 Hz, 9.2 Hz), 2.96–2.98 (1H, q, J = 4.1 Hz), 3.24–3.27 (1H, dd, J = 5.5 Hz, 14.0 Hz), 3.56–3.60 (1H, dd, J = 3.3 Hz, 14.0 Hz), 3.74–3.77 (1H, dd, J = 4.4 Hz, 5.5 Hz), 3.82–3.85 (1H, dd of AB, J = 4.4 Hz, 10.5 Hz), 3.90–3.93 (1H, dd of AB, J = 4.1 Hz, 10.5 Hz), 3.94–3.96 (1H, t, J = 3.8 Hz); ^{13}C NMR($CDCl_3$) δ −5.3, −5.2, 18.5, 22.7, 26.55, 35.2, 39.2, 62.8, 70.2, 71.8, 79.8, 80.8, 173.8; HRMS ($M+H^+$) calcd. 333.2204, found 333.2216.

*4.1.3. Preparation of iminocyclitol **12***

A solution of 7 mg of **9** (0.02 mmol) in 2 ml pyridine was cooled down to 0°C and then 0.1 ml Ac_2O was added. The mixture was stirred for 5.0 h at room temperature. The solvent was then removed under vacuum and dried further under high vacuum. The residue was purified by silica gel chromatography (CH_3Cl/MeOH = 9:1) to afford the silyl ether **12** (7.8 mg, 90% yield). ^{1}H NMR ($CDCl_3$) δ 0.08 (3H, s), 0.09 (3H, s), 0.91 (9H, s), 2.01 (3H, s), 2.08 (3H, s), 2.09 (3H, s), 2.43 (3H, s), 3.04–3.06 (1H, q, J = 3.6 Hz), 3.16–3.20 (1H, m), 3.20–3.23 (1H, dd of AB, J = 3.0 Hz, 7.2 Hz), 3.57–3.61 (1H, ddd, J = 1.8 Hz, 6.6 Hz, 12.0 Hz), 3.82–3.86 (1H, dd of AB, J = 3.6 Hz, 10.5 Hz), 3.86–3.89 (1H, dd of AB, J = 4.0 Hz, 10.5 Hz), 4.92–4.94 (1H, dd of AB, J = 3.1 Hz, 4.9 Hz), 5.13–5.15 (1H, t, J = 2.6 Hz), 6.15 (1H, br.); ^{13}C NMR($CDCl_3$) δ −5.60, −5.57, 18.1, 20.95, 21.04, 23.3, 25.8, 34.0, 37.0, 61.2, 66.6, 68.7, 79.2, 79.3, 170.5, 170.6, 171.0; HRMS ($M+H^+$) calcd. 417.2415, found 417.2420.

*4.1.4. Preparation of **13***

The silyl ether **10** (7 mg) was dissolved in 0.5 ml of a mixture solvent ($AcOH/H_2O$/THF = 5:1:3) and stirred for 8 h at 50°C. After the reaction was completed, the solvent was removed under vacuum and the residue was purified by silica gel chromatography (EtOAc/MeOH = 4:1) to yield **13** (4.0 mg, 80%). ^{1}H NMR ($CDCl_3$) δ 2.00 (3H, s), 2.09 (3H, s), 2.11 (3H, s), 2.53 (3H, s), 3.01–3.05 (1H, q, J = 4.0 Hz), 3.18 (1H, m), 3.27–3.31 (1H, dd of AB, J = 8.0 Hz, 13.5 Hz), 3.55–3.59 (1H, dd, J = 3.7 Hz, 14.0 Hz), 3.73–3.76 (1H, dd of AB, J = 5.1 Hz, 11.7 Hz), 3.77–3.81 (1H, dd of AB, J = 4.0 Hz, 11.6 Hz), 4.97–5.01 (1H, dd, J = 2.2 Hz, 3.0 Hz), 5.11–5.13 (1H, dd, J = 2.2 Hz, 3.6 Hz); ^{13}C NMR ($CDCl_3$) δ 20.85, 20.89, 22.6, 34.9, 37.4, 60.4, 68.9, 70.2, 80.55, 80.61, 171.95, 172.07, 173.6; HRMS ($M+H^+$) calcd. 303.1551, found 303.1546.

