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Effect of a novel synthesized sulfonamido-based gallate-SZNTC on chondrocytes metabolism in vitro

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ABSTRACT

The ideal therapeutic agent for treatment of osteoarthritis (OA) should have not only potent anti-inflammatory effect but also favorable biological properties to restore cartilage function. Gallic acid (GA) and its derivatives are anti-inflammatory agents reported to have an effect on OA (Singh et al., 2003) [1]. However, GA has much weaker antioxidant effects and inferior bioactivity compared with its derivatives. We modified GA with the introduction of sulfonamide to synthesize a novel sulfonamido-based gallate named sodium salt of 3,4,5-trihydroxy-N-[4-(thiazol-2-ylsulfamoyl)-phenyl]-benzamide (SZNTC) and analyzed its chondro-protective and pharmacological effects. Comparison of SZNTC with GA and sulfathiazole sodium (ST-Na) was also performed. Results showed that SZNTC could effectively inhibit the Interleukin-1 (IL-1)-mediated induction of metalloproteinase-1 (MMP-1) and MMP-3 and could induce the expression of tissue inhibitor of metalloproteinase-1 (TIMP-1), which demonstrated ability to reduce the progression of OA. SZNTC can also exert chondro-protective effects by promoting cell proliferation and maintaining the phenotype of articular chondrocytes, as evidenced by improved cell growth, enhanced synthesis of cartilage specific markers such as aggrecan, collagen II and Sox9. Expression of the collagen I gene was effectively down-regulated, revealing the inhibition of chondrocytes dedifferentiation by SZNTC. Hypertrophy that may lead to chondrocyte ossification was also undetectable in SZNTC groups. The recommended dose of SZNTC ranges from 3.91 μg/ml to 15.64 μg/ml, among which the most profound response was observed with 7.82 μg/ml. In contrast, its source products of GA and ST-Na have a weak effect in the bioactivity of chondrocytes, which indicated the significance of this modification. This study revealed SZNTC as a promising novel agent in the treatment of chondral and osteochondral lesions.

1. Introduction

Cartilage defects are usually characterized by a structural breakdown due to trauma or disease, which may lead to chronic disabilities. After injury, catabolic factors are activated, such as pro-inflammatory cytokines that can inhibit chondrogenic differentiation and induce a gradual self-destruction of cartilage finally resulting in secondary osteoarthritis (OA) [2]. Interleukin-1 (IL-1), a well-known monocyte/macrophage product, inhibits the synthesis of proteoglycans and collagen and enhances their degradation [3,4]. IL-1 is known to mediate up-regulation of metalloproteinases (MMPs) and down-regulation of metalloproteinase-1 (TIMP-1) [5], which combined may simulate cartilage senescence and destruction in OA patients. The ideal therapeutic agent for OA would not only reduce joint inflammation but would also maintain normal cartilage function [6]. Gallic acid (GA) and its derivatives are a group of polyphenol compounds that have been known to have strong anti-oxidant [7] and anti-inflammatory [1,8,9] properties through the modulation of several important pharmacological and biochemical pathways. Gallic acid has been reported to induce apoptosis of rheumatoid arthritis (RA) fibroblast-like synoviocytes (FLS) by regulating the expression of apoptosis-related proteins and reducing the expression of pro-inflammatory mediators, such as pro-inflammatory cytokines, chemokines, COX-2 and MMP-9 [10]. Another investigation revealed that gallic acid attenuates pro-inflammatory and pro-oxidant effects [11], In addition, the bioactivity of GA is compromised because it is much more hydrophilic than its esters, resulting in much weaker anti-oxidant effects than

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its esters in cell systems [7]. GA has also been reported to suppress cell proliferation [12]. Therefore, the introduction of certain lipophilic compounds onto GA may improve its bioactivity and pharmacological effects.

The sulfonamide family, possessing a broad spectrum of synthetic bacteriostatic antibiotics, has been commonly used in the last century in human and veterinary medicine for therapeutic and prophylactic purposes for their ability to easily penetrate through membranes and into body fluids and tissues [13]. Recently, a new series of arylsulfonamido-based hydroxamates were synthesized and evaluated and were reported to be effective in blocking ex vivo cartilage degradation without side effects on cytotoxicity [14]. These compounds contained several phenyl groups and a sulfonamide group, which may illustrate the need to synthesize GA derivatives with the addition of sulfonamide groups. The work of Lin et al. showed favorable bioactivities of GA derivatives [15]. Thus, GA modified with sulfonamide may exhibit easy absorption properties that may promote its pharmacological activity.

In this study, we synthesized a sulfonamido-based gallate, sodium salt of 3,4,5-trihydroxy-N-[4-(thiazol-2-ylsulfamoyl)-phenyl]-benzamide (SZNTC) and tested its effect on IL-1-stimulated chondrocytes and the restoration of chondrocytes. Comparison of SZNTC with its substrates, GA and sulfathiazole sodium (ST-Na), was also performed. We hypothesized that GA-sulfonamide composites may enhance the bioactivity of GA. This study may be helpful in developing a new agent for the treatment of OA.

2. Materials and methods

2.1. The synthesis of SZNTC

Electrospray ionization mass spectrometry (ESI-MS) was recorded on a Shimadzu LC-MS 2010A. ^{1}H and ^{13}C NMR spectra were obtained from a Bruker Advance III 300 at 400 and 125 MHz, respectively.

The compound sodium salt of 3,4,5-trihydroxy-N-[4-(thiazol-2-ylsulfamoyl)-phenyl]-benzamide (SZNTC) was prepared from gallic acid (GA) and sulfathiazole sodium (ST-Na). The synthetic strategy is presented in Fig. 1 in detail. After reactions, distilled water was added to the mixture, and then the raw product precipitated and was separated by vacuum filtration. The raw product was recrystallized in a THF-methanol solvent system.

