BMB Reports - Manuscript Submission

Manuscript Draft

Manuscript Number: BMB-21-108

Title: Avenanthrimide C as a novel candidate of treatment to alleviate osteoarthritic pathogenesis

Article Type: Article

Keywords: Osteoarthritis; Avenanthrimide C; matrix-metalloproteinases; p38 kinase; c-Jun N-terminal kinase (JNK)

Corresponding Author: Je-Hwang Ryu

Authors: Thanh-Tam Tran[1], Won-Hyun Song[1], Gyuseok Lee[1], Hyung Seok Kim[2], Daeho Park[3], Yun Hyun Huh[3], Je-Hwang Ryu[1,1,*]

Institution: [1]School of Dentistry, Chonnam National University,
[2]Department of Forensic Science, Chonnam National University Medical School,
[3]School of Life Sciences, Gwangju Institute of Science and Technology,

Avenanthramide C as a novel candidate to alleviate osteoarthritic pathogenesis

Thanh-Tam Tran[1], Won-Hyun Song[2], Gyuseok Lee[2], Hyung Seok Kim[3], Daeho Park[4], Yun Hyun Huh[4] and Je-Hwang Ryu[1,2*]

[1]Department of Pharmacology and Dental Therapeutics, School of Dentistry, Chonnam National University, Gwangju 61186, Republic of Korea

[2]Hard-tissue Biointerface Research Center, School of Dentistry, Chonnam National University, Gwangju 61186, Republic of Korea

[3]Department of Forensic Science, Chonnam National University Medical School, Jeonnam-do 58128, Republic of Korea

[4]School of Life Sciences, Gwangju Institute of Science and Technology, Gwangju 61005, Republic of Korea

[*] **Corresponding Authors**:

Je-Hwang Ryu, PhD, Hard-tissue Biointerface Research Center, Department of Pharmacology and Dental Therapeutics, School of Dentistry, Chonnam National University, Gwangju 61186, Republic of Korea, Tel: +82-62-530-4863; Fax: +82-62-530-2543; E-mail: jesryu@jnu.ac.kr

Running title: Avn-C for OA treatment

ABSTRACT

Osteoarthritis (OA) is a degenerative disorder that can result in the loss of articular cartilage. No effective treatment against OA is currently available. Thus, interest in natural health products to relieve OA symptoms is increasing. However, their qualities such as efficacy, toxicity, and mechanism are poorly understood. In this study, we determined the efficacy of avenanthramide (Avn)-C extracted from oats as a promising candidate to prevent OA progression and its mechanism of action to prevent the expression of matrix-metalloproteinases (MMPs) in OA pathogenesis. Interleukin-1 beta (IL-1β), a proinflammatory cytokine as a main causing factor of cartilage destruction, was used to induce OA-like condition of chondrocytes *in vitro*. Avn-C restrained IL-1β-mediated expression and activity of MMPs, such as MMP-3, -12, and -13 in mouse articular chondrocytes. Moreover, Avn-C alleviated cartilage destruction in experimental OA mouse model induced by destabilization of the medial meniscus (DMM) surgery. However, Avn-C did not affect the expression of inflammatory mediators (*Ptgs2* and *Nos*) or anabolic factors (*Col2a1*, *Aggrecan*, and *Sox9*), although expression levels of these genes were upregulated or downregulated by IL-1β, respectively. The inhibition of MMP expression by Avn-C in articular chondrocytes was mediated by p38 kinase and c-Jun N-terminal kinase (JNK) signaling, but not by ERK or NF-κB. Interestingly, Avn-C added with SB203580 and SP600125 as specific inhibitors of p38 kinase and JNK, respectively, enhanced its inhibitory effect on the expression of MMPs in IL-1β treated chondrocytes. Taken together, these results suggest that Avn-C is an effective candidate to prevent OA progression and a natural health product to relieve OA pathogenesis.

Key words: Osteoarthritis (OA), Avenanthramide C (Avn-C), matrix-metalloproteinases (MMPs), p38 kinase, and c-Jun N-terminal kinase (JNK)

INTRODUCTION

Osteoarthritis (OA) is the most prevalent degenerative arthritis. It is characterized by a progressive destruction of articular cartilage accompanying phenotypes of whole-joint disease, such as cartilage destruction, synovitis, osteophyte formation, and subchondral bone sclerosis (1). In the cartilage, chondrocytes are unique resident cells that can produce cartilage-specific extracellular matrix (ECM) components as well as various catabolic and anabolic factors. OA pathogenesis is mainly caused by an imbalance between anabolic and catabolic factors due to dedifferentiation and apoptosis of chondrocytes (2).

Various biochemical signals in chondrocytes can lead to the imbalance by modulating proinflammatory cytokine production, the cessation of ECM synthesis, and the irreversible degradation of the ECM by the action of matrix-metalloproteinases (MMPs) (3, 4). Aging and mechanical stresses are potential OA-causing factors that can promote the expression of pro-inflammatory cytokines as well as pro-inflammatory mediators such as cyclooxygenase-2 (COX-2) encoded by *Ptgs2* and inducible nitric oxide synthase (iNOS) encoded by *Nos2* in chondrocytes (5). Interleukin-1 beta (IL-1β), IL-6, and tumor necrosis factor alpha (TNF-α) are well-known proinflammatory cytokines causing OA. They are responsible for the production of matrix-degrading enzymes such as MMPs and ADAMTS (aggrecanases). Previous studies have shown that hypoxia-inducible factor (HIF)-2α encoded by *Epas1* (2) and zinc importer ZIP8 encoded by *Slc39a8* (6) play crucial roles in OA pathogenesis by upregulating MMPs (such as MMP-3, MMP-12, and MMP-13) and ADAMTS.