*4.1.5. Sulfation of compound **13***

Under argon protection, 15 mg of SO_3·pyridine complex and 3 mg (0.01 mmol) of compound **13** were dissolved in 0.5 ml of pyridine. The mixture was stirred for 6 h at room temperature. After no starting material was left, the solvent was removed and the residue was purified by silica gel chromatography (EtOAc/MeOH = 2:1) to afford 3.1 mg of **12** (82%). ^{1}H NMR (CD_3OD) δ 1.94 (3H, s), 2.04 (3H, s), 2.05 (3H, s), 2.50 (3H, s), 3.11 (1H, m), 3.25 (2H, m), 3.50–3.54 (1H, dd of AB, J = 4.0 Hz, 17.6 Hz), 4.13–4.16 (1H, dd of AB, J = 3.8 Hz, 14.0 Hz), 4.17–4.21 (1H, dd of AB, J = 3.0 Hz, 13.6 Hz), 4.89–4.92 (1H, dd, J = 2.6 Hz, 5.2 Hz), 5.04–5.07 (1H, t, J = 3.3 Hz); ^{13}C NMR (CD_3OD) δ 21.0(2), 22.8, 35.0, 38.2, 66.7, 68.0, 68.8, 80.5(2), 172.15, 172.23, 173.8; MS$(M-H)^-$ 381.

*4.1.6. α-Iminocyclitol 6-sulfate **8***

2 mg (0.005 mmol) of protected α-iminocyclitol 6-sulfate **14** was dissolved in 2 ml of MeOH and then two drops of a 25% MeONa solution were added to the solution. The reaction was completed in 30 min and was quenched by acidic resin (Dowex 50-H^+). The solvent was then removed and the residue was purified by reverse phase column (Lichroprep® RP-18) to afford 1.8 mg (85%) of the product. ^{1}H NMR (D_2O) δ 1.94 (3H, s), 2.48 (3H, s), 3.00 (1H, br.), 3.17 (1H, br.), 3.32–3.56 (1H, dd of AB, J = 6.2 Hz, 14.4 Hz), 3.47–3.51 (1H, dd of AB, J = 4.8 Hz, 14.4 Hz), 3.78–3.81 (1H, t, J = 5.3 Hz), 3.98–4.00 (1H, t, J = 5.3 Hz), 4.16–4.19 (1H, dd of AB, J = 4.4 Hz, 11.0 Hz), 4.21–4.25 (1H, dd of AB, J = 4.1 Hz, 11.4 Hz); ^{13}C NMR (D_2O) δ 22.7, 35.3, 38.4, 66.3, 67.8, 68.4, 77.7, 79.0, 175.5; HRMS $(M-H)^-$ calcd. 297.0762, found 297.0749.

*4.1.7. β-Iminocyclitol 6-silyl ether **15***

45 mg (0.2 mmol) of β-iminocyclitol **2** and 60 µl of triethylamine (0.4 mmol) were dissolved in 2 ml of DMF. The mixture was cooled down to 0°C. 71 µl (0.3 mmol) of TBDMSOTf was then added dropwise to the reaction system. The reaction was completed in 1.0 h. After removal of the solvent, the residue was purified by silica gel chromatography (CH_3Cl/MeOH = 10:1) to afford 62 mg of **15** (90%). ^{1}H NMR ($CDCl_3$) δ 0.098 (3H, s), 0.103 (3H, s), 0.91 (12H, s), 1.97 (3H, s), 2.39 (3H, s), 2.48–2.50 (1H, m), 2.74–2.77 (1H, m), 3.14–3.16 (1H, dt, J = 2.9 Hz, 11.0 Hz), 3.64–3.68 (1H, dd, J = 5.0 Hz, 15.0 Hz),

3.72–3.76 (1H, ddd, $J = 2.0$ Hz, 7.0 Hz, 14.0 Hz), 3.76–3.80 (2H, m), 4.14 (1H, dd, $J = 4.0$ Hz, 6.6 Hz); ^{13}C NMR ($CDCl_3$) δ −5.53, −5.48, 18.1, 21.5, 23.1, 25.8, 25.9, 35.2, 67.6, 77.9, 78.5, 171.4.