SZNTC has the following properties: pale yellow powder, yield 59%, m.p. > 255 °C, MS-ESI: m/z: 529.2$[M-H]^{-}$, ^{1}H–NMR (400 MHz, DMSO) δ 10.22(s, 1H, –CO–NH), 7.91 (m, 4H, 2 × Ar–H), 6.94(s, 2H, Ar–H), 6.73 (s, 1H, Py–H), and 2.23(s, 6H, 2 × $-CH_3$). ^{13}C-NMR (125 MHz, DMSO) δ 165.99, 156.30, 145.57, 143.39, 137.29, 129.05, 124.42, 118.99, 107.84 and 22.95.

SZNTC was dissolved in dimethylsulfoxide (DMSO, Sigma, USA). ST-Na and GA were dissolved in double distilled water respectively. The stock solutions were stored at −4 °C.

2.2. Articular chondrocytes culture

Articular chondrocytes were harvested from knee joint cartilage slices of 1-week-old New Zealand rabbits by enzymatic digestion. In brief, cartilage slices from two rabbits were dissociated enzymatically with 0.25% trypsin (Solarbio, China) for 30 min and then with 2 mg/ml collagenase type II (Gibco, USA) in alpha-modified Eagle's medium (α-MEM, Gibco, USA) for 3 h. After centrifugation, the chondrocytes were resuspended. Cells were cultured with alpha-modified Eagle's medium (α-MEM, Gibco, USA) containing 20% (v/v) fetal bovine serum (FBS) (Gibco, USA) and 1% (v/v) penicillin/streptomycin (Solarbio, China) in a 5% CO_2 humidified incubator at 37 °C with the culture medium replaced every other day after plating. Articular chondrocytes at passage 2 were used for further studies.

2.3. Cytotoxicity assay

Cytotoxicity in chondrocytes was assessed by the 3-(4,5)-dimethylthiahiazo(-z-y1)-3,5-di-phenytetrazolium-romide (MTT, Gibco, USA) method. Articular chondrocytes were cultured with 200 μl of culture medium in 96-well microplates with preliminary treatment of various concentrations of SZNTC, ST-Na and GA for 3 d. Twenty microliter of 5 mg/ml MTT was added, and the plates were incubated in the dark at 37 °C for 4 h. After removal of the MTT, cells were treated with 150 μl dimethyl sulfoxide (DMSO, Gibco, USA) to dissolve the formazan product. The absorbance was determined at 570 nm using an enzyme-labeled instrument (Thermo Fisher Scientific, UK).

As determined by MTT analysis, the concentrations of SZNTC at 3.91, 7.82 and 15.64 μg/ml; of ST-Na at 1.95, 3.9 and 7.8 μg/ml; and of GA at 1.95, 3.9 and 7.8 μg/ml, among which a peak was presented, were chosen for further investigation.

2.4. IL-1 induced chondrocytes

To investigate the effects of SZNTC, ST-Na and GA on Interleukin-1 (IL-1) on the induction of chondrocytes, five groups were divided: (1) control group, chondrocytes without any treatment;

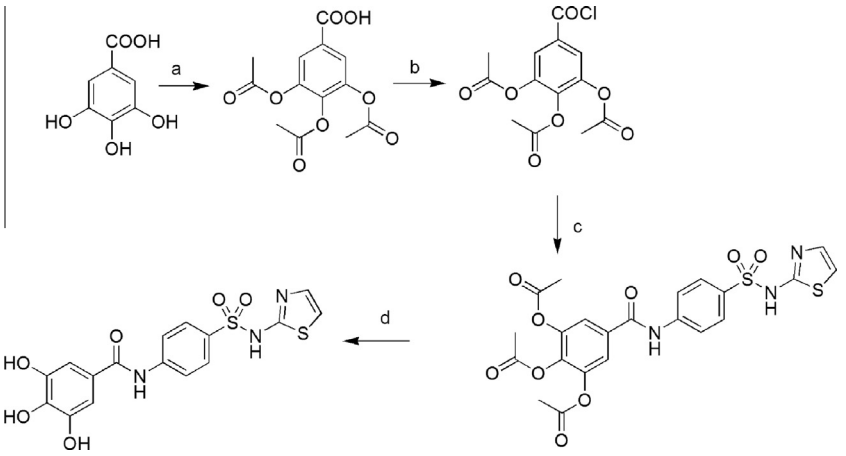

Fig. 1. Schematic description of R synthesis route and synthetic procedures of SZNTC. Reagents and conditions: (a) acetyl oxide, oil bath, 120 °C (b) $SOCl_2$, oil bath, 80 °C (c) sulfathiazole sodium, THF, pyridine, ice bath (d) HCl, THF, 60 °C.

(2) OA model group, chondrocytes treated with 10 ng/ml IL-1β (10 ng/ml, Gibco, USA); (3) SZNTC treatment groups, chondrocytes pre-incubated with various concentrations of SZNTC (3.91, 7.82 and 15.64 μg/ml) for 1 h followed by stimulation with IL-1β for 24 h; (4) ST-Na treatment groups, chondrocytes pre-incubated with various concentrations of ST-Na (1.95, 3.9 and 7.80 μg/ml) for 1 h followed by stimulation with IL-1β for 24 h; (5) GA treatment groups, chondrocytes pre-incubated with various concentrations of GA (1.95, 3.9 and 7.80 μg/ml) for 1 h followed by stimulation with IL-1β for 24 h. The concentrations of SZNTC, ST-Na and GA were derived from the cytotoxicity assay.

