J Clin Immunol (2011) 31:1131–1142
DOI 10.1007/s10875-011-9576-9

A Benzamide-Linked Small Molecule HS-Cf Inhibits TNF-α-Induced Interferon Regulatory Factor-1 in Porcine Chondrocytes: A Potential Disease-Modifying Drug for Osteoarthritis Therapeutics

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Received: 1 June 2011 / Accepted: 19 July 2011 / Published online: 20 August 2011

Abstract
Background Using tumor necrosis factor-alpha (TNF-α)-activated porcine chondrocytes as a screening tool, we aim to synthesize and identify small-molecule inhibitors preserving immunomodulatory effects as therapeutics for osteoarthritis (OA).
Methods Chondrocytes were isolated from pig joints. A minilibrary of 300 benzamide-linked small molecules was established. The levels of inducible nitric oxide synthase (iNOS) and nitric oxide (NO) were measured by Western blot and Griess reaction, respectively. Proteoglycan degradation in cartilage explants was determined by histochemistry analysis. The activation of transcription factors and protein kinases was determined by electrophoretic mobility shift assays or Western blots. Zymography and real-time reverse transcriptase-polymerase chain reaction were used to determine enzyme activity and expression of matrix metalloproteinases (MMPs) and aggrecanases, respectively.
Results Bioassay screening of benzamide-linked small molecules revealed that 2-hydroxy-*N*-[3-(trifluoromethyl)phenyl] benzamide (HS-Cf) was a potent inhibitor of NO production and iNOS expression in TNF-α-stimulated porcine chondrocytes. HS-Cf suppressed TNF-α-induced activity of MMP-13 and expressions of several aggrecanases and prevented TNF-α-mediated reduction of collagen II. Histochemistry analysis confirmed that HS-Cf could prevent TNF-α-induced degradation and release of proteoglycan/aggrecan in cartilage explants. Such effects by HS-Cf were likely through suppressing TNF-α-induced interferon regulatory factor-1 (IRF-1) but not nuclear factor-kappaB signaling. The significance of IRF-1 was further confirmed by short hairpin knockdown studies.
Conclusions In a minilibrary containing 300 small molecules, we identified a benzamide-linked small molecule, HS-Cf, that through down-regulating TNF-α-induced IRF-1 activity suppressed chondrocyte activation and prevented cartilage

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destruction. HS-Cf might be a potential disease-modifying drug for OA therapeutics.

Keywords Osteoarthritis · small molecule · chondrocytes · tumor necrosis factor-alpha · inflammation · interferon regulatory factor-1

Abbreviations

OA	Osteoarthritis
TNF-α	Tumor necrosis factor-alpha
HS-Cf	2-Hydroxy-*N*-[3-(trifluoromethyl)phenyl] benzamide
iNOS	Nitric oxide synthase
NO	Nitric oxide
MMPs	Matrix metalloproteinases
ADAMTS	Aggrecanases like a disintegrin and metalloproteinase with thrombospondin motifs
IRF-1	Interferon regulatory factor-1
NF-κB	Nuclear factor-kappaB
MAPK	Mitogen-activated protein kinase
JNK	c-Jun N-terminal kinase
ERK	Extracellular signal-regulated kinase
AP-1	Activator protein-1

Introduction

Osteoarthritis (OA) is a degenerative disease with low-grade inflammation of the joints in aged people [1]. Many factors such as aging, obesity, trauma, genetic predisposition, and endocrine are related to the pathophysiological events along the process of OA development [2–4]. Several catabolic factors, including proinflammatory cytokines, matrix metalloproteinases (MMPs), and nitric oxide (NO) are commonly recognized molecules responsible for joint damage in OA [5]. Regarding proinflammatory cytokines, both interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF-α) are considered to be the major ones contributing to the process of development of OA [6].

The extracellular matrix components such as collagen and proteoglycan (aggrecan) are important structural bases for integrity of cartilage [7]. Among many different types of collagens, type II collagen is the most important and is often used as an indicator of cartilage metabolism in OA [8]. MMP-13 (collagenase-3), the enzyme that preferentially cleaves collagen II and preserves much higher potency than collagenase-1, is considered to be the critical proteinase that damage cartilage in OA joints [9]. Importantly, the co-localization of MMP-13 expression and collagen II degradation in active joint lesions further strengthens its significance in OA pathogenesis [10]. In addition to MMP-13, other MMPs and aggrecanases like a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) enzymes are also contributing to cartilage destruction [11]. The significance of nitric oxide synthase (iNOS)–NO system in OA pathogenesis has been demonstrated in experimental OA dogs [12, 13]. The production of MMP-13 and NO can be efficiently induced by both TNF-α and IL-1 in chondrocytes [6].

The goal of a disease-modifying anti-OA drug has been a critical challenge, as it must induce its immunomodulatory effects while causing very limited toxicity [14–16]. Small molecules designed to target specific signaling pathways and/or mechanisms have great potential in manipulating various cellular functions for therapeutic purposes [17]. In this report, we constructed a minilibrary using a solution-phase synthesis through coupling core amino compounds with carboxylic acids via amide bond formation. The simplified structural core compound 3-(trifluoromethyl)aniline was selectively coupled with several carboxylic acids. Through coupling the benzamide-linked small molecules library with cell-based assays, we demonstrated that a small molecule, 2-hydroxy-*N*-[3-(trifluoromethyl)phenyl]benzamide (HS-Cf), with very limited cytotoxicity was able to inhibit TNF-α-induced activation of chondrocytes by suppressing MMPs and ADAMTSs expression, MMP-13 enzyme activity, iNOS–NO production, and proteoglycan degradation. The mechanisms were likely through inhibiting TNF-α-induced activation of interferon regulatory factor-1 (IRF-1) but not nuclear factor-kappaB (NF-κB), activation protein-1 (AP-1) or several other examined transcriptional factors signaling pathways.

Materials and Methods

Reagents and Antibodies

TNF-α was purchased from R & D (Canandaigua, NY, USA). Polyclonal antisera against iNOS, IRF-1, and IκBα were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyclonal anti-collagen II antibodies were purchased from Chemicon International (Temecula, CA, USA) and aggrecan neoepitope antibody (NB100-74350) was from Novus Biologicals, (Littleton, CO, USA). The antibodies recognizing phosphorylated extracellular signal-regulated kinases (ERKs), phosphorylated p38, and phosphorylated c-Jun N-terminal kinase (JNK) were purchased from Cell Signaling (Danvers, MA, USA). The reagents used in the amide bond formation benzamide-linked small molecules were core amine, carboxylic acid, *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide, and 1-HOBt. The required concentrations of small molecules for individual experiments were made by further dilution of the stock preparation with culture medium when needed. Unless otherwise specified, the rest of the reagents were purchased from Sigma-Aldrich Chemical Company.

Isolation and Culture of Porcine Chondrocytes

Porcine cartilage was obtained from the hind leg joints of pigs. The preparation of chondrocytes from cartilage was performed according to our previous report [18]. After enzymatic digestion of articular cartilage with 2 mg/ml protease in serum-free Dulbecco's modified Eagle's medium (DMEM)/antibiotics, the specimens were then digested overnight with 2 mg/ml collagenase I and 0.9 mg/ml hyaluronidase in DMEM containing 10% fetal bovine serum (FBS). The cells were collected, passed through a cell strainer (Becton Dickinson, Mountain View, CA, USA), and cultured in DMEM containing 10% FBS and antibiotics for 3–4 days before use.