*4.1.8. Benzylation of **15***

70 mg (0.21 mmol) of silyl ether **15** was dissolved in 3 ml of DMF under argon, the solution was then cooled to 0°C, 24 mg of 60% NaH (0.56 mmol) was added. The mixture was stirred for 10 min before 101 µl of BnBr (0.46 mmol) was added. The temperature was then increased to 25°C. The reaction was completed after 12 h. The reaction mixture was then poured into 20 ml of ice-water. The resulting mixture was extracted with EtOAc. The organic phase was then dried over anhydrous Na_2SO_4 and concentrated. The residue was purified by silica gel chromatography (hexane/EtOAc = 1:2) to afford 97 mg of fully protected iminocyclitol **16** (90%). ^1H NMR ($CDCl_3$) δ 0.061 (6H, s), 0.87 (9H, s), 1.68 (3H, s), 2.36 (3H, s), 3.06 (1H, m), 3.16–3.20 (1H, d, $J = 14.0$ Hz), 3.43–3.48 (1H, ddd, $J = 2.2$ Hz, 5.9 Hz, 14.0 Hz), 3.58–3.61 (1H, dd, $J = 4.8$ Hz, 9.2 Hz), 3.71–3.75 (1H, dd, $J = 4.4$ Hz, 14 Hz), 3.77–3.81 (1H, dd, $J = 6.6$ Hz, 13.8 Hz), 3.96 (1H, s), 4.53–4.57 (4H, m), 7.27–7.36 (10H, m); HRMS $(M+H)^+$ calcd. 513.3148, found 513.3165.

*4.1.9. β-Iminocyclitol **17***

25.8 mg (0.05 mmol) of **16** was dissolved in 3 ml of THF. The mixture was cooled down to 0°C. 76 µl of TBAF solution (1.0 M in THF) was added dropwise. The mixture was stirred for 4 h at room temperature until the reaction was completed. The solvent was removed and the residue was purified by silica gel chromatography (MeOH/EtOAc = 1:10) to give 16 mg of **17** in 80% yield. ^1H NMR ($CDCl_3$) δ 1.73 (3H, s), 2.37 (3H, s), 2.45 (1H, m), 2.81 (1H, s), 2.92–2.95 (1H, q, $J = 5.5$ Hz), 3.07–3.10 (1H, d, $J = 14.3$ Hz), 3.56–3.60 (1H, dd, $J = 9.5$ Hz, 5.1 Hz), 3.66–3.70 (1H, ddd, $J = 1.8$ Hz, 7.3 Hz, 13.9 Hz), 3.75–3.79 (1H, dd, $J = 5.9$ Hz, 9.6 Hz), 3.87–3.92 (1H, dd, $J = 4.1$ Hz, 10.3 Hz), 3.93 (1H, br.), 4.55 (2H, s), 4.57–4.60 (1H, d, $J = 11.8$ Hz), 4.64–4.66 (1H, d, $J = 11.8$ Hz), 6.25–6.28 (1H, d, $J = 5.2$ Hz), 7.28–7.34 (10H, m); HRMS $(M+H)^+$ calcd.399.2284, found 399.2279.

*4.1.10. Sulfation of **15***

Compound **17** (14 mg, 0.035 mmol) and SO_3·pyridine (56 mg, 10 eq.) were dissolved in 2 ml of pyridine. The mixture was stirred for 8 h at room temperature. After the reaction was completed, the solvent was removed and the residue was purified by silica gel chromatography (MeOH/EtOAc = 1:4) to afford 13 mg of **18** (80%). ^1H NMR (CD_3OD) δ 1.83 (3H, s), 2.52 (3H, s), 2.89 (1H, br.), 3.05 (1H, br.), 3.56–3.59 (1H, dd, $J = 3.3$Hz, 11.4 Hz), 3.64–3.69 (1H, dd, $J = 4.4$ Hz, 8.0 Hz), 3.75–3.79 (1H, dd, $J = 5.1$ Hz, 8.0 Hz), 4.13–4.15 (1H, d, $J = 3.3$ Hz), 4.47–4.53 (4H, m), 4.65–4.68 (1H, d, $J = 1.8$ Hz), 4.79–4.80 (1H, d, 9.5 Hz), 7.23–7.34 (10H, m); ^{13}C NMR (CD_3OD) 22.7, 41.0, 41.5, 55.3, 69.2, 69.8, 73.0, 74.6, 83.7, 128.85, 128.95, 129.07, 129.6, 129.49, 129.53, 139.4, 139.6, 175.2; MS $(M+Na)^+$ 501.

*4.1.11. β-Iminocyclitol 6-sulfate **7***

The benzyl ether-protected iminocyclitol **18** (20 mg, 0.042 nmol) was dissolved in 3 ml of MeOH–H_2O (1:1). 30 mg of Pd(OH)/C was added. The mixture was hydrogenated under 1.0 atm of H_2 pressure for 12 h. After the reaction was completed, the solvent was removed and the residue was purified by silica gel chromatography (CH_3Cl/MeOH/H_2O = 6:4:0.7) to afford 9.3 mg of **7** (75%). ^1H NMR (CD_3OD) δ 1.99 (3H, s), 2.98 (3H, s), 3.41 (1H, br.), 3.54–3.62 (2H, m), 3.91–3.95 (1H, dd of AB, $J = 5.9$ Hz, 12.3 Hz), 3.98–4.01 (1H, dd of AB, $J = 5.1$ Hz, 12.5 Hz), 4.38–4.40 (1H, d, $J = 2.9$ Hz), 4.53 (1H, s); MS $(M-H)^-$ 297.