2.5. Cell proliferation analysis and biochemical assay

Chondrocytes were then continuously treated with SZNTC, ST-Na and GA for 2, 4 and 6 d with the culture medium changed every 2 d. Cells treated for 2, 4 and 6 d were digested with proteinase K (Sigma, USA) for the following biochemical assay. Intracellular glycosaminoglycan (GAG) secretion was assayed with 1,9-dimethylmethylene blue (DMMB; Sigma, USA) dye, and the DNA content was quantified by Hoechst 33258 dye (Sigma, USA) assessment. The absorbance value of total intracellular DNA content in each sample was measured with a spectrofluorometer using Hoechst 33258 dye at 460 nm with calf thymus DNA as a standard. The total intracellular glycosaminoglycan (GAG) secretion was quantified spectrophotometrically at 525 nm with chondroitin sulfate (Sigma, USA) as a standard. Finally, the GAG content was normalized to the total DNA content of the chondrocytes.

2.6. Morphological examination

Cells of the control, SZNTC, ST-Na and GA groups were removed from the incubator at 6 d respectively, and were then fixed in 95% alcohol for subsequent Hematoxylin-eosin (HE, JianCheng Biotech, China) staining. Cells were incubated with a nuclear dye for 3 min and then with a cytoplasmic dye for 5 s. Subsequently, the cells were rinsed by PBS, naturally dried and sealed with neutral gum. Cells were then examined and photographed utilizing an inverted phase contrast microscope (Zeiss Corporation, Germany).

Another portion of the chondrocytes was used for the detection of actin filaments. In brief, cells were fixed with 4% paraformaldehyde (PFA, Sigma, USA) for 10 min at room temperature. After a rinse with PBS, cells were treated with 0.5% Triton X-100 (Sigma Aldrich, USA) for 5 min. Cells were treated with rhodamine phalloidin (Invitrogen, USA) for 30 min at room temperature in the dark to label the cellular matrix. After double-staining with Hoechst 33258 (Beyotime, USA) away from light for 5 min, fluorescence was detected with a laser scanning confocal microscope (Nikon A1, Japan).

2.7. Safranin O staining

Histology was performed to assess the synthesis of glycosaminoglycans (GAGs) using Safranin O staining. The cells after being fixed by 95% alcohol for 30 min were successively incubated with 0.1% Safranin O (Sigma, USA) for 10 min. Subsequently, the cells were rinsed with tap water and then dried naturally. Eventually, the cells were sealed with neutral gum, observed and photographed by an inverted phase contrast microscope (Zeiss Corporation, Germany).

2.8. Cell viability assay

Cell viability was determined with a live-dead viability assay kit (Invitrogen, USA). In brief, cells were quickly rinsed with PBS, and then, 1 μM calcein-AM and 1 μM PI were added to the cell cultures and incubated in the dark for 5 min at 37 °C. After rinsing with PBS, the images were captured using a laser scanning confocal microscope (Nikon A1, Japan).

2.9. Immunohistochemical staining

The secretion of collagen types I and II, MMP-1 and TIMP-1 were performed immunohistochemically with an immunohistochemical staining kit (Bioss, China). To visualize protein, cells were fixed in 4% (w/v) paraformaldehyde and treated with Triton X-100. To exclude endogenous peroxidase activity, cells were incubated with 3% H_2O_2 for 10 min at room temperature. Cells were blocked with normal goat serum for 10 min at room temperature. After a 1:200 dilution of rat anti-rabbit antibody (collagen type I and II) was added, cells were then incubated with the second antibody and biotin labeled horseradish peroxidase. Subsequently, the antibody binding was visualized with a 3,3′-diaminobenzidine tetrahydrochloride (DAB) kit (Boster, China) before brief counterstaining with hematoxylin. Eventually, cells were gradually dehydrated, sealed with neutral gum, observed and photographed with an inverted phase contrast microscope (Zeiss Corporation, Germany).

2.10. Real-time quantitative PCR (qRT-PCR) analysis

The genetic information was detected by qRT-PCR for type I, II and X collagen, aggrecan, Sox9, MMP-1, MMP-3 and TIMP-1. Total intracellular RNA was extracted with an RNA isolation kit (Tiangen Biotechnology; Beijing, China) according to the manufacturer's instructions. Approximately 300 ng of total RNA was used as a template and reverse transcribed into cDNA using a reverse transcription kit (Fermentas Company, USA). The qRT-PCR reactions were performed using a Quantitative PCR Detection System (Realplex 4, Eppendorf Corporation, USA) with a FastStart Universal SYBR Green Master (Mix, Roche company, Germany) under the condition of 10 min at 95 °C, 15 s at 95°Cand 1 min at 60 °C. The primers used for PCR were designed as follows (Table 1). The melting curve data were collected to verify PCR specificity. Each gene was analyzed in triplicate to diminish operation errors. The relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method using glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Table 1
Primer sequences used in qRT-PCR experiments.

mRNA	Forward primer	Reverse primer
GAPDH	5′-GTCATCATCTCAGCCCCCTC-3′	5′-GGATGCGTTGCTGACAATCT-3′
Aggrecan	5′-TTGCCTTTGTGGACACCAGT-3′	5′-GAGCCAAGGACGTAAACCCA-3′
Type I collagen	5′-CCCAGCCACCTCAAGAGAAG-3′	5′-CGGGGCTCTTGATGTTCTCA-3′
Type II collagen	5′-TCCGGAAACCAGGACCAAAG-3′	5′-CTTTGTCACCACGGTCACCT-3′
Type X collagen	5′-CTACGCTGAGCGGTACCAAA-3′	5′-GGCTTCCCAGTGGCTGATAG-3′
Sox9	5′-GACGCACATCTCGCCCAAC-3′	5′-TCTCGCTTCAGGTCAGCCTT-3′
MMP-1	5′-GGCATTGGAGGGGATGTTCA-3′	5′-GGCTGACTGGGATTTTGGGA-3′
MMP-3	5′-TTCCAACCCTGCTACTGCTG-3′	5′-TCACCTCCAAGCCAAGGAACA-3′
TIMP-1	5′-CTGCGGGTACTCCCACAAAT-3′	5′-CCTAGGAGGAGCTGGTCTGT-3′

2.11. Statistical analysis

Results were presented as the means ± SD. Significant differences were determined using one way analysis of variance (ANOVA) followed by Dunnett's post hoc test. The level of significance was set to $P < 0.05$.