Numerous signaling pathways are involved in inflammatory responses and ECM degradation in OA pathogenesis. It has been reported that phosphorylated levels of mitogen-activated protein (MAP) kinases, including extracellular signal-regulated kinase (ERK), p38 kinase, and c-Jun N-terminal kinase (JNK), are upregulated in osteoarthritic cartilage (7). In fact, MAP kinases play a crucial role in OA pathogenesis by promoting cytokine-mediated MMP expression. For instance, IL-1β can stimulate the induction of MMP-3 and MMP-13 via activation of p38 kinase and JNK in articular chondrocytes (7). ERK activity is predominantly required for the expression of MMP-1 and MMP-3 in IL-1β-stimulated SW1353 human

chondrosarcoma cells (8). It has been reported that IL-1β expression in osteoarthritic cartilage and synovial fluids is mediated by nuclear factor-κB (NF-κB) (9). NF-κB dimers are sequestered by an inhibitor of κB (IκB) in the cytoplasm in an inactive form. Signal-induced degradation of IκB initiates the activation of NF-κB, leading to its translocation into the nucleus and up-regulation of inflammation-related genes, including NOS2 and COX-2 (9, 10).

Avenanthramides (Avns) are unique polyphenolic compounds exclusively found in oats (11). It has been scientifically verified that oats (*Avena sativa* L.) have health-promoting effects including cholesterol-lowering potential of β-glucan (12). Oats also have anti-oxidant effects due to the presence of phenolic compounds Avns (13). Avns have been isolated in three major isoforms: Avn-A, -B, and -C. They have shown essential functions in several biological activities, including anti-oxidant, anti-proliferative, anti-histamine, and anti-inflammatory functions (14). Among them, previous studies have largely focused on the high anti-oxidant effects of Avn-C to demonstrate the potential of using this natural health product as an anti-cancer drug (15). Avn-C also has anti-inflammatory effects by blocking the expression of pro-inflammatory cytokines such as IL-6 and TNF-α through decreased NF-κB activity in endothelial cells (16) and skeletal muscle cells (17). In addition, Avn-C can prevent lung inflammation under hypoxia by inhibiting SIRT1-mediated COX-2 expression in lung cancer cell lines (18).

In the current study, we determined the potential of using Avn-C to prevent OA pathogenesis. Avn-C markedly blocked cartilage destruction by restraining the expression and activities of MMPs in articular chondrocytes. The inhibitory effects of Avn-C on cartilage destruction were mediated by p38 kinase and JNK signaling. Results of this study suggest possible functions and underlying molecular mechanisms of Avn-C in alleviating OA pathogenesis.

RESULTS

Avn-C reduces catabolic gene expression during IL-1β-mediated *in vitro* OA-like pathogenesis

To determine effects of Avn-C on OA pathogenesis, we induced an *in vitro* arthritis condition in mouse articular chondrocytes by treating cells with IL-1β, a main factor causing arthritis. Expression levels of well-known catabolic and pro-inflammatory factors of OA pathogenesis as well as anabolic factors were examined by reverse transcription-polymerase chain reaction **(**RT-PCR). Avn-C did not recover IL-1β-mediated reduction of anabolic factors such as *Aggrecan* and *Col2a1,* main constituents of cartilage ECM, or *Sox9,* a major transcription factor of *Col2a1* (Fig. 1A). Moreover, Avn-C treatment did not inhibit IL-1β-mediated induction of pro-inflammatory factors *Il-6*, *Ptgs2*, or *Nos2* (Fig. 1B). On the other hand, transcript levels of *Mmp-3, -9, -12, -13,* and *Adamts-4* were dramatically increased by IL-1β, showing a well-induced OA pathologic condition. They were significantly reduced by Avn-C in a dose-dependent manner in articular chondrocytes (Fig. 1C). In consistent with a previous study (2), IL-1β did not induce the expression of *Mmp-14* or *Adamts-5* (Fig. 1C). We focused on inhibitory effects of Avn-C on the expression of *Mmp-3*, *-12*, and *-13* as these genes were inhibited the most effectively by Avn-C. Both transcriptional levels and protein levels of MMP-3, -12, and -13 were inhibited by Avn-C in IL-1β-treated mouse articular chondrocytes as determined by quantitative real-time (qRT)-PCR (Fig. 2A) and Western blotting (Fig. 2B), respectively. Matrix-degrading activities of secreted MMP-3, -12, and -13 were also significantly reduced by Avn-C treatment (Fig. 2C). These data show that Avn-C can inhibit IL-1β-induced *in vitro* OA pathogenesis by restraining the expression of catabolic factors, especially MMP-3, -12, and -13.

Avn-C blocks destabilization of the medial meniscus (DMM)-induced cartilage destruction

We attempted to determine *in vivo* effects of Avn-C in the surgical model of DMM, a type of experimental OA model in mice. After 200 mM Avn-C or dimethyl sulfoxide (DMSO) as a control was administered by intra-articular injection every week for six weeks to DMM or sham (control) mice, cartilage destruction was histologically analyzed by Safranin-O staining (Fig. 3A) and scored according to the Osteoarthritis

Research Society International (OARSI) system (Fig. 3B). Two control groups of sham mice injected with DMSO or Avn-C showed relatively intact cartilages, whereas DMM mice with Avn-C treatment presented less cartilage destruction than DMSO-injected DMM mice which exhibited severe progression of cartilage destruction (Fig. 3A). The OARSI grade of Avn-C-injected DMM mice was decreased about 55% compared to that of DMSO-injected DMM mice (Fig. 3B). It has been reported that the more severe the cartilage degeneration, the thicker the subchondral bone plate (SBP) (19). SBP thickness of articular cartilage was increased in DMM mice. However, it was slightly inhibited in Avn-C injected DMM mice compared to DMSO-injected DMM mice (Fig. 3B). In addition, osteophyte formation seemed to be slightly prevented by Avn-C in DMM-induced OA mice model, although such prevention was not significant (Fig. 3B). These data indicate that Avn-C has a protective effect on DMM-induced cartilage destruction.