Cytotoxicity Analysis

Evaluation of potential cytotoxic effects from amide-forming compounds was performed by using 3-[4,-dimethylthiazol-2-y]-2,5-diphenyl-tetrazolium bromide (MTT) colorimetric assays as described [19]. In brief, chondrocytes at 5×10^4/ml in 100 μl volume were incubated in the presence or absence of HS-Cf for 48 h. Then, 100 μl of MTT (5 mg/ml in H_2O) was added, and cells were incubated at 37°C for 6 h followed by the addition of 100 μl of dimethyl sulfoxide (DMSO). After incubation at 37°C for another 30 min, the content of dissolved reduced MTT crystals was measured with a plate reader (TECAN, Grodig, Austria). The measurement of the concentrations of the released lactate dehydrogenase (LDH), as an indicator of damage to the plasma membrane, was performed according to the manufacturer's instructions (Roche, Indianapolis, IN, USA). The percent cytotoxicity was calculated as ([sample value−medium control]/[high control−medium control])×100. Individual sample values were the averages of the absorbance values in treated culture supernatants after subtraction of the absorbance values in background control in triplicate. Similarly, the average absorbance values of untreated cell culture supernatants, used as the medium control, were calculated. Equal amount of cells treated with 1% Triton X-100 was taken as the high control.

Measurement of NO Concentrations

The measurement of NO release was reflected by determination of its stable end product, nitrite, in supernatants [18]. The Griess reaction was performed with the concentrations of nitrite measured by a spectrophotometer. In brief, an aliquot (100 μl) of culture supernatant was incubated with 50 μl of 0.1% sulfanilamide in 5% phosphoric acid and 50 μl of 0.1% *N*-1-naphthyl-ethylenediamine dihydrochloride. After 10 min of incubation at room temperature, the absorbance was measured at 550 nm wavelength with a plate reader (Tecan, Grodig, Australia).

Western Blotting

ECL Western blotting (Amersham-Pharmacia, Arlington Heights, IL, USA) was performed as described [20]. Briefly, equal amounts of whole cellular extracts were analyzed on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to the nitrocellulose filter. For immunoblotting, the nitrocellulose filter was incubated with Tris-buffered saline with 1% Triton X-100 containing 5% non-fat milk for 1 h and then blotted with antibodies against specific proteins for another 2 h at room temperature. After washing with milk buffer, the filter was incubated with rabbit anti-goat IgG or goat anti-rabbit IgG conjugated to horseradish peroxidase at a concentration of 1:5,000 for 30 min. The filter was incubated with the substrate and then exposed to X-ray film (GE Healthcare, Buckinghamshire, UK).

Nuclear Extract Preparation and Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extract preparation and EMSA analysis were performed as detailed in our previous report [20]. The oligonucleotides containing NF-κB-binding site, IRF-1-binding site, and other transcription factor-binding sites were purchased from Promega or Santa Cruz and used as DNA probes. The DNA probes were radiolabeled with [γ-^{32}P]ATP using the T4 kinase (Promega). For the binding reaction, the radiolabeled probe was incubated with 4 μg of nuclear extracts. The binding buffer contained 10 mM Tris–HCl (pH 7.5), 50 mM NaCl, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol, 1 mM $MgCl_2$, 4% glycerol, and 2 μg poly(dI-dC). The reaction mixture was left at room temperature to proceed with binding reaction for 20 min. The final reaction mixture was analyzed in a 6% non-denaturing polyacrylamide gel with 0.5×Tris/borate/EDTA as an electrophoresis buffer.

Real-Time Reverse Transcriptase-Polymerase Chain Reaction (Real-Time RT-PCR)

Total RNA was isolated after lysing the cells by Trizol reagent (Invitrogen; Carlsbad, CA, USA) according to the manufacturer's protocol. RNA samples were treated with DNase I (Roche, Indianapolis, IN, USA) before reverse transcription. Reverse transcription was performed in a 20-μl mixture containing 2 μg of total RNA, 10× RT buffer (Invitrogen), random hexomer (Invitrogen), mixture of dNTP (Promega; Madison, WI, USA), and Moloney Murine Leukemia Virus Reverse Transcriptase (Invitrogen) based on the description of applying Superscript First-Strand Synthesis System (Invitrogen). After reverse transcription of RNA to complementary DNA (cDNA), the obtained template cDNA samples were subjected to PCR reactions. Real-time measure-

ment of MMP-1, MMP-3, MMP-13, ADAMTS4, ADAMTS5, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) messenger RNA (mRNA) was performed according to the manufacturer's instructions (power SYBR Green PCR Master Mix, Applied BioSystems, Foster City, CA, USA). In brief, 10 ng of cDNA was amplified in a total volume of 20 μl mixture consisting of 1× Master Mix and the gene-specific primers, which were added at a final concentration of 100 nM. The primer sequences for MMP-1, MMP-3, MMP-13, ADAMTS4, ADAMTS5, and GAPDH were designed by us or described by other researchers [21, 22], and they were as follows: 5′-AAG CAG ACA TAA TGA TAT CCT TTG TCA-3′ and 5′-TGA GCA TCC CCT CCA ATA CCT-3′ (for MMP-1); 5′-AGA AGT TCC TGG GGT TGG AGG T-3′ and 5′-TCT TGG AGA ATG TAA GCG GAG T-3′ (for MMP-3); 5′-GAT CCC CAT TTT GAT GAT GAT GAA-3′ and 5′-GTC TTC ATC TCC TGG ACC ATA GAG AGA-3′ (for MMP-13); 5′-CAG GGT CCC ATG TGC AAC GT-3′ and 5′-CAT CTG CCA CCA CCA GGG TCT-3′ (for ADAMTS4); 5′-TTC GAC ATC A AG CCA TGG CAA CTG-3′ and 5′-AAG ATT TAC CAT TAG CCG GGC GG-3′ (for ADAMTS5); 5′-GCA ACT CCG ACC TTG TCA TC-3′ and 5′-AGC GTA GGT CTT GGT GAA GC-3′ (for TIMP-1); 5′-GTA GTG ATC AGG GCC AAA GC-3′ and 5′-TTC TCT GTG ACC CAG TCC AT-3′ for (TIMP-2); 5′-CGT GTC TAT G AT GGC AAG ATG-3′ and 5′-CAG GCG TAG TGT TTG GAC TG-3′ for (TIMP-3); and 5′-GTC ATC CAT GAC AAC TTC GG-3′ and 5′-GCC ACA GTT TCC CAG AGG-3′ (for GAPDH). The reactions were performed for 50 cycles with 95°C for denaturation and 60°C for annealing and extension on the ABI Prism 7000 Sequence Detection system (Applied BioSystems). The data were collected and the changes in gene expression following stimulation with AGEs in the presence or absence of inhibitors were calculated with the following formula: fold changes $= 2^{-\Delta(\Delta C_t)}$, where $\Delta C_t = C_{t\ \text{targeted gene}} - C_{t\ \text{GAPDH}}$, and $\Delta(\Delta C_t) = \Delta C_{t\ \text{stimulated}} - \Delta C_{t\ \text{control}}$.