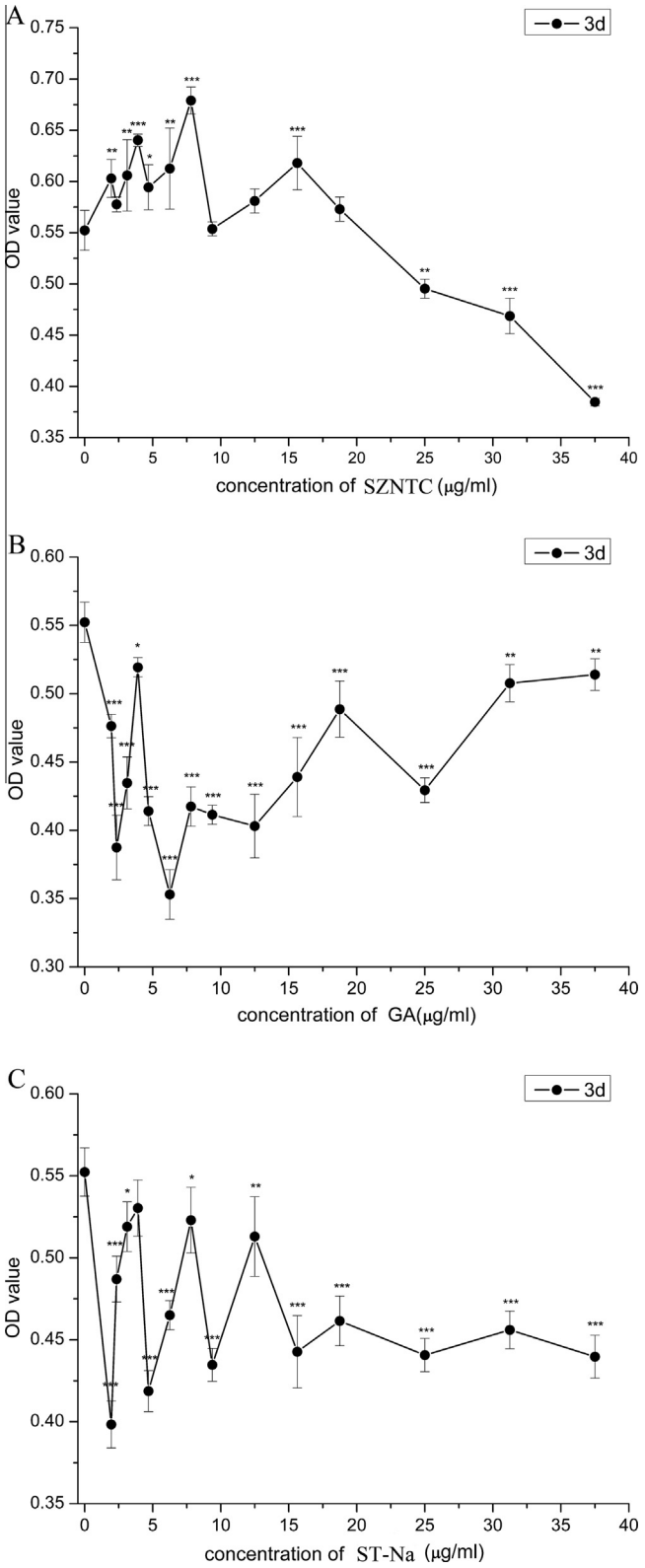

Fig. 2. Cytotoxicity of SZNTC, GA and ST-Na on chondrocytes after 3 d (mean ± SD, $n = 4$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