Avn-C reduces the expression of MMPs in experimental mouse OA cartilages

Next, to examine the *in vivo* expression of MMPs, we performed immunohistochemical analysis using cartilage tissue sections from experimental OA mouse models which we analyzed in Fig. 3. Expression levels of MMP-3 (Fig. 3C), MMP-12 (Fig. 3D), and MMP-13 (Fig. 3E) were definitely reduced in Avn-C treated DMM cartilages than in sham and DMSO-treated DMM mice, consistent with *in vitro* results from IL-1β-mediated arthritic chondrocytes shown in Fig. 1C. Altogether, these data indicate that Avn-C has a protective effect on DMM-induced cartilage destruction by preventing the expression of matrix-degrading enzymes such as MMP-3, -12, and -13.

Avn-C inhibits IL-1β signaling by blocking p38 MAP kinase and JNK signaling pathways

To determine the molecular mechanisms involved in the protective effect of Avn-C on the pathogenesis of arthritis, we examined signaling pathways involved in IL-1β mediated MMP expression. It has been reported that MAP kinases including ERK, p38 kinase, and JNK are activated by IL-1β and the activation of these signaling pathways is involved in the pathogenesis of arthritis, including degradation of cartilage ECM, inflammation, and apoptosis of chondrocytes (20). The maximal activation of all three MAP kinases

in 15 ~ 30 min after IL-1β treatment was verified in current experiments by determining phosphorylation on Thr202/Tyr204 of ERK, Thr180/Tyr182 of p38 kinase, and Thr183/Tyr185 of JNK (Fig. 4A). Pre-treatment with Avn-C significantly blocked p38 kinase and JNK signaling induced by IL-1β, but not ERK signaling (Fig. 4B). NF-κB is a major transcription factor that controls gene expression involved in the production of cytokines such as IL-6 and TNF-α (21). IL-1β also activated NF-κB signaling in mouse chondrocytes as determined by the degradation of IκB in 15 ~ 30 min after IL-1β stimulation (Fig. 4A). However, degradation of IκB was sustained by the addition of Avn-C in IL-1β stimulated articular chondrocytes, indicating that Avn-C did not modulate IL-1β-mediated NF-κB signaling (Fig. 4B). Next, we examined whether IL-1β-induced p38 kinase and JNK signaling regulated the expression of MMP-3, -12, and -13. Treatment with SB203580, a specific inhibitor of p38 kinase, blocked IL-1β-induced expression of *Mmp-3* and *-13*, but not *Mmp-12* (Fig. 4C). SP600125, a specific inhibitor of JNK, more effectively prevented the expression of all three *Mmps* (Fig. 4C). In addition, we tested synergistic effects of Avn-C on the inhibition of each signaling pathway with specific inhibitors. Co-treatment with Avn-C and SB203580 or SP600125 more dramatically inhibited the expression of all three *Mmps* tested in this study (Fig. 4D). Interestingly, *Mmp-12* expression was also inhibited by co-treatment with Avn-C and SB200125, although IL-1β -induced *Mmp-12* upregulation was not blocked by only p38 kinase inhibition (Figs. 4C and D). These data support that the protective effect of Avn-C on OA pathogenesis is due to inhibition of p38 kinase and JNK signaling pathways causing expression of MMPs.

DISCUSSION

OA is typically characterized by progressive degradation and loss of articular cartilage resulting from the action of mechanical and biochemical factors (1, 22). Although various therapeutic approaches have been attempted to prevent OA progression, no effective treatment is currently available. The use of natural health product by OA patients to alleviate symptoms is rising globally. In this study, we evaluated the efficacy of Avn-C extracted from oats as a promising candidate to prevent OA progression. Here, we found that Avn-C prevented cartilage destruction in experimental OA mouse model induced by DMM surgery and inhibited the expression of MMPs, including MMP-3, -12, and -13, by regulating p38 kinase and JNK signaling.

Previous papers have reported that Avn-C exhibits anti-inflammatory effects by inhibiting the expression of pro-inflammatory cytokines via NF-kB signaling in skeletal muscle cells (17) and anti-oxidant effects on the inhibition of colon cancer cell growth (15, 18). However, in our current study, Avn-C had no effect on the expression of inflammatory mediators such as *Ptgs2* or *Nos2* during OA pathogenesis (Fig. 1B). Moreover, Avn-C did not affect NF-kB activation stimulated by IL-1β (Fig. 4B). However, Avn-C effectively inhibited *Mmps* and *Adamts4* expression in OA cartilage.