Gelatin Zymography

Gelatin zymography was performed according to the description [16] with modification. The culture supernatant (16 μl) was mixed with 4 μl buffer containing 4% SDS, 0.15 M Tris (pH 6.8), and 20% glycerol that contained 0.05% bromophenol blue and analyzed on a 10% polyacrylamide gel with copolymerized 0.1% gelatin (Sigma). After electrophoresis, gels were washed with 2.5% Triton X-100 three times for 20 min each. After incubation with the gelatinase buffer (50 mM Tris–HCl [pH 7.6], 10 mM $CaCl_2$, 50 mM NaCl, and 0.05% Brij-35) at 37°C for 24 h, the gel was stained with 0.1% Coomassie blue, and the clear bands indicating genatinolytic activity were visualized under the background of uniform light blue staining. The localization of MMP-13 was identified as judged by the molecular weight of the standards and the report from other researchers [23] under Alpha EaseFC Softwave (Alpha Innotech Corp, USA).

Short Hairpin RNA Knockdown

To reduce IRF-1 expression, several IRF-1-specific short hairpin RNA (shRNA) constructs were designed, synthesized, and obtained from the National RNAi Core Facility in Taiwan as described similarly in our recent report [24]. One of the IRF-1-specific shRNA constructs (clone ID: TRCN0000229685) or a control green fluorescence protein (GFP) shRNA construct (clone ID: TRCN0000072202) was co-transfected with the package and envelop plasmids to 293T cells to generate recombinant lentivirus carrying specific shRNA. The lentivirus carrying different shRNA constructs at MOI 2 were used to infect chondrocytes in the presence of 8 μg/ml polybrene. Cells were used for experiments 6 days after infection.

Preparation of Cartilage Explants

The preparation of cartilage explants was performed according to our previous report with mild modification [18]. In brief, articular cartilage from the femur head of hind limb joint of pigs was excavated by a stainless steel dermal-punch 3 mm in diameter (Aesculap, Tuttlingen, Germany) and weighed. After dissection, each cartilage explant was placed in a 24-well plate and cultured for 24 h in DMEM containing antibiotics and 10% FBS. After resting for 72 h in serum-free DMEM, the cartilage explants were used for further study.

Analysis of Cartilage Degradation

Cartilage degradation was assessed by measuring the amount of proteoglycan released into the culture medium as described [18]. In brief, culture medium was added to 1,9-dimethylmethylene blue (DMB) solution (Sigma) in which the metachromatic dye can bind sulfated glycosaminoglycan (GAG), a major component of proteoglycan. The amount of the formation of GAG–DMB complex was measured in a 96-well plate using a plate reader (TECAN) at a wavelength of 595 nm. The loss of GAG was calculated and expressed as the total GAG (micrograms) released per milligram (wet weight) of the cartilage weight.

Safranin O Staining and Measurement of the Aggrecan NITEGE (G1 Fragment of the Proteoglycan/Aggrecan) Neoepitope

Cartilage explants were mounted in embedding medium (Miles Laboratories, Naperville, IL, USA) and rapidly frozen at −80°C. Serial but incontinuous microscopic sections (7 μm) of cartilage explants were cut at −20°C

on a Microm cryostat and mounted on Superfrost Plus glass slides (Menzel-Gläser, Braunschweig, Germany). Tissue sections were then stained with Safranin O/fast green countered with Weigert's iron hematoxylin to assess the changes of proteoglycan content [18]. In parallel, the expression of aggrecan NITEGE neoepitope recognized by NITEGE antibodies in tissue sections was determined as described by other researchers [25].

Statistical Analysis

When necessary, the results were expressed as the mean ± standard deviation. The statistical analysis was performed using one-way analysis of variance with Bonferroni post hoc tests for multiple comparisons and Student's *t* test for comparison between two groups. $P<0.05$ was considered significant.

Results

Benzamide-Linked Small Molecules Inhibited iNOS–NO Production in TNF-α-Stimulated Porcine Chondrocytes

To meet the purpose of identifying disease-modifying anti-OA drugs preserving immunomodulatory effects and yet containing limited cytotoxicity, a minilibrary of benzamide-linked small molecules was synthesized and established, and their suppressive activities in TNF-α-induced produc-