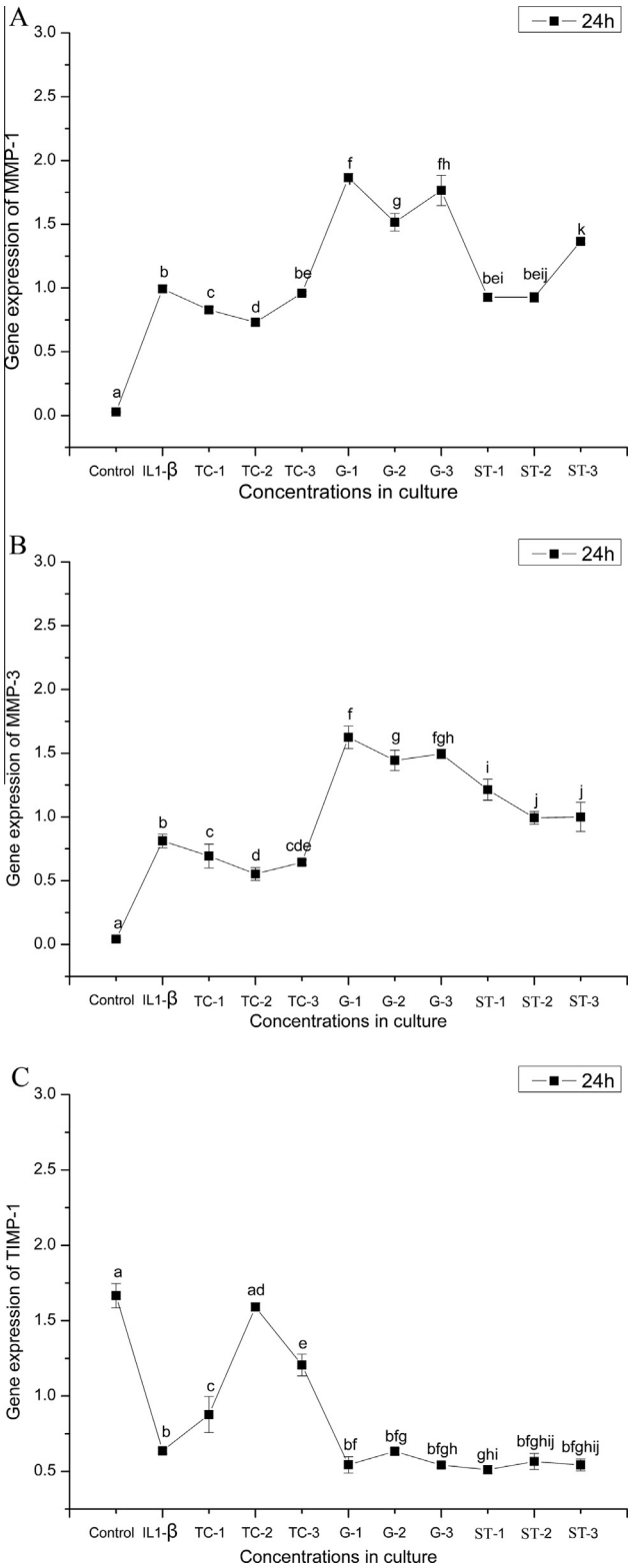

Fig. 3. Quantitative comparison of ECM-related gene expression of MMP-1 (A), MMP-3 (B) and TIMP-1 (C) by qRT-PCR. The chondrocytes were cultured with different concentrations of SZNTC, GA and ST-Na on the induction by IL-1β for 24 h (Control: 0 μg/ml; IL-1β: 10 ng/mL; TC-1: 3.91 μg/ml; TC-2: 7.82 μg/ml; TC-3: 15.64 μg/ml; G-1: 1.95 μg/ml; G-2: 3.9 μg/ml; G-3: 7.8 μg/ml; ST-1: 1.95 μg/ml; ST-2: 3.9 μg/ml; ST-3: 7.8 μg/ml) ($n = 3$ for each experiment). The gene expression levels in SZNTC, GA and ST-Na media relative to the control group were analysed by the $2^{-\Delta\Delta CT}$ method using GAPDH as the internal control. The data represent the mean ± SD of three independent culture experiments. Bars with different letters are significantly different from each other at $P < 0.05$.

3. Results

3.1. Cytotoxicity assay

This study examined the cytotoxicity of different drugs on articular chondrocytes by MTT assay. Articular chondrocytes were treated with SZNTC, ST-Na or GA in increasing concentrations (1.95–37.5 μg/ml). As shown in Fig. 2, SZNTC concentrations ranging from 1.95 to 18.75 μg/ml were comparable to the control and therefore nontoxic to cells. However, SZNTC exhibited an inhibitive effect on chondrocytes at the concentrations ranging from 25 to 37.5 μg/ml. Comparatively, ST-Na and GA exhibited insignificant or inhibitive effects on chondrocyte growth at different levels ranging from 1.95 to 37.5 μg/ml. Hence, concentrations of SZNTC over the range 3.90–15.62 μg/ml which can significantly improve the cell proliferation were used in subsequent assays, whereas concentrations of ST-Na and GA ranging from 1.95 to 7.80 μg/ml and 1.95 to 7.80 μg/ml, respectively, were utilized.

3.2. Effects of SZNTC, ST-Na and GA on IL-1β-induced chondrocytes

To investigate the effects of SZNTC, ST-Na and GA on arthritis, chondrocytes were pre-incubated with SZNTC, ST-Na and GA for 1 h prior to stimulation with IL-1β for 24 h. Chondrocytes stimulated by IL-1β exhibited upregulation of MMP-1 and MMP-3 gene expression, and downregulation of TIMP-1 expression. In contrast, SZNTC inhibited the IL-1β-mediated induction of MMP-1 and MMP-3 gene expression and induced the expression of TIMP-1. However, ST-Na and GA could not effectively prevent the induction of MMP-1 and MMP-3 gene expression or upregulate TIMP-1 expression (Fig. 3). We next examined the effects of IL-1β, SZNTC, ST-Na and GA on protein secretion of MMP-1 and TIMP-1 in chondrocytes. Treatment with IL-1β resulted in the upregulation of MMP-1and the downregulation of TIMP-1 at the protein level. In agreement with the results of RT-PCR, SZNTC (but not ST-Na and GA) could downregulate MMP-1 expression and upregulate TIMP-1 expression, as demonstrated with higher levels of staining