The proinflammatory cytokine IL-1β is one of the most critical catabolic factors in the development of OA. It can induce the release of inflammatory mediators and MMPs. Excessive amount of IL-1β is found in cartilage, synovial fluid, the synovial membrane, and the subchondral bone (23). Herein, IL-1β was used to develop an *in vitro* OA inflammatory microenvironment. Results of the present study confirmed that IL-1β dramatically stimulated the expression of catabolic factors and inflammatory mediators, such as *IL-6, Ptgs2, Nos2, Mmps,* and *Adamts* that play crucial roles in the degeneration of articular cartilage. Of these genes, expression levels of *Mmp-3, -9, -12, -13, and Adamts4* were significantly inhibited by Avn-C in a dose-dependent manner in IL-1β-mediated *in vitro* mouse OA-like pathogenic articular chondrocytes. *In vivo* results from mouse experimental OA model induced by DMM supported the inhibitory effect of Avn-C on OA progression by inhibiting the expression of MMPs such as MMP-3, -12, and -13.

OA is considered as a whole joint disease with pathological changes, including synovial inflammation,

subchondral bone sclerosis, osteophyte formation, and cartilage destruction. Avn-C prevented pathological changes of the whole joint. We scored the grade of cartilage degeneration according to the OARSI system. DMM induced OA cartilage showed an average OARSI grade of 2.4 ($p < 0.005$), denoting increased subchondral sclerosis and osteophyte formation (Fig. 3B). However, Avn-C injection decreased the severity of OA phenotypes induced by DMM, which showed an OARSI grade 1.1 with thinner subchondral bone plate than in DMM control mice (Fig. 3B).

A previous study has demonstrated that HIF-2α as a critical transcription factor plays an essential role in IL-1β-induced MMP expression during OA pathogenesis (2). Our current results were consistent with results of the previous study showing induction of MMPs except MMP-2, -14, and -15 by IL-1β treatment or HIF-2α overexpression (2). The 5'-flanking regions of *Mmp-2, -14,* and *-15* are distinct from those of other MMPs, although these genes have HIF-2α binding sites on their promoter regions. This indicates that *Mmp-2, -14,* and *-15* are regulated by other factors rather than HIF-2α (24, 25). However, in the current study, inhibitory effect of Avn-C on IL-1β-induced MMPs expression did not seem to be mediated by HIF-2α because other catabolic factors such as *Ptgs2* and *Nos2* were not regulated by Avn-C (Fig. 1B). Anabolic factors such as *Col2a1*, *Aggrecan,* and *Sox9* were not affected by Avn-C either (Fig. 1A), although these anabolic genes were suppressed by IL-1β. These results provide a possibility that Avn-C can inhibit certain downstream signaling pathways among multiple signaling pathways activated by IL-1β. Thus, we considered various signaling pathways involved in IL-1β induced MMP production. It is well-known that proinflammatory cytokines such as IL-1β, TNF-α, and IL-6 are responsible for the production of MMPs by upregulating NF-kB and MAP kinases (ERK, p38 kinase, and JNK) (23, 26, 27). Of them, p38 kinase and JNK signaling, but not ERK or NF-kB, were responsible for the inhibitory effect of Avn-C on IL-1β induced MMPs production in mouse articular chondrocytes. In addition, Avn-C enhanced effects of specific inhibitors of p38 kinase (SB203580) and JNK (SP600125). These results support that Avn-C can be used to relieve OA progression as a natural health product. Our current study demonstrates that Avn-C has potential to alleviate OA progression.

MATERIALS AND METHODS

Experimental OA model by DMM surgery in mice

Cartilage destruction in mice was induced by DMM surgery. Sham-operated mice as controls were also prepared. At two weeks after DMM operation, mice were injected with 10 μl of 200 μM Avn-C or DMSO every week for six weeks by intra-articular injection. Knee joints were processed for histological analysis at 8 weeks after DMM surgery. All experiments were approved by Chonnam National University Animal Care and Use Committee.

Histological analysis

Mouse joint tissues were decalcified with 0.5 M EDTA (pH 8.0) for two weeks, embedded in paraffin, and sectioned at 5 μm in thickness. Cartilage destruction was evaluated by safranin O staining and was scored according to the OARSI grade system. Lateral sections were placed on slides for immunohistochemical staining and incubated in 3% H_2O_2 for 10 min to block endogenous peroxidase activity. Sections were then incubated with 0.1% trypsin for 30 min at 37°C to retrieve antigen. After blocking with 1% BSA for 30 min, slides were incubated with rabbit anti-MMP-3 (Abcam, Cambridge, UK), rabbit anti-MMP-12 (Epitomics, California, USA), and rabbit anti-MMP-13 (Abcam) followed by staining using the EnVision+System-HRP (Dako, Denmark) and AEC+substrate (Dako). Sections were counter-stained with Hematoxylin (Dako).

Primary culture of articular chondrocytes

Primary chondrocytes were isolated from femoral condyles and tibial plateaus of postnatal day 5 mice. The articular cartilage was pre-incubated for 2 h at 37°C with 0.2% trypsin and 0.2% type II collagenase and further digested with 0.2% type II collagenase for an additional 90 min. Chondrocytes were maintained as a monolayer in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and appropriate antibiotics. On culture day 3, cells were treated as indicated for each experiment. Chondrocytes were treated with indicated concentration of IL-1β (GeneScript, Piscataway, NJ, USA) for 24 h with or

without Avn-C (SI-033-053-1, Sigma–Aldrich, St Louis, MO, USA) dissolved in DMSO, PD98059 (ERK inhibitor), SB203580 (p38 inhibitor), or SP6000125 (JNK inhibitor) (Santa Cruz, CA, USA).