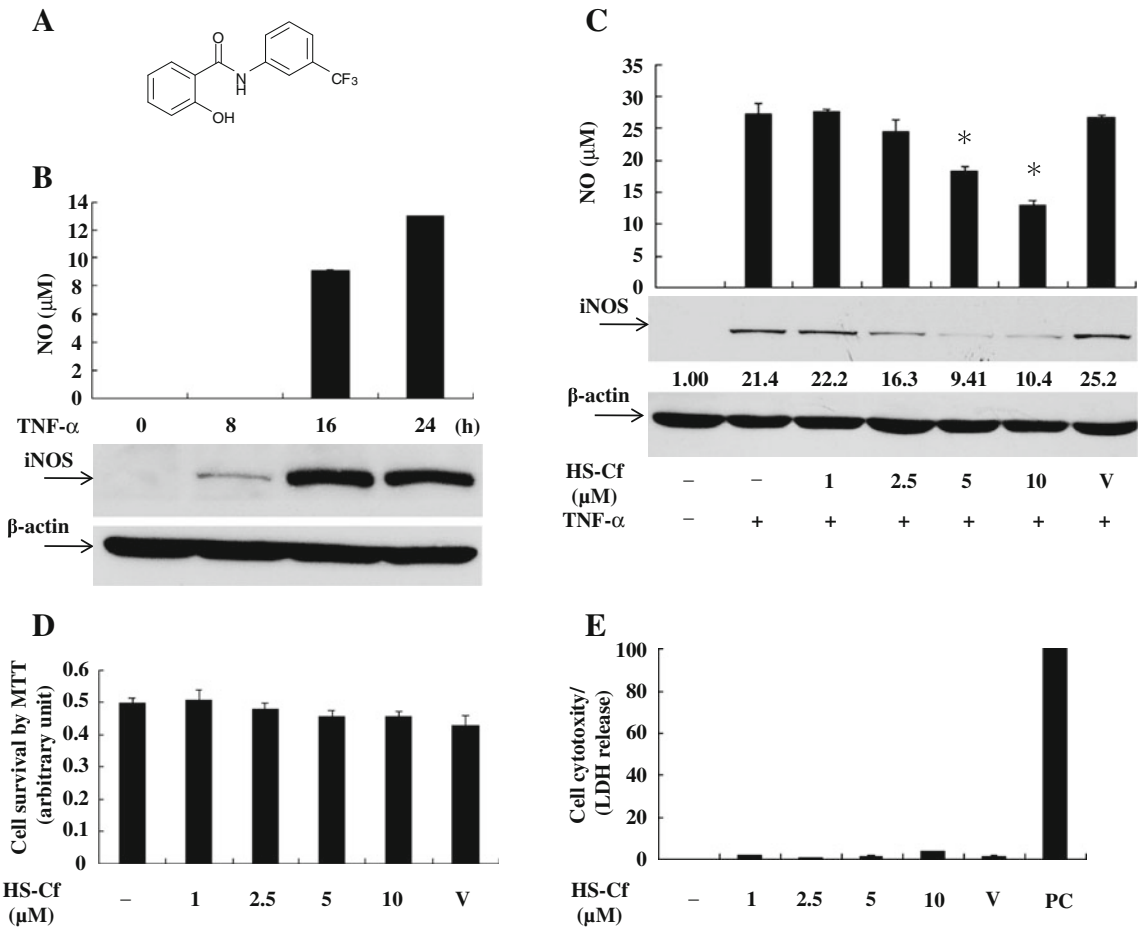

Fig. 1 HS-Cf dose-dependently inhibited iNOS–NO production in TNF-α-stimulated porcine chondrocytes. Structure of 2-hydroxy-*N*-[3-(trifluoromethyl)phenyl]benzamide (HS-Cf) was shown in **a**. In **b**, chondrocytes, 3×10^6 in total for each condition, were treated with 5 ng/ml TNF-α for different time periods, and the expressions of iNOS and β-actin were determined by Western blotting. Supernatants were collected and the concentrations of NO were determined by Griess reaction. In **c**, porcine chondrocytes were pretreated with various doses of HS-Cf or the solvent, DMSO, for 24 h and then stimulated with TNF-α for 24 h. The expression of iNOS and the production of NO were determined. To determine potential cytotoxic effects of HS-Cf, chondrocytes were treated with various concentrations of HS-Cf for 48 h. The cells and culture supernatants were collected for the measurement of possible cell death with MTT (**d**) and LDH release assays (**e**) as described in "Materials and Methods". *PC* Equal amount of cells treated with 1% Triton X-100 was taken as the positive control. The representative data out of at least three independent experiments are shown. *$P<0.05$ compared to the TNF-α-stimulated in the absence of HS-Cf treatment. *V* vehicle (DMSO)

tion of NO were determined. When chondrocytes were pretreated with 10-μM various benzamide-linked small molecules, the TNF-α-induced productions of NO were suppressed by some compounds (data not shown). After preliminary comparisons of potency and cytotoxicity among those potential compounds preserving the inhibitory effects on TNF-α-induced NO production, HS-Cf (Fig. 1a) was chosen for further studies. In Fig. 1b, we showed that in addition to the induction of NO production, TNF-α also potently induced the expression of iNOS in porcine chondrocytes. When chondrocytes were pretreated with various concentrations of HS-Cf, we observed a dose-dependent suppression of TNF-α-induced production of NO and expression of iNOS. The suppressive effects could be readily observed at a concentration of 2.5 μM of HS-Cf (Fig. 1c). By both MTT and LDH release assays, we confirmed that at the examined concentrations, HS-Cf did not have significantly detectable cytotoxic effects in porcine chondrocytes (Fig. 1d, e).

HS-Cf Suppressed TNF-α-Induced MMP-13 Expression and Activity as well as Prevented TNF-α-Mediated Collagen II Reduction

Because MMP-13 is directly responsible for damaging cartilage matrix, we examined the effects of HS-Cf on TNF-α-induced MMP-13 activity. By zymographic and real-time RT-PCR analysis, the results showed that TNF-α-induced MMP-13 enzyme activity (Fig. 2a) and mRNA expression (Fig. 2b) were suppressed by HS-Cf. Since collagen II is preferentially cleaved by MMP-13, we determined whether HS-Cf could affect TNF-α-mediated reduction of collagen II. TNF-α treatment effectively reduced the levels of collagen II (Fig. 2c). As shown in Fig. 2d, HS-Cf treatment prevented TNF-α-mediated decrease of collagen II. Altogether, the results indicated that HS-Cf could provide cartilage protection at least by down-regulating TNF-α-induced MMP-13 expression and enzyme activity as well as by preventing TNF-α-mediated decrease of collagen II in chondrocytes.

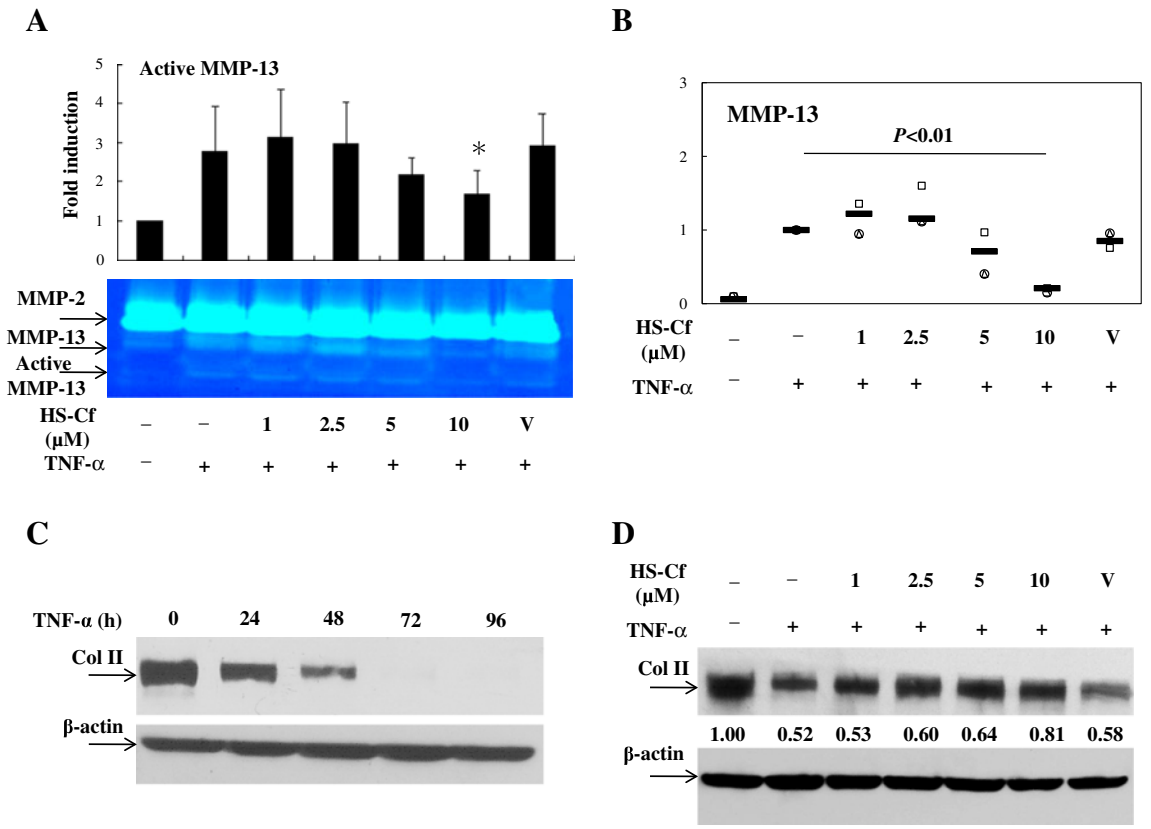

Fig. 2 HS-Cf suppressed TNF-α-induced MMP-13 gene expression and enzyme activity as well as prevented TNF-α-mediated reduction of collagen II. Chondrocytes were pretreated with various doses of HS-Cf for 24 h and then stimulated with 5 ng/ml TNF-α for another 24 h. The activities of MMP-13 released into the culture supernatants were determined by gelatin zymography, and the representative and pooled data from at least three independent experiments are shown (**a**). In **b**, after treatment, real-time RT-PCR was applied to measure the levels of MMP-13 mRNA. In **c**, the total cell lysates from chondrocytes treated with 5 ng/ml TNF-α for various time points were collected for determination of the levels of collagen II by Western blots. In **d**, chondrocytes were pretreated with various doses of HS-Cf for 24 h and stimulated with 5 ng/ml TNF-α for another 48 h, and then the levels of collagen II in total cell lysates were determined by Western blot. The results from at least three independent experiments are shown. *$P<0.05$ compared to the TNF-α-stimulated in the absence of HS-Cf treatment. *V* vehicle (DMSO)