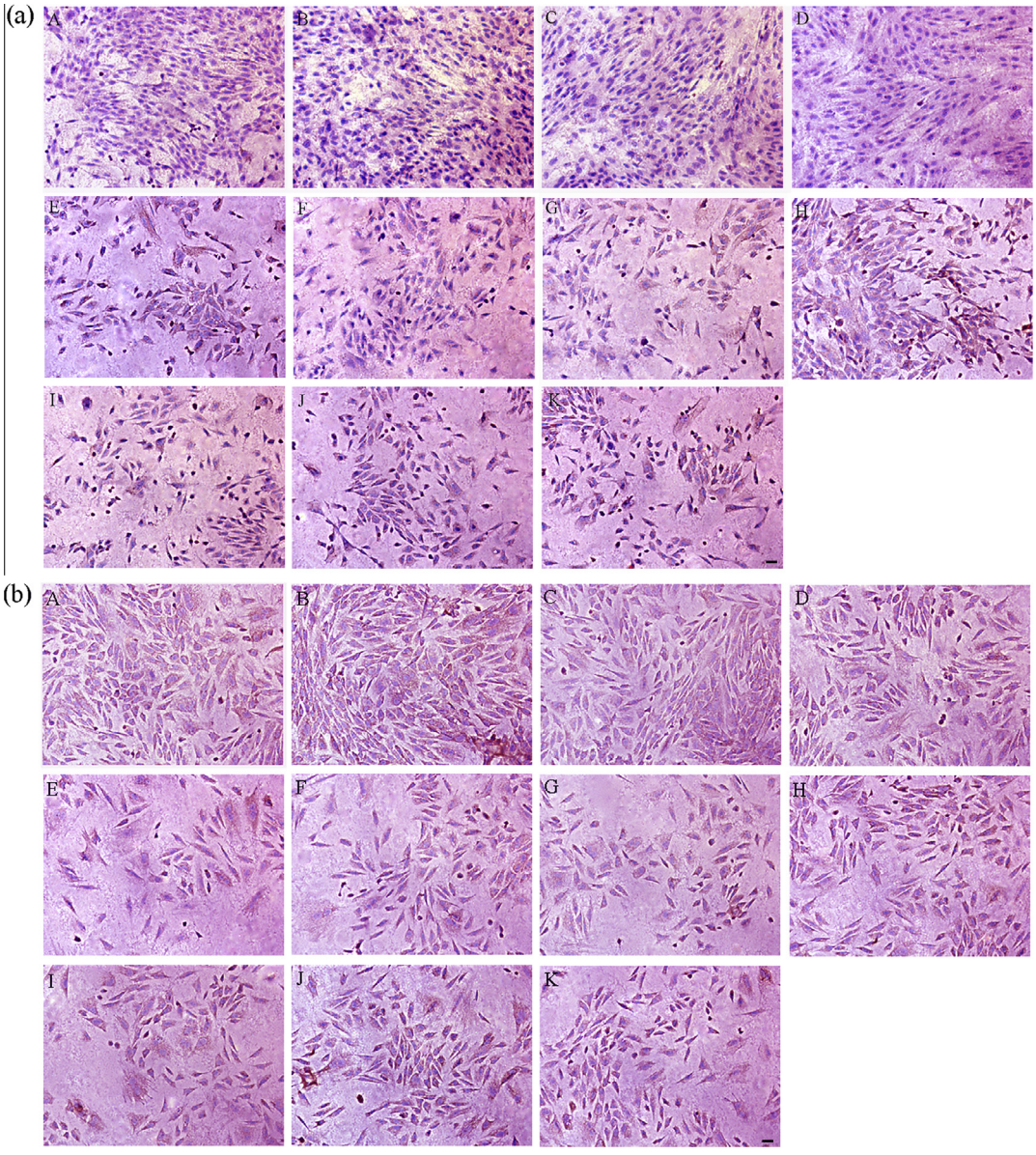

Fig. 4. Immunohistochemical staining images revealed the presence of MMP-1 (a) and TIMP-1(b). Chondrocytes cultured in vitro with different concentrations of SZNTC, GA and ST-Na on the induction by IL-1β for 24 h: (A) 3.91 μg/ml; (B) 7.82 μg/ml; (C) 15.64 μg/ml; (D) control (0 μg/ml); (E) 1.95 μg/ml; (F) 3.9 μg/ml; (G) 7.8 μg/ml; (H) IL-1β (10 ng/ml); (I) 1.95 μg/ml; (J) 3.9 μg/ml; (K) 7.8 μg/ml; cell seeding density: 2×10^4/ml (original magnification × 100). Scale bar = 200 μm.

for MMP-1 and lower levels of staining for TIMP-1. These results suggested that the IL-1β-mediated induction of MMPs and downregulation of TIMP-1 were effectively blocked by SZNTC whereas these effects could not be prevented by ST-Na or GA (Fig. 4, Table 2).

3.3. Cell proliferation

In this study, the cell proliferation in experimental groups and control groups was analyzed by measurements of DNA content. Comparatively, cells cultured with SZNTC grew faster than those in the control group ($P < 0.05$), as shown by DNA values obviously higher than the control in the same culture period. Oppositely, cells treated with ST-Na and GA grew slower than those in the control group ($P < 0.05$), which agreed with DNA values results. Furthermore, among all SZNTC groups, the highest cell proliferation was achieved at a concentration of 7.82 μg/ml. These data indicated that SZNTC exhibited the strongest promoting effect on chondrocyte growth, especially at the concentration of 7.82 μg/ml (Fig. 5A).

3.4. Secretion of GAGs

To determine if extracellular GAG production was affected by SZNTC, ST-Na and GA, biochemical assays were performed after 2, 4 and 6 d of culture. Results of intracellular GAG production treated by different concentrations of SZNTC, ST-Na and GA (Fig. 5B) showed that GAGs gradually accumulated in all groups. Comparatively, GAG production in culture media treated with SZNTC was significantly improved over that in the control at the same time point, whereas reduced tendency was obviously observed in ST-Na and GA groups compared with the control. Particularly, SZNTC at a concentration of 7.82 μg/ml exhibited the strongest promotion GAG synthesis among the three concentrations.

The Safranin O-positive stain (Fig. 6b) in the SZNTC group indicated that GAGs were abundant and homogeneously distributed around the chondrocytes. Comparison of ST-Na and GA groups with the control revealed indigent GAGs. The result of Safranin O staining conformed to GAG production by biochemical analysis (Fig. 5B).

Table 2
MMP-1 and TIMP-1 expression.