4.2. Cell and tissue culture

Human cartilage was obtained from autopsy services, tissue banks and from the Division of Orthopedic Surgery at Scripps Clinic. Briefly, articular cartilage was harvested from the femoral condyles and the tibial plateaus. All tissue samples were graded according to the Mankin scale [8]. Chondrocytes were isolated from the cartilage by collagenase digestion and maintained in high-density monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) containing 2% fetal bovine serum. The experiments reported here were performed with first-passage cells. The human chondrosarcoma SW 1353 cell line was obtained from ATCC (catalog no. HTB-94) and maintained in DMEM containing 2% fetal bovine serum.

4.3. Assay for hexosaminidase activity

4-Methylumbelliferyl-*N*-acetyl-β-D-glucosamine (TRC, Toronto, ON, Canada) at a final concentration of 0.1 mM in 0.1 M sodium citrate buffer pH 4.5 was used as a substrate for total hexosaminidase activity. 4-Methylumbelliferyl-6-sulfate-*N*-acetyl-β-D-glucosamine (TRC) at a final concentration of 0.1 mM in 0.1 M sodium citrate buffer pH 4.5 was used as a substrate for HexA activity. Human placental hexosaminidase, cell culture supernatants or cell lysates were diluted in 0.1 M sodium citrate buffer, pH 4.5, and incubated with the substrate for 2 h at 37°C. The reaction was stopped by the addition of 0.5 M sodium glycine buffer, pH 10.5. Hexosaminidase activity was measured by the release of 4-methylumbelliferone, utilizing fluorometry (VersaFluor Fluorometer, Bio-Rad, Hercules, CA, USA) with the excitation wavelength of 360 nm and the emission wavelength of 460 nm.

4.4. Hyaluronic acid measurement

The concentration of hyaluronic acid in cell culture supernatants and in cell-associated extracts was measured by competitive ELISA with hyaluronan binding protein [28] as a capture molecule and biotinylated hyaluronic acid as a competitor [29]. Purified hyaluronic acid from human umbilical cord (Sigma) was used as a standard. Cell-associated hyaluronic acid was extracted with 4 M guanidine chloride, precipitated with 5% potassium acetate and then redissolved in phosphate-buffered saline.

4.5. Metabolic labeling with [^{35}S]sulfate

Production and accumulation of sulfated GAGs was measured using radioactive [^{35}S]sulfate. Briefly, cells were plated in 96-well plates and incubated for 24 h in serum-free DMEM containing [^{35}S]sulfate (20 μCi/ml) with or without hexosaminidase inhibitor. After incubation, the culture supernatants were harvested and sulfated GAGs were precipitated with dimethylene blue. Simultaneously, cell-associated sulfated GAGs were extracted with 4 M guanidine chloride and precipitated with 5% potassium acetate in ethyl alcohol. The measurement of [^{35}S]sulfate incorporation was performed separately for free and cell-associated fractions of the sulfated GAGs.

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References

[1] R.C. Lawrence, C.G. Helmick, F.C. Arnett, R.A. Deyo, D.T. Felson, E.H. Giannini, S.P. Heyse, R. Hirsch, M.C. Hochberg, G.G. Hunder, M.H. Liang, S.R. Pillemer, V.D. Steen, F. Wolfe, Estimates of the prevalence of arthritis and selected musculoskeletal disorders in the United States, Arthritis Rheum. 41 (1998) 778–799.

[2] S.E. Gabriel, C.S. Crowson, M.E. Campion, W.M. O'Fallon, Direct medical costs unique to people with arthritis, J. Rheumatol. 24 (1997) 719–725.

[3] L.M. March, C.J. Bachmeier, Economics of osteoarthritis: a global perspective, Baillieres Clin. Rheumatol. 11 (1997) 817–834.

[4] R.D. Altman, C.J. Lozada, Practice guidelines in the management of osteoarthritis, Osteoarthritis Cartilage 6 (Suppl. A) (1998) 22–24.