HS-Cf Inhibited TNF-α-Induced MMP-1, MMP-3, ADAMTS4, and ADAMTS5 Expression

In addition to MMP-13, other MMPs and aggrecanases enzymes are also contributing to cartilage destruction. The ADAMTS are a group of extracellular, multidomain proteases and are capable of causing collagen processing as procollagen N-proteinase and causing cleavage of the matrix proteoglycans and aggrecans [11]. We examined whether HS-Cf might affect TNF-α-induced MMP-1, MMP-3, ADAMTS4, and ADAMTS5 expressions. Chondrocytes were pretreated with different doses of HS-Cf and then treated with 5 ng/ml TNF-α, and the cellular RNA was prepared for real-time RT-PCR analysis. As shown in Fig. 3, although the intensity varied, in the presence of HS-Cf, the TNF-α-induced expressions of these enzymes were suppressed in chondrocytes. In contrast, HS-CF did not affect the expression of TIMP-1, TIMP-2, and TIMP-3.

HS-Cf Protected Against TNF-α-Induced Proteoglycan/Aggrecan Degradation in Cartilage Explants

To further address the chondroprotective effect of HS-Cf as a reflection of its anti-inflammatory property, we investigated whether HS-Cf could prevent TNF-α-induced degradation of cartilage matrix. When the cartilage explants were stained, a significant reduction of Safranin O-positive proteoglycan and an increase of the cleavage products of aggrecan (NITEGE) were observed in TNF-α-treated samples. Such TNF-α-induced events were successfully prevented by HS-Cf pretreatment in a dose-dependent manner (Fig. 4, upper and middle panels). Consistently, we demonstrated that treatment with HS-Cf was effective to prevent TNF-α-mediated release of proteoglycan into the culture supernatants of cartilage explants (Fig. 4, lower panels).

HS-Cf Could Not Suppress TNF-α-Stimulated NF-κB and AP-1 Signaling Pathways

We next investigated the mechanisms of HS-Cf-mediated inhibition of iNOS expression in TNF-α-activated chondrocytes. Because both NF-κB and AP-1 families of transcription factors play crucial roles in TNF-α-mediated activation of chondrocytes and are also important in regulating iNOS and MMP-13 expression, we determined whether the chondroprotective effects of HS-Cf were mediated through regulating these transcription factors.

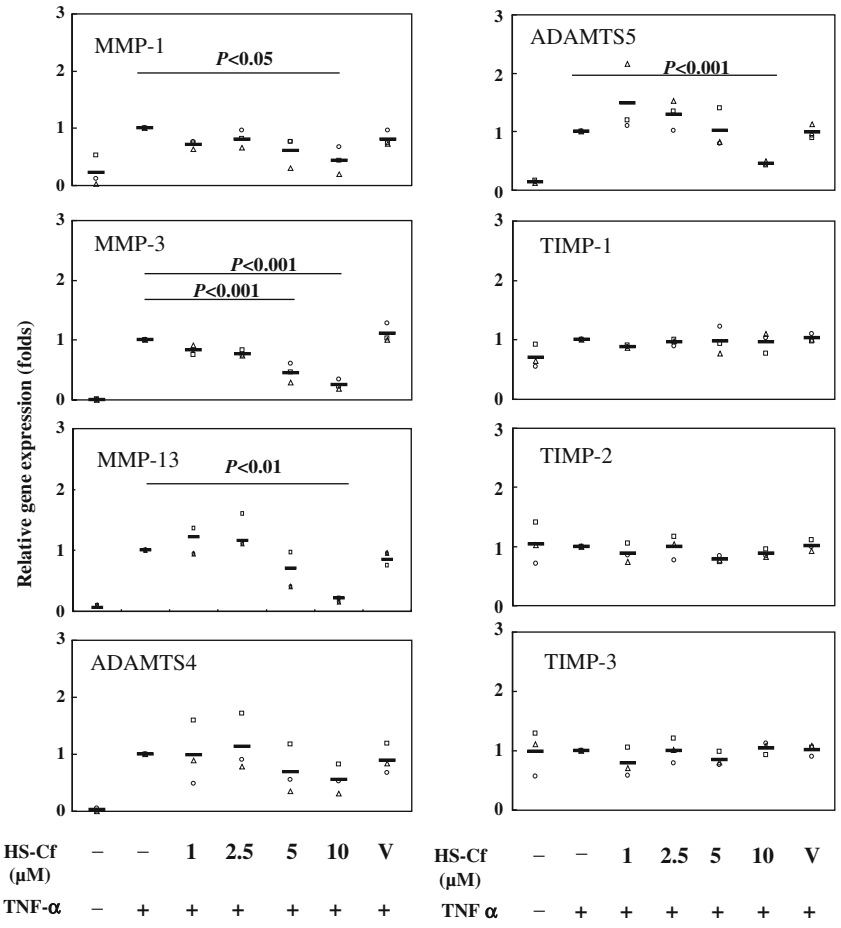

Fig. 3 HS-Cf reduced TNF-α-stimulated MMP-1, MMP-3, ADAMTS4, and ADAMTS5 mRNA expression. Porcine chondrocytes treated with various doses of HS-Cf for 2 h were stimulated with TNF-α for 4 h. The cells were collected, total RNA prepared, and the expression of mRNA was determined by real-time RT-PCR. The relative expression of MMP-1, MMP-3, ADAMTS4, and ADAMTS5 mRNA was normalized to GAPDH, with subsequent normalization to the TNF-α-stimulated sample in each experiment. Significance of the difference between sample groups was calculated using one-way analysis of variance with Bonferroni post hoc tests. The results from three independent experiments are shown