RT-PCR and qRT-PCR

Total RNA was isolated from cultured articular chondrocytes using TRI reagent (TR118, Molecular Research Center Inc., Cincinnati, OH, USA). The RNA was reverse transcribed to cDNA. The resulting cDNA was amplified by PCR and quantified by qRT-PCR. qRT-PCR was performed using SYBR premix Ex Taq (RR420, TaKaRa, Japan). All qRT-PCR reactions were performed in duplicate and the threshold cycle value of each gene was normalized against that of *glyceraldehyde-3 phosphate dehydrogenase (Gapdh)* as an internal control. Primers and experimental condition are summarized in Table 1.

Western blot analysis

Cultured mouse articular chondrocytes were washed with cold PBS and lysed in lysis buffer containing 50 mM Tris-HCl, pH8.0, 150 mM NaCl, 5 mM NaF, 1% NP-40, 0.2% SDS, 0.5% deoxycholate, a protease inhibitor cocktail, and a phosphatase inhibitor cocktail (Roche, Basel, Switzerland). Cell lysates were loaded and separated by SDS-PAGE. Proteins were transferred to nitrocellulose membranes. After blocking with 5% skim milk at room temperature for 1 h, membranes were incubated with primary antibodies overnight at 4℃ with indicated antibodies. The following antibodies were used: rabbit anti-MMP-3 (Abcam), rabbit anti-MMP-12 (Epitomics), rabbit anti-MMP-13 (Abcam), mouse anti-ERK1 (BD Biosciences, NJ, USA), rabbit anti-phospho-Erk1/2 (Cell Signaling Technology, Danvers, MA, USA), rabbit anti-JNK (Cell Signaling Technology), mouse anti-phospho-JNK (Cell Signaling Technology), rabbit anti-p38 (Santa Cruz), mouse anti-phospho-p38 (Cell Signaling Technology), and rabbit anti-IκBα (Santa Cruz). Membranes were then incubated with horseradish peroxidase conjugated secondary antibodies, anti-mouse (Sigma–Aldrich) or anti-rabbit IgG (Sigma–Aldrich), and detected using an ECL solution (GE Healthcare, Pittsburgh, PA, USA). Images were detected using ImageSaver6 software via an

EZ-capture MG system (ATTO, Tokyo, Japan). Protein level was normalized against the level of β-actin (Sigma–Aldrich) as a loading control.

Enzyme-linked immunosorbent assay (ELISA)

To quantify activities of MMP-3, -12, and -13 in the culture medium, primary chondrocytes were cultured without serum. They were then treated with IL-1β and indicated dose of Avn-C. Supernatants were collected after 24 h and stored at -80˚C until ELISA. ELISA kits #71130, #71157 and #71156 for analyzing substrate degrading activities of MMP-3, -12, and -13, respectively, were purchased from Anaspec (CA, USA). ELISAs were performed according to the manufacturer's specifications using duplicate wells for each sample.

Statistical analysis

All experiments were repeated at least three times. Data obtained from qRT-PCR and enzymatic activity assays were first tested for conformation to a normal distribution using the Shapiro-Wilk test, followed by analysis with Student's t-test (pairwise comparisons) or analysis of variance with post-hoc tests (multiple comparisons) as appropriate. The *n*-value is the number of independent experiments or mice. Threshold for significance was set at the 0.05 level of probability ($p < 0.05$).

ACKNOWLEDGMENTS

This work was supported by grants (NRF-2019R1A5A2027521 and 2021R1A2C300572711) of the National Research Foundation of Korea (NRF) funded by the Korea government (MSIT), the Korea Healthcare Technology R&D Project of the Korea Health Industry Development Institute (HR14C0008), and Chonnam National University Hospital Biomedical Research Institute (BCRI18012).

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

REFERENCES

1. Loeser RF, Goldring SR, Scanzello CR, Goldring MB (2012) Osteoarthritis: a disease of the joint as an organ. Arthritis Rheum 64, 1697–1707
2. Yang S, Kim J, Ryu JH et al (2010) Hypoxia-inducible factor-2alpha is a catabolic regulator of osteoarthritic cartilage destruction. Nat Med 16, 687–693
3. Goldring MB, Goldring SR (2007) Osteoarthritis. J Cell Physiol 213, 626–634
4. Hashimoto M, Nakasa T, Hikata T, Asahara H (2008) Molecular network of cartilage homeostasis and osteoarthritis. Med Res Rev 28, 464–481
5. Roman-Blas JA, Jimenez SA (2006) NF-kappaB as a potential therapeutic target in osteoarthritis and rheumatoid arthritis. Osteoarthritis Cartilage 14, 839–848
6. Kim JH, Jeon J, Shin M et al (2014) Regulation of the catabolic cascade in osteoarthritis by the zinc-ZIP8-MTF1 axis. Cell 156, 730-43
7. Mengshol JA, Vincenti MP, Coon CI, Barchowsky A, Brinckerhoff CE (2000) Interleukin-1 induction of collagenase 3 (matrix metalloproteinase 13) gene expression in chondrocytes requires p38, c-Jun N-terminal kinase, and nuclear factor kappaB: differential regulation of collagenase 1 and collagenase 3. Arthritis Rheum 43, 801-811
8. Zeng Li, Xiaofeng Rong, Rong-Heng Li and X Wu (2017) Icariin inhibits MMP-1, MMP-3 and MMP-13 expression through MAPK pathways in IL-1β-stimulated SW1353 chondrosarcoma cells. Molecular medicine reports 15, 2853-2858
9. Chang SH, Mori D, Kobayashi H et al (2019) Excessive mechanical loading promotes osteoarthritis through the gremlin-1-NF-κB pathway. Nat Commun 10, 1442
10. Roman-Blas JA, Jimenez SA (2006) NF-kappaB as a potential therapeutic target in osteoarthritis and rheumatoid arthritis. Osteoarthritis Cartilage 14, 839-848
11. Collins FW (1989) Oat phenolics: avenanthramides, novel substituted N-cinnamoylanthranilate alkaloids from oat groats and hulls. Journal of Agricultural and Food Chemistry 37, 60–66