	MMP-1 expression Positive area	TIMP-1 expression Positive area
A (3.91 μg/ml)	3.5%	67.1%
B (7.82 μg/ml)	2.1%	83.5%
C (15.64 μg/ml)	2.5%	79.2%
D (0 μg/ml)	1.6%	84.7%
E (1.95 μg/ml)	41.4%	29.3%
F (3.9 μg/ml)	30.6%	33.6%
G (7.8 μg/ml)	34.9%	28.3%
H (10 ng/ml)	23.2%	40.1%
I (1.95 μg/ml)	30.4%	33.7%
J (3.9 μg/ml)	27.5%	38.4%
K (7.8 μg/ml)	32.3%	35.6%

3.5. Cell morphology

We assessed the morphology of articular chondrocytes with an inverted microscope after treatment with SZNTC (3.91, 7.82 and 15.64 μg/ml), ST-Na (1.95, 3.9 and 7.8 μg/ml) and GA (1.95, 3.9 and 7.8 μg/ml) (Fig. 6a). There was no significant difference in cartilaginous morphology in all any group after 6 d of culture. Compared with the control, the chondrocytes in the presence of SZNTC grew better and had a distinctive proliferation tendency that gradually increased with time. In addition, at the concentration of 7.82 μg/ml, SZNTC could better enhance the proliferation of chondrocytes over that of the other two concentrations. Nevertheless, these effects were not exhibited by the other two experimental groups treated with ST-Na and GA. Fig. 7b shows the actin filaments of chondrocytes by staining with rhodamine phalloidin/Hoechst 33258, which was in agreement with the HE analysis. The cells in the SZNTC treated groups grew in clumps with a densely distributed ECM. In the ST-Na and GA groups, fewer cells and less ECM were present compared with the control.

3.6. Cell viability assay

Viable cells and dead cells were determined using calcein-AM/PI staining (Fig. 7a). The results demonstrated that SZNTC exerted potent effects, whereas ST-Na and GA demonstrated an inhibitory effect on chondrocytes survival under identical culture conditions. Calcein-AM/PI staining images displayed that survival in SZNTC groups was higher than observed in the control; however, viable

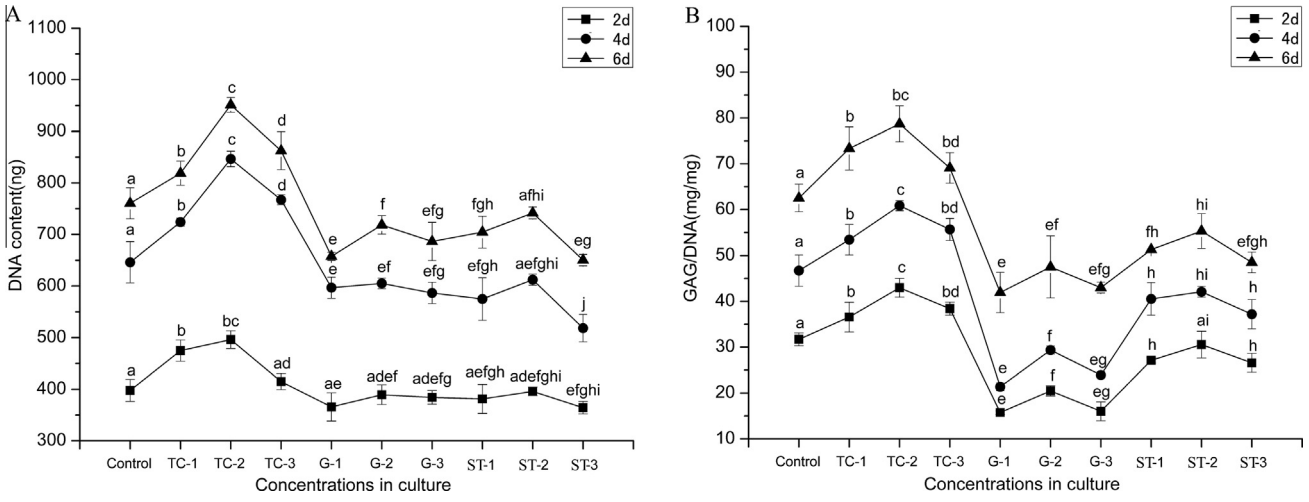

Fig. 5. Quantification of cell proliferation (DNA) and matrix production (glycosaminoglycan (GAG)) of cells by biochemical assays: (A) the proliferation of chondrocytes cultured with different concentrations of SZNTC, GA and ST-Na in vitro for 2, 4 and 6 d (control: 0 μg/ml; TC-1: 3.91 μg/ml; TC-2: 7.82 μg/ml; TC-3: 15.64 μg/ml; G-1: 1.95 μg/ml; G-2: 3.9 μg/ml; G-3: 7.8 μg/ml; ST-1: 1.95 μg/ml; ST-2: 3.9 μg/ml; ST-3: 7.8 μg/ml); (B) GAG (mg) normalized to DNA (mg). Data from four independent experiments were evaluated, and the mean ± SD is shown. Bars with different letters are significantly different from each other at $P < 0.05$.

chondrocytes in the ST-Na and GA groups was significantly less than that in the control. Consistent with the result of cell proliferation (Fig. 5A), more viable cells were found than dead cells in the SZNTC groups, implying that SZNTC could better support cell growth compared with ST-Na and GA. Among the SZNTC groups, SZNTC at a concentration of 7.82 μg/ml was superior to others, as evidenced by more viable cells.

3.7. Secretion of type I and type II collagen

Expression of type I and type II collagen in the cytoplasm at different levels with and without drugs-treated culture media was shown in Fig. 8 and Table 3. Strongly positive staining with large areas was evident for cartilage-specific type II collagen and only very sparse and light staining was seen for type I collagen in the SZNTC groups compared with control groups after incubation for 6 d, confirming the maintenance of the chondrocytic phenotype after treatment with SZNTC. In addition, in the ST-Na and GA groups, not only was collagen II staining weaker than the control but collagen I staining was also stronger than the control. These results indicated that SZNTC may more effectively inhibit de-differentiation of chondrocytes cultured in vitro than ST-Na and GA.