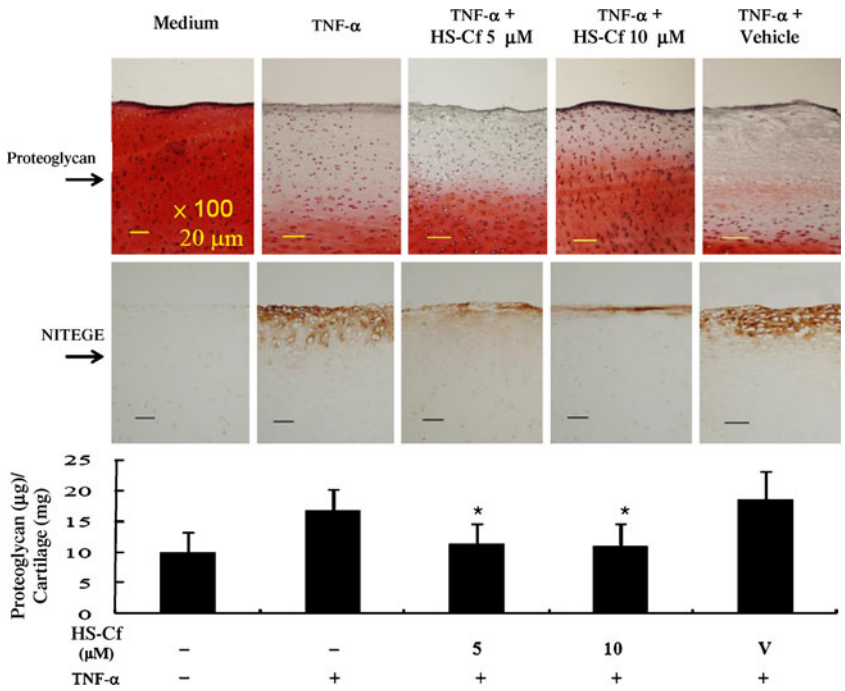

Fig. 4 HS-Cf protected against TNF-α-induced degradation of proteoglycan and aggrecan. Porcine cartilage blocks were cultured in 24-well plates and pretreated or not with 5 or 10 μM HS-Cf for 24 h and then stimulated with 5 ng/ml TNF-α for another 72 h. The retained proteoglycan in cartilage explants was monitored by Safranin O staining (*upper panels*). In parallel, the intensity of aggrecan staining was examined (*middle panels*). Meanwhile, the release of proteoglycan into the culture medium was determined and normalized to the cartilage weight as described in "Materials and Methods" (*lower panels*). The representative data from three independent experiments using different donor cartilage blocks are shown. *P value <0.05 denotes the statistical significance as compared to the TNF-α-stimulated in the absence of HS-Cf treatment

Chondrocytes stimulated with TNF-α expressed strong DNA-binding activity of NF-κB and such a DNA-binding activity could be competed by wild-type but not by mutant κB oligonucleotides (Fig. 5a). Meanwhile, TNF-α stimulation also caused transient decrease of IκBα, an inhibitor of NF-κB (Fig. 5a, lower panels). By EMSA analysis, the results suggested that the TNF-α-induced NF-κB DNA-binding activity was not suppressed by HS-Cf (Fig. 5b). Consistently, HS-Cf did not affect TNF-α-mediated degradation of IκBα (Fig. 5b, lower panel). When the effect of HS-Cf on TNF-α-induced AP-1 activation was examined, the results showed that HS-Cf had no effect on TNF-α-induced AP-1 DNA-binding activity (Fig. 5c, upper panel). In parallel, the TNF-α-induced activation of upstream MAPKs, including JNK, p38, and ERK, was not suppressed by HS-Cf (Fig. 5c, lower panels).

HS-Cf Suppressed TNF-α-Stimulated IRF-1

To our surprise that both NF-κB and AP-1 signaling pathways were not affected by HS-Cf, we then focused on examining other transcriptional factors that bind iNOS promoter and might potentially be targeted by HS-Cf. The activation of IRF-1, which is also critical in many inflammatory responses, was investigated [26]. The results revealed that the levels of IRF-1 were significantly induced by TNF-α treatment in porcine chondrocytes (Fig. 6a). Pretreatment with HS-Cf resulted in a dose-dependent reduction of TNF-α-induced IRF-1 expression (Fig. 6b). In addition, as treatment with TNF-α induced DNA-binding activity of IRF-1 (Fig. 6c), such IRF-1 DNA-binding activity was effectively suppressed in the presence of HS-Cf (Fig. 6d). As additional controls, HS-Cf had no effects of DNA-binding activities of several transcriptional factors, including SP-1 and Oct-1 (Fig. 6d, lower panels).

Knockdown of IRF-1 by Short Hairpin RNA Inhibited TNF-α-Induced Activation of iNOS and MMP-13

To confirm that IRF-1 is indeed critical in TNF-α-induced cartilage damage, the effects of IRF-1 knockdown by short hairpin RNA approach was conducted. Chondrocytes were infected by lentivirus carrying IRF-1-specific shRNA or GFP shRNA construct and then stimulated or not with TNF-α, and the expressions of iNOS and IRF-1 were determined by Western blotting. As shown in Fig. 7a, interference of IRF-1 expression decreased TNF-α-induced iNOS and IRF-1 expressions. Meanwhile, reduction of IRF-1 also decreased TNF-α-induced NO production (Fig. 7b). Using zymography, we also demonstrated decrease of TNF-

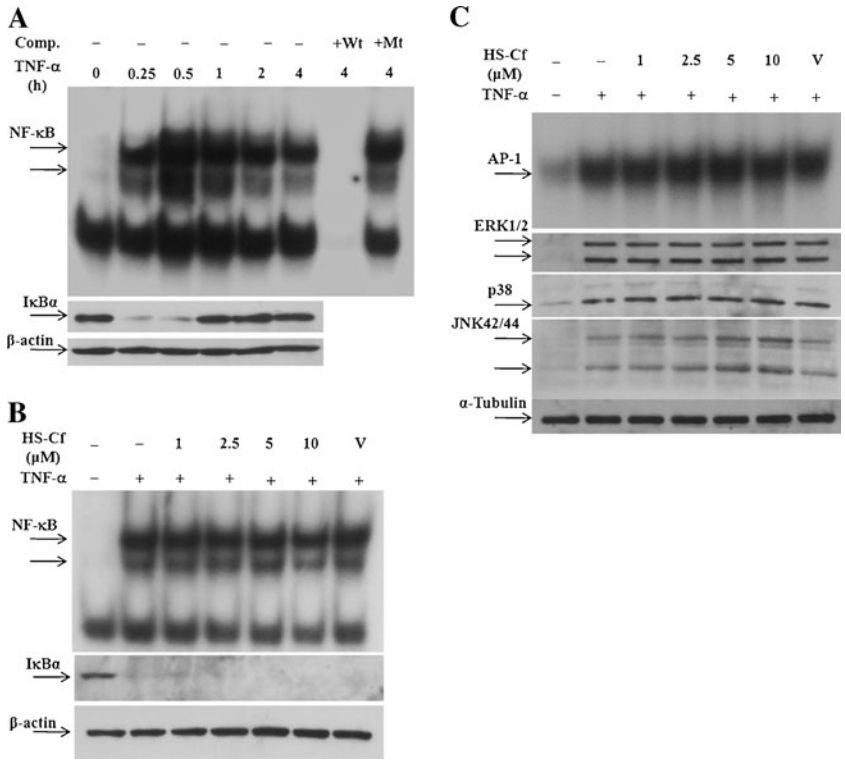

Fig. 5 HS-Cf had no effects on TNF-α-stimulated NF-κB and AP-1 signaling pathways. Porcine chondrocytes were cultured overnight with serum-free medium followed by treatment with 5 ng/ml TNF-α for different time periods. The nuclear extracts were prepared from collected cells and the DNA-binding activity of NF-κB was determined by EMSA. For determining the specificity of the DNA-binding complex, a 100-fold molar excess of non-radiolabeled wild-type (*Wt*) or mutant (*Mt*) competitive oligonucleotides was pre-incubated with nuclear extracts for 30 min before the addition of radiolabeled oligonucleotides. The expression of IκBα and β-actin in total cell lysates was determined by Western blotting (**a**). Similar to **a**, the effects of HS-Cf on TNF-α-induced DNA-binding activity of NF-κB and degradation of IκBα were determined by EMSA or by Western blot, respectively (**b**). In **c**, the nuclear extracts of cells treated with 5 ng/ml TNF-α in the presence of various doses of HS-Cf were prepared and analyzed for AP-1 DNA-binding activity with EMSA. The expression of phosphorylated ERK1/2, p38, JNK42/44, and α-Tubulin was determined by Western blotting. *Comp*. stands for competitive oligonucleotides. The results from at least three independent experiments are shown

α-induced MMP-13 enzyme activity by IRF-1 interference (Fig. 7c).