12. Othman RA, Moghadasian MH, Jones PJ (2011) Cholesterol-lowering effects of oat β-glucan. Nutr Rev 69, 299-309
13. Peterson, David & Hahn, Martha & Emmons, Cheryld (2002) Oat avenanthramides exhibit antioxidant activities in vitro. Food Chemistry 79, 473-478
14. Emmons CL, Peterson DM, Paul GL (1999) Antioxidant capacity of oat (Avena sativa L.) extracts. 2. In vitro antioxidant activity and contents of phenolic and tocol antioxidants. J Agric Food Chem 47, 4894–4898
15. Guo W, Nie L, Wu D et al (2010) Avenanthramides Inhibit Proliferation of Human Colon Cancer Cell Lines In Vitro. Nutrition and cancer 62, 1007-1016
16. Guo W, Wise ML, Collins FW, Meydani M (2008) Avenanthramides, polyphenols from oats, inhibit IL-1beta-induced NF-kappaB activation in endothelial cells. Free Radic Biol Med 44, 415–429
17. Kang C, Shin WS, Yeo D, Lim W, Zhang T, Ji LL (2018) Anti-inflammatory effect of avenanthramides via NF-κB pathways in C2C12 skeletal muscle cells. Free Radic Biol Med 117, 30–36
18. Lim W, Kang C (2020) Avenanthramide C suppresses hypoxia-induced cyclooxygenase-2 expression through sirtuin1 activation in non-small-cell lung cancer cells. Anim Cells Syst 24, 79-83
19. Nakasa T, Adachi N, Kato T, Ochi M (2014) Correlation between subchondral bone plate thickness and cartilage degeneration in osteoarthritis of the ankle. Foot Ankle Int 35, 1341-1349
20. Saklatvala J (2007) Inflammatory signaling in cartilage: MAPK and NF-kappaB pathways in chondrocytes and the use of inhibitors for research into pathogenesis and therapy of osteoarthritis. Curr Drug Targets 8, 305-313
21. Liu T, Zhang L, Joo D, Sun SC (2017) NF-κB signaling in inflammation. Signal Transduct Target Ther 2,17023

22. Guilak F (2011) Biomechanical factors in osteoarthritis. Best Pract Res Clin Rheumatol 25, 815-823
23. Kapoor M, Martel-Pelletier J, Lajeunesse D, Pelletier JP, Fahmi H (2011) Role of proinflammatory cytokines in the pathophysiology of osteoarthritis. Nat Rev Rheumatol 7, 33-42
24. Yun S, Dardik A, Haga M et al (2002) Transcription factor Sp1 phosphorylation induced by shear stress inhibits membrane type 1-matrix metalloproteinase expression in endothelium. J Biol Chem 277, 34808–34814
25. Pan MR, Hung WC (2002) Nonsteroidal anti-inflammatory drugs inhibit matrix metalloproteinase-2 via suppression of the ERK/Sp1-mediated transcription. J Biol Chem 277, 32775–32780
26. Pulai JI, Chen H, Im HJ et al (2005) NF-kappa B mediates the stimulation of cytokine and chemokine expression by human articular chondrocytes in response to fibronectin fragments. J Immunol 174, 5781-5788
27. Loeser RF, Erickson EA, Long DL (2008) Mitogen-activated protein kinases as therapeutic targets in osteoarthritis. Curr Opin Rheumatol 20, 581-586

FIGURE LEGENDS

Fig. 1. Avn-C reduces the expression of catabolic factors during IL-1β-mediated *in vitro* OA-like pathogenesis. Primary cultured mouse articular chondrocytes were treated with 2 ng/ml IL-1β for 24 h in the presence of the indicated concentration of Avn-C. Expression level of each gene was then analyzed by RT-PCR. (A) Anabolic factors of OA pathogenesis: *Aggrecan*, *Col2a1,* and *Sox9*. (B) Pro-inflammatory factors: *Il-6, Ptgs2, and Nos2*. (C) Catabolic factors: *Mmp-3, -9, -12, -13, -14, Adamts4,* and *Adamts5*.

Fig. 2. Avn-C inhibits the expression and activity of MMPs in IL-1β-treated chondrocytes. Cells were

cultured without serum and treated with 2 ng/ml IL-1β for 24 h in the presence of the indicated concentration of Avn-C. (A) Transcriptional levels of *Mmp-3, -12, and -13* were analyzed by qRT-PCR (n = 3). (B) Protein levels were examined by Western blot analysis (n = 3). (C) Cultured media were subjected to ELISA to measure activities of MMP-3, -12, and -13 (n = 4). Values are presented as means ± SEM (*, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.005$).

Fig. 3. Avn-C blocks DMM-induced cartilage destruction. Mice were subjected to sham operation or DMM surgery and injected with DMSO (vehicle) or Avn-C into knee joints (sham with DMSO; n = 7, DMM with DMSO; n = 7, sham with Avn-C; n = 11, and DMM with Avn-C; n = 11). (A) DMM-induced experimental OA was analyzed by safranin-O staining for the cartilage section. Scale bars, 100 μm. (B) OA parameters were scored, including OARSI grade for cartilage destruction, thickening of the subchondral bone plate (SBP), and osteophyte maturity. (C-E) Immunohistochemical staining for MMP-3 (C), MMP-12 (D), and MMP-13 (E) in cartilage tissues from sham- or DMM-operated mice with intra-articular injection of 10 μl of DMSO or 200 μM Avn-C. Scale bars, 25 μm. Values are presented as means ± SEM (*NS*, not significant; **, $p < 0.01$; and ***, $p < 0.005$).