3.8. Gene expression

The effects of SZNTC, ST-Na and GA on chondrocyte ECM synthesis were further inspected through examination of gene expression of collagen I, collagen II, collagen X, Sox9, aggrecan (a proteoglycan composed of GAGs) after 2, 4 and 6 d of culture. As shown in Fig. 9, cartilage specific gene expressions, such as aggrecan, collagen II and Sox9, were significantly boosted by SZNTC at concentrations ranging from 3.91 to 15.64 μg/ml but were markedly reduced by ST-Na and GA. Moreover, the highest collagen II, aggrecan and Sox9 expressions in the SZNTC group were accompanied with the 7.82 μg/ml concentration. The presence of SZNTC up-regulated collagen II, aggrecan and Sox9 expressions, suggesting

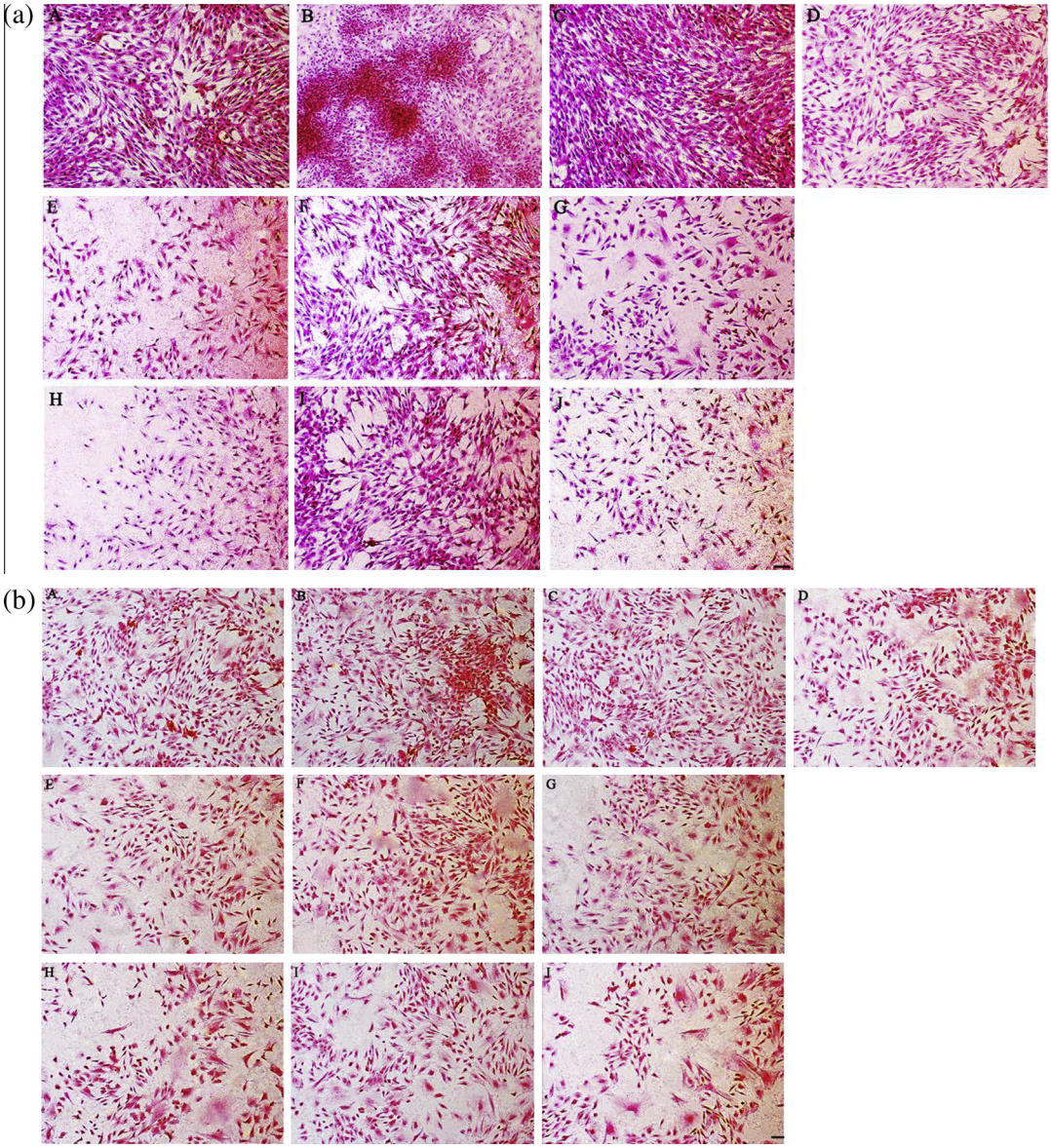

Fig. 6. Hematoxylin-eosin staining and Safranin O/fast green staining images respectively showing the morphology (a) and GAG production (b) of chondrocytes cultured in vitro with different concentrations of SZNTC, GA and ST-Na for 6 d: (A) 3.91 μg/ml; (B) 7.82 μg/ml; (C) 15.64 μg/ml; (D) control (0 μg/ml); (E) 1.95 μg/ml; (F) 3.9 μg/ml; (G) 7.8 μg/ml; (H) 1.95 μg/ml; (I) 3.9 μg/ml; (J) 7.8 μg/ml; cell seeding density: 2×10^4/ml (original magnification × 100). Scale bar = 200 μm.

that SZNTC either delayed or prevented the chondrocytes from dedifferentiating into a hypertrophic phenotype; correspondingly, ST-Na and GA may insignificantly influence the differentiation of chondrocytes. At the same time, collagen X expression was scarcely detectable in all groups, suggesting that cell hypertrophy was not prominent.

SZNTC at different concentrations induced lower collagen I expression, whereas higher or similar collagen I expressions were surveyed in the ST-Na and GA group when compared with the control group after being cultured for 2, 4 and 6 d. Moreover, the levels of collagen I at a concentration of 7.82 μg/ml were lower than that of the other two concentrations in the SZNTC group. These results further hinted that SZNTC could inhibit the dedifferentiation of chondrocytes but ST-Na and GA could not do so.

Therefore, SZNTC ranging from 3.91 to 15.64 μg/ml could upregulate the synthesis of cartilage specific markers while downregulating dedifferentiation related genes. The effects of ST-Na and GA are the opposite of SZNTC. Among all of the groups, SZNTC at