Discussion

Several important parameters related to the damage of cartilage such as iNOS–NO and MMP-13 and the stimulant TNF-α were chosen as tools to study and to identify potential anti-OA compounds. The readouts in this report included the levels of collagen II and the degradation of proteoglycan/aggrecan that are all highly correlated with the damage of structural integrity of cartilage. Thus, the study of the potential immunomodulatory effects of small molecules here is of clinical relevance. By screening a benzamide-linked small molecules minilibrary, we identified HS-Cf as the potential candidate for anti-OA therapeutics. Although the best effects of HS-Cf were seen in the higher doses (5 or 10 μM), the results indicated that HS-Cf could dose-dependently suppress TNF-α-induced iNOS–NO production, mRNA expression of several MMPs and ADAMTS, and enzyme activity of MMP-13 in chondrocytes and prevent degradation and release of proteoglycan/aggrecan in cartilage explants. Molecular approaches revealed that the immunomodulatory mechanisms of HS-Cf were likely to be through the suppression of IRF-1 but not NF-κB or AP-1 signaling pathway. The conclusion was further supported by the observation that knocking-down IRF-1 expression by shRNA approach reduced TGF-α-induced iNOS expression, NO production, and MMP-13 enzyme activity. This study therefore provides evidence of chondroprotective effects and mechanisms of a benzamide-linked small molecule, HS-Cf, as well as its potential application in TNF-α-induced damage of cartilage in joints (Fig. 8).

IRFs are a group of transcriptional factors regulating genes induced by interferons, proinflammatory cytokines, and other stimuli and play pivotal roles in immune

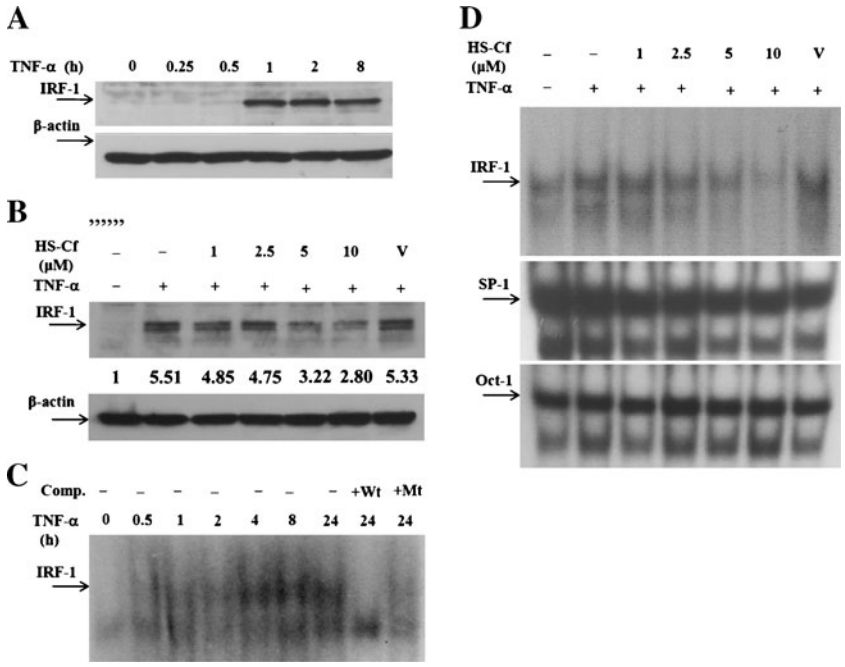

Fig. 6 HS-Cf suppressed TNF-α-stimulated IRF-1 signaling. Porcine chondrocytes were cultured overnight with serum-free medium followed by treatment with 5 ng/ml TNF-α for different time periods. The expression of IRF-1 and β-actin was determined by Western blotting (**a**). Chondrocytes were pretreated with various doses of HS-Cf or the solvent, DMSO, for 24 h and then stimulated with 5 ng/ml TNF-α for another 2 h. The total cell lysates were prepared to measure the levels of IRF-1 by Western blot (**b**). The *numbers* shown are the values of signal intensity measured by a densitometer. The nuclear extracts of cells treated with 5 ng/ml TNF-α for various periods of times were prepared and analyzed for IRF-1 DNA-binding activity with EMSA (**c**). For determining the specificity of the DNA-binding complex, a 100-fold molar excess of non-radiolabeled wild-type (*Wt*) or mutant (*Mt*) competitive oligonucleotides was pre-incubated with nuclear extracts for 30 min before the addition of radiolabeled oligonucleotides. In **d**, the effects of HS-Cf on TNF-α-induced DNA-binding activity of IRF-1 and two other transcription factors, including SP-1 and Oct-1, were also determined by EMSA. *Comp.* stands for competitive oligonucleotides. The results from at least three independent experiments are shown

responses and oncogenesis [26]. Regarding IRF-1, the first IRF discovered, in addition to its well-known role mediating anti-viral responses [27], many other effects have also been reported. In cultured rat glioma cell line C6 cells, activation of IRF-1 is critical in TNF-α-stimulated NO production [28]. Lipopolysaccharide-induced activation of cyclooxygenase-2 (Cox-2) is abrogated in IRF-1-deficient macrophages, and blocking IRF-1 appears to provide hepatic protection [29]. However, the role of IRF-1 may vary in response to different stimuli in different systems. For example, Tsung et al. [30] are unable to detect induction of IRF-1 by LPS stimulation in cultured hepatocytes; however, under the same conditions, stimuli such as interferon-gamma (IFN-γ), IFN-β, TNF-α, and IL-1β are capable of inducing IRF-1. Only very few reports examine the role of IRF-1 in chondrocytes. The only retrievable report studying IRF-1 in chondrocytes from Medline indicates that articular chondrocytes from both wild-type and IRF-1-deficient mice produce similar levels of NO in response to IL-1 or LPS stimulation [31]. In contrast, under the same system, the production of NO from peritoneal macrophages is much less in IRF-1-deficient mice [31]. In this particular study, the effects of TNF-α as a stimulant used in our study were not examined. Our observation that HS-Cf could suppress TNF-α-induced activation of IRF-1 might suggest its significance in TNF-α-mediated damage of cartilage.