Fig. 4. Avn-C inhibits IL-1β signaling by blocking p38 MAP kinase and JNK. (A) Primary cultured mouse articular chondrocytes were treated with 2 ng/ml IL-1β for the indicated time. Phosphorylation of ERK, p38 kinase, and JNK was determined by Western blot analysis. Degradation of IκBα was detected to determine the activation of NF-κB. (B) Cultured cells were treated with IL-1β for 15 min in the presence of Avn-C at indicated concentration and then subjected to Western blot analysis. (C) Chondrocytes were treated with IL-1β in the presence of indicated dose of p38 kinase inhibitor, SB203580, or JNK inhibitor, SP600125. *Mmp-3, -12,* and *-13* expression levels were analyzed by RT-PCR. (D) Cells were co-treated with IL-1β, Avn-C, and SB203580 or SP600125. Transcriptional levels of *Mmp-3, -12,* and *-13* were determined by RT-PCR.

Table 1. PCR primers and conditions

Gene	Strand	Sequences (5'-3')	Size (bp)	[a]AT (°C)	Origin
Mmp-2	[b]S	CCAACTACGATGATGAC	233	60	Mo
	[c]As	ACCAGTGTCAGTATCAG			
Mmp-3	S	CTGTGTGTGGTTGTGTGCTCATCCTAC	349	60	Mo
	As	GGCAAATCCGGTGTATAATTCACAATC			
Mmp-9	S	TGCACTGGGCTTAGATCATTCC	428	60	Mo
	As	CCGTCCTTGAAGAAATGCAGAG			
Mmp-12	S	CCCAGAGGTCAAGATGGATG	482	60	Mo
	As	GGCTCCATAGAGGGACTGAA			
Mmp-13	S	TGATGGACCTTCTGGTCTTCTGGC	449	60	Mo
	As	CATCCACATGGTTGGGAAGTTCTG			
Mmp-14	S	GTGCCCTAGGCCTACATCCG	580	60	Mo
	As	TTGGGTATCCATCCATCACT			
Mmp-15	S	GAGAGATGTTTGTGTTCAAGGG	260	60	Mo
	As	TGTGTCAATGCGGTCATAGGG			
Adamts4	S	ACTTCCTGGACAATGGTTATGGGC	307	62	Mo
	As	ATGAAGTCCTTGAGCTGGTCCACG			
Adamts5	S	GCCATTGTAATAACCCTGCACC	292	60	Mo
	As	ATGTCCACCAAAGGCCCAAA			
Aggrecan	S	GAAGACGACATCACCATCCAG	581	60	Mo
	As	TCAGTCCCATCCGTAACCTTTG			
Col2a1	A	CACACTGGTAAGTGGGGCAAGACCG	148	60	Mo
	As	GGATTGTGTTGTTTCAGGGTTCGGG			
Sox9	S	GCGCGTGCAGCACAAGAAGGACCACCCGGATT ACAAGA	380	62	Mo
	As	CGAAGGTCTCGATGTTGGAGATGACGTCGCTGC TCAGCC			
Il-6	S	GAAGCCATAGTCGTGCCTGT	351	60	Mo
	As	AGGACATGCATGAAGAGCCC			

Ptgs2	S	CCAAACCAGCAGACTCATACTCATAG	387	62	Mo
	As	CATCTCTCTGCTCTGGTCAATGGAG			
Nos2	S	TCACTGGGACAGCACAGAAT	366	62	Mo
	As	TGTGTCTGCAGATGTGCTGA			
Gapdh	S	TCACTGCCACCCAGAAGA	432	60	Mo
	As	TGTAGGCCATGAGGTCCA			