[5] M.C. Hochberg, R.D. Altman, K.D. Brandt, B.M. Clark, P.A. Dieppe, M.R. Griffin, R.W. Moskowitz, T.J. Schnitzer, Guidelines for the medical management of osteoarthritis. Part I. Osteoarthritis of the hip, Arthritis Rheum. 38 (1995) 1535–1540.

[6] M.C. Hochberg, R.D. Altman, K.D. Brandt, B.M. Clark, P.A. Dieppe, M.R. Griffin, R.W. Moskowitz, T.J. Schnitzer, Guidelines for the medical management of osteoarthritis. Part II. Osteoarthritis of the knee, Arthritis Rheum. 38 (1995) 1541–1546.

[7] S. Inerot, D. Heinegard, L. Audell, S.-E. Olsson, Articular cartilage proteoglycan in aging and osteoarthritis, Biochem. J. 169 (1978) 143–156.

[8] H.J. Mankin, L. Lippiello, Biochemical and metabolic abnormalities in articular cartilage from osteoarthritic human hips, J. Bone Joint Surg. (Am.) 52 (1970) 424–434.

[9] H.J. Mankin, L. Lippiello, The glycosaminoglycans of normal and arthritic cartilage, J. Clin. Invest. 50 (1971) 1712–1719.

[10] A.H. Plaas, F.R. Nelson, S. Wong-Palms, L.A. West, Glycosaminoglycan sulfation in human osteoarthritis. Disease-related alterations at the non-reducing termini of chondroitin and dermatan sulfate, J. Biol. Chem. 273 (1998) 12642–12649.

[11] D. Scott, P.J. Coleman, R.M. Mason, J.R. Levick, Glycosaminoglycan depletion greatly raises the hydraulic permeability of rabbit joint synovial lining, Exp. Physiol. 82 (1997) 603–606.

[12] R.C. Billinghurst, L. Dahlberg, M. Ionescu, A. Reiner, R. Bourne, C. Rorabeck, P. Mitchell, J. Hambor, O. Diekmann, H. Tschesche, J. Chen, H. Van Wart, A.R. Poole, Enhanced cleavage of type II collagen by collagenases in osteoarthritic articular cartilage, J. Clin. Invest. 99 (1997) 1534–1545.

[13] T. Kikuchi, T. Sakuta, T. Yamaguchi, Intra-articular injection of collagenase induces experimental osteoarthritis in mature rabbits, Osteoarthritis Cartilage 6 (1998) 177–186.

[14] B. Beekman, B. van El, J.W. Drijfhout, H.K. Ronday, J.M. TeKoppele, Highly increased levels of active stromelysin in rheumatoid synovial fluid determined by a selective fluorogenic assay, FEBS Lett. 418 (1997) 305–309.

[15] A.J. Freemont, V. Hampson, R. Tilman, P. Goupille, Y. Taiwo, J.A. Hoyland, Gene expression of matrix metalloproteinases 1, 3, and 9 by chondrocytes in osteoarthritic human knee articular cartilage is zone and grade specific, Ann. Rheum. Dis. 56 (1997) 542–549.

[16] E.C. Arner, C.E. Hughes, C.P. Decicco, B. Caterson, M.D. Tortorella, Cytokine-induced cartilage proteoglycan degradation is mediated by aggrecanase, Osteoarthritis Cartilage 6 (1998) 214–228.

[17] M.W. Lark, E.K. Bayne, J. Flanagan, C.F. Harper, L.A. Hoerrner, N.I. Hutchinson, I.I. Singer, S.A. Donatelli, J.R. Weidner, H.R. Williams, R.A. Mumford, L.S. Lohmander, Aggrecan degradation in human cartilage. Evidence for both matrix metalloproteinase and aggrecanase activity in normal, osteoarthritic, and rheumatoid joints, J. Clin. Invest. 100 (1997) 93–106.

[18] S. Tanaka, C. Hamanishi, H. Kikuchi, K. Fukuda, Factors related to degradation of articular cartilage in osteoarthritis: A review, Semin. Arthritis Rheum. 27 (1998) 392–399.

[19] G.M. Keyszer, A.H. Heer, J. Kriegsmann, T. Geiler, A. Trabandt, M. Keysser, R.E. Gay, S. Gay, Comparative analysis of cathepsin L, cathepsin D, and collagenase messenger RNA expression in synovial tissues of patients with rheumatoid arthritis and osteoarthritis, by in situ hybridization, Arthritis Rheum. 38 (1995) 976–984.