Inhibition of IRF-1 but not NF-κB or several other examined transcriptional factors suggests a relative specificity of HS-Cf-mediated immunomodulatory effects and mechanisms. A similar mechanism is also observed in other examples. According to Inoue et al. [32], they identify a compound named KE-758 that protects joints from damage in an adjuvant-induced arthritis model in rats. In this system, KE-758 suppresses iNOS expression and inhibits only the activation of IRF-1 but not NF-κB although both IRF-1 and NF-κB activities are efficiently induced by LPS stimulation [32]. A report from Cheng et al. [33] demonstrates that the inhibition of IL-1β-induced iNOS and Cox-2 expression by selenomethionine involves only p38 but not NF-κB signaling pathway in human chondrocytes. In cultured human chondrocytes, our study also demonstrates that AP-1 but not the NF-κB signaling pathway is involved in all-*trans* retinoic acid-mediated

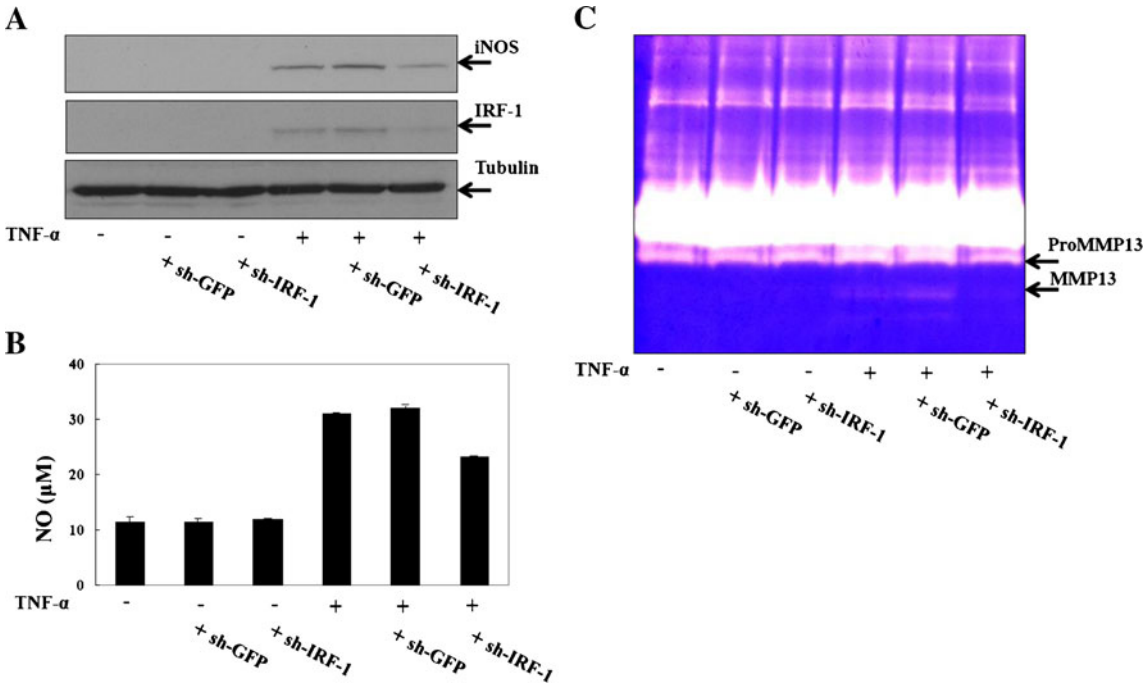

Fig. 7 shRNA interference of IRF-1 expression reduced TNF-α-stimulated iNOS and MMP-13. Porcine chondrocytes infected by lentivirus carrying IRF-1-specific shRNA (*sh-IRF-1*) or GFP shRNA (*sh-GFP*) were treated with TNF-α for 24 h. The cells were collected, total cell lysate prepared, and the expressions of iNOS, IRF-1, and β-actin were determined by Western blotting (**a**). The concentrations of NO in collected supernatants were determined by Griess reaction (**b**). The activities of MMP-13 released into the culture supernatants were determined by gelatin zymography, and the representative and pooled data from at least three independent experiments are shown (**c**). The results from at least three independent experiments are shown

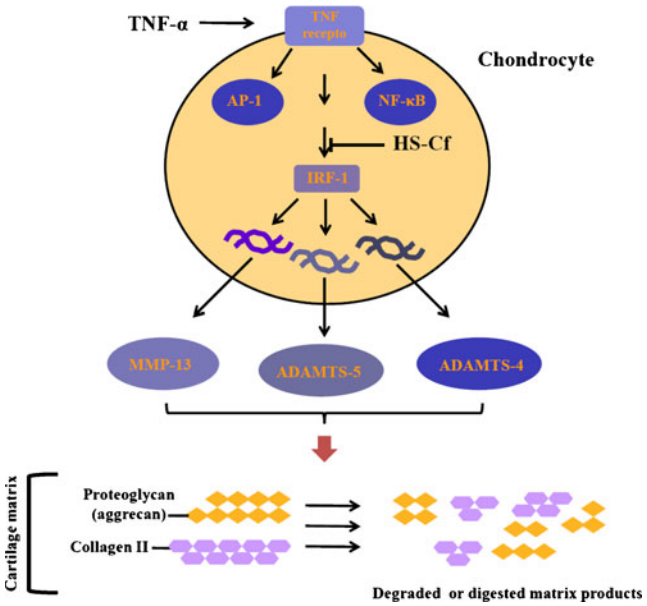

Fig. 8 The role of IRF-1 in TNF-α-mediated damage of cartilage. TNF-α stimulation activated at least three signaling pathways, including NF-κB, AP-1, and IRF-1. The small molecule HS-Cf suppressed only IRF-1 but not NF-κB or AP-1 signaling pathways. Suppression of IRF-1 prevented TNF-α-mediated activation of MMP-13, ADAMTS4, and ADAMTS5 as well as the degradation of proteoglycan, aggrecan, and collagen II, three major components of cartilage matrix, through direct and indirect mechanisms

suppression of IL-1-induced production and enzyme activity of MMPs [20]. Collectively, although NF-κB appears to be critical in proinflammatory cytokine-mediated pathogenesis of OA [34], the inhibition of this signaling pathway may not be absolutely necessary to show protective effects in OA.

While establishing a benzamide-linked small molecule minilibrary, to clarify whether the bioactivity is mainly associated with the core benzamide-linked small molecules, a structurally simplified salicylic acid was used as a control for comparison purposes. The chondroprotective bioactivity of HS-Cf demonstrated in this report might be due to either the lipid part or the hydrophilic head of the compound. In spite of the success in elucidating the structure–activity relationship through molecular bioassays, the mechanisms observed might not fully account for the subtle bioactivity of HS-Cf. Thus, HS-Cf can be taken as a potential hit and may act as a lead structure for further screening and identifying other new hit compounds or even other lead compounds. Our approach will therefore be of help in exploring new potential compounds based on the concept of lead optimization. It is anticipated that the results from this report will bring more in vitro and in vivo studies to confirm the therapeutic benefits of benzamide-linked small molecules like HS-Cf in patients with OA and inflammation-mediated joint disorders.

Acknowledgments The authors' work was supported in part by grants from the National Science Council (NSC 98-2314-B-400-002-MY3 and NSC 100-2314-B-016-014) and from the Tri-Service General Hospital (TSGH-C98-08-S02), Taiwan, Republic of China.

Competing Interests The authors declare that they have no competing interests.

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