[a]AT,annealing temperature; [b]S,sense primer; [c]As,antisense primer

Figure 1

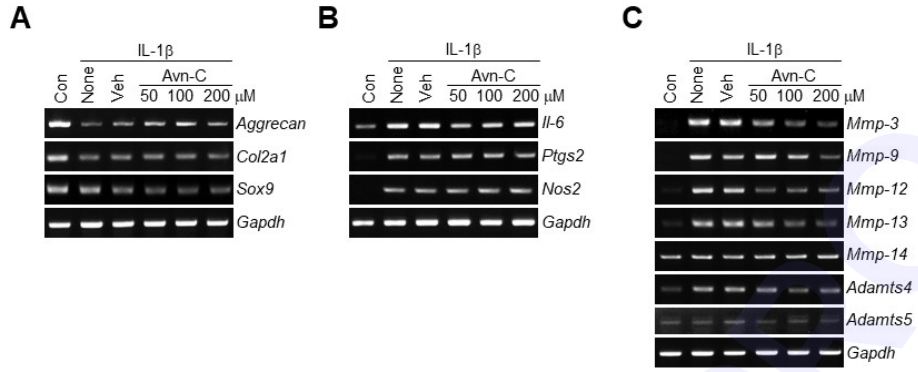

Fig. 1. Figure 1

Figure 2

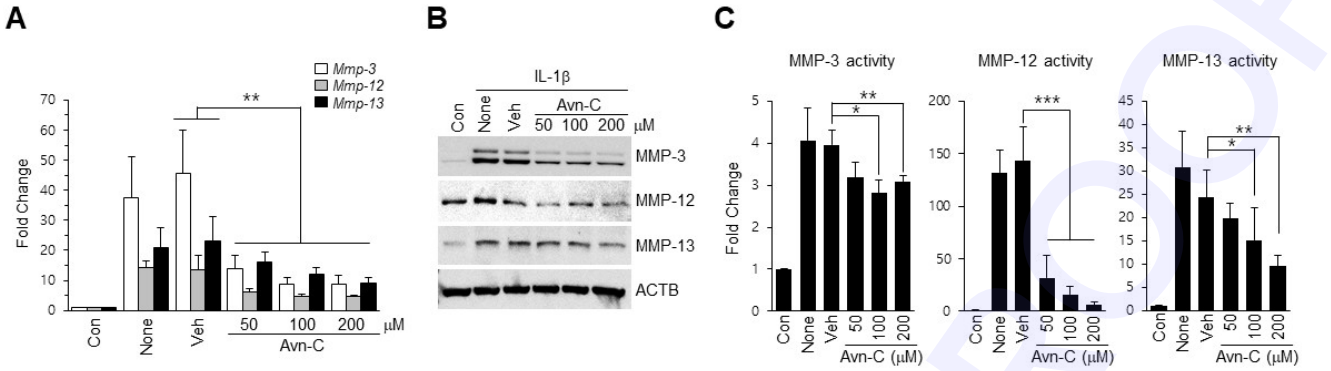

Fig. 2. Figure 2

Figure 3

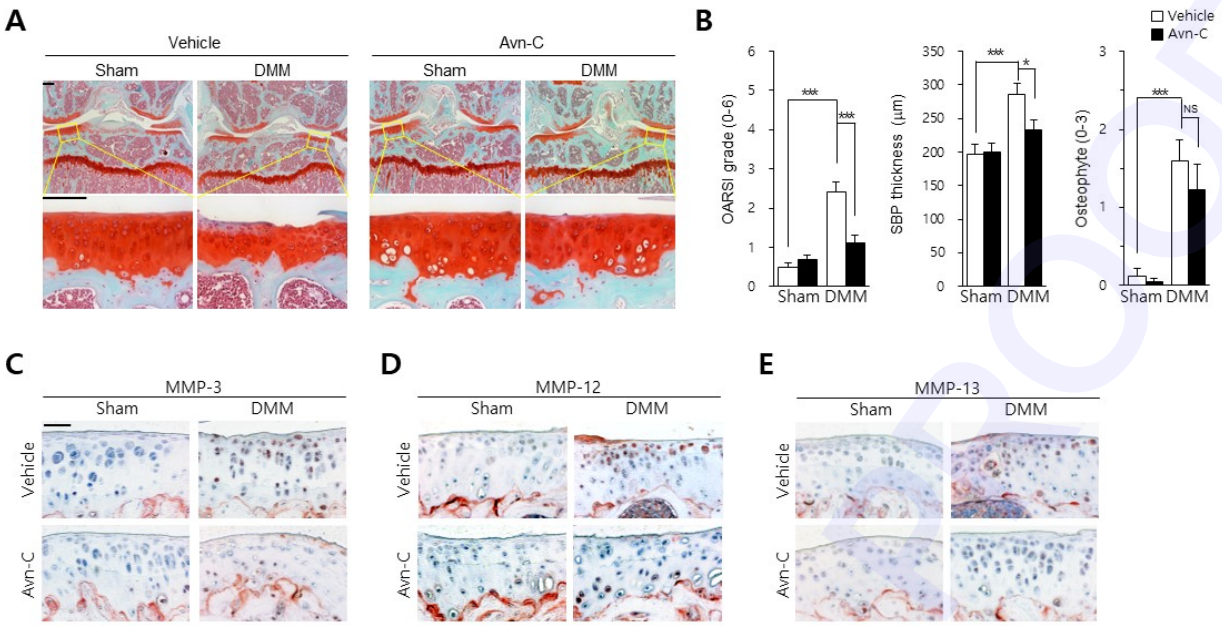

Fig. 3. Figure 3

Figure 4

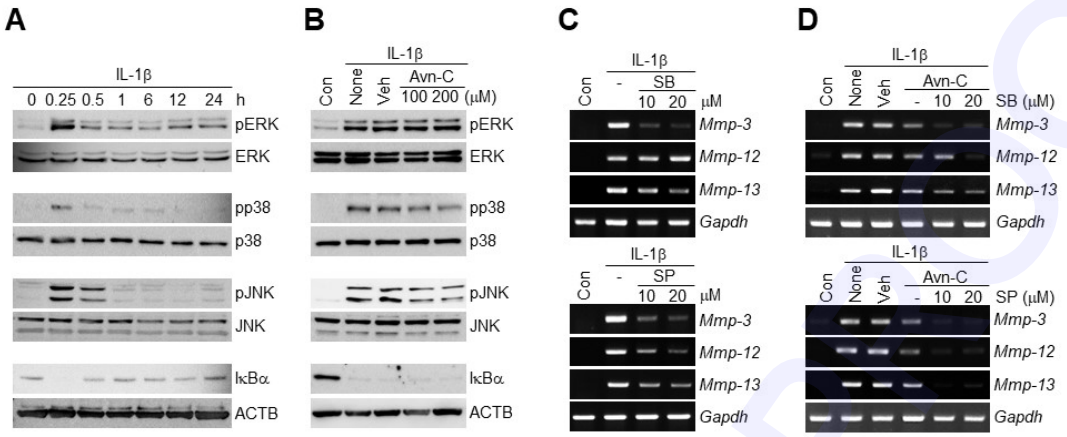

Fig. 4. Figure 4