[20] R. Lemaire, G. Huet, F. Zerimech, G. Grard, C. Fontaine, B. Duquesnoy, R.M. Flipo, Selective induction of the secretion of cathepsins B and L by cytokines in synovial fibroblast-like cells, Br. J. Rheumatol. 36 (1997) 735–743.

[21] Y. Iwata, J.S. Mort, H. Tateishi, E.R. Lee, Macrophage cathepsin L, a factor in the erosion of subchondral bone in rheumatoid arthritis, Arthritis Rheum. 40 (1997) 499–509.

[22] B.G. Winchester, Lysosomal metabolism of glycoconjugates, Subcell. Biochem. 27 (1996) 191–238.

[23] H. Kresse, J. Glossl, Glycosaminoglycan degradation, Adv. Enzymol. Relat. Areas Mol. Biol. 60 (1987) 217–311.

[24] A.R. Shikhman, D.C. Brinson, M. Lotz, Profile of glycosaminoglycan-degrading glycosidases and glycoside sulfatases secreted by human articular chondrocytes in homeostasis and inflammation, Arthritis Rheum. 43 (2000) 1307–1314.

[25] C.S. Rye, S.G. Withers, Glycosidase mechanisms, Curr. Opin. Chem. Biol. 4 (2000) 573–580.

[26] T. Kajimoto, K.K.-C. Liu, R.L. Pederson, Z. Zhong, Y. Ichikawa, J.A.Jr. Porco, C-H. Wong, Enzyme-catalyzed aldol condensation for asymmetric synthesis of azasugars; synthesis, evaluation and modeling of glycosidase inhibitors, J. Am. Chem. Soc. 114 (1991) 6187–6196.

[27] G. Legler, Glycosidase inhibition by basic sugar analogs and the transition state of enzymatic glycoside hydrolysis, in: A.E. Stütz (Ed.), Iminosugars as Glycosidase Inhibitors: Nojirimycin and Beyond, Wiley-VCH, Weinheim, 1999, pp. 49–56.

[28] Y. Takaoka, T. Kajimoto, C.-H. Wong, Inhibition of *N*-acetylglucosaminyl-transfer enzymes: Chemical-enzymatic synthesis of new five-membered acetamido azasugars, J. Org. Chem. 58 (1993) 4809–4812.

[29] M. Takebayashi, S. Hiraruma, Y. Kanie, T. Kajimoto, O. Kanie, C.-H. Wong, A versatile synthetic strategy for the preparation and discovery of new iminocyclitols as inhibitors of glycosidases, J. Org. Chem. 64 (1999) 5280–5291.

[30] S. Knapp, D. Vocadlo, Z. Gao, B. Kirk, J. Lou, S.G. Withers, NAG-thiazoline, an *N*-acetyl-β-hexosaminidase inhibitor that implicates acetamido participation, J. Am. Chem. Soc. 118 (1996) 6804–6805.

[31] C.G. Wermuth, Designing prodrugs and bioprecusors, in: G. Jolles, K.R.H. Wooldridge (Eds.), Drug Design: Fact or Fantasy? Academic Press, London, 1984, pp. 47–72.
[32] C.R. Parish, C. Freeman, K.J. Brown, D.J. Francis, W.B. Cowden, Identification of sulfated oligosaccharide-based inhibitors of tumor growth and metastasis using novel in vitro assays for angiogenesis and heparanase activity, Cancer Res. 59 (1999) 3433–3441.
[33] M. Cantz, H. Kresse, Sandhoff disease: Defective glycosaminoglycan catabolism in cultured fibroblasts and its correction by β-*N*-acetylhexosaminidase, Eur. J. Biochem. 47 (1974) 581.
[34] K. Suzuki, K. Sango, R.L. Prola, C. Langaman, Mice deficient in all forms of lysosomal β-hexosaminidase show mucopolysaccharide-like pathology, J. Neuropathol. Exp. Neurol. 56 (1997) 693.
[35] H. Maeda, H. Fujita, Y. Sakura, K. Miyazaki, M. Goto, A competitive enzyme-linked immunosorbent assay-like method for measurement of urinary hyaluronan, Biosci. Biotechnol. Biochem. 63 (1999) 892–895.
[36] B. Yang, B.L. Yang, P.F. Goetnick, Biotinylated hyaluronic acid as a probe for identifying hyaluronic acid-binding proteins, Anal. Biochem. 228 (1995) 299–306.
[37] M. Sinnott, Catalytic mechanism of enzymic glycosyl transfer, Chem. Rev. 90 (1990) 1171–1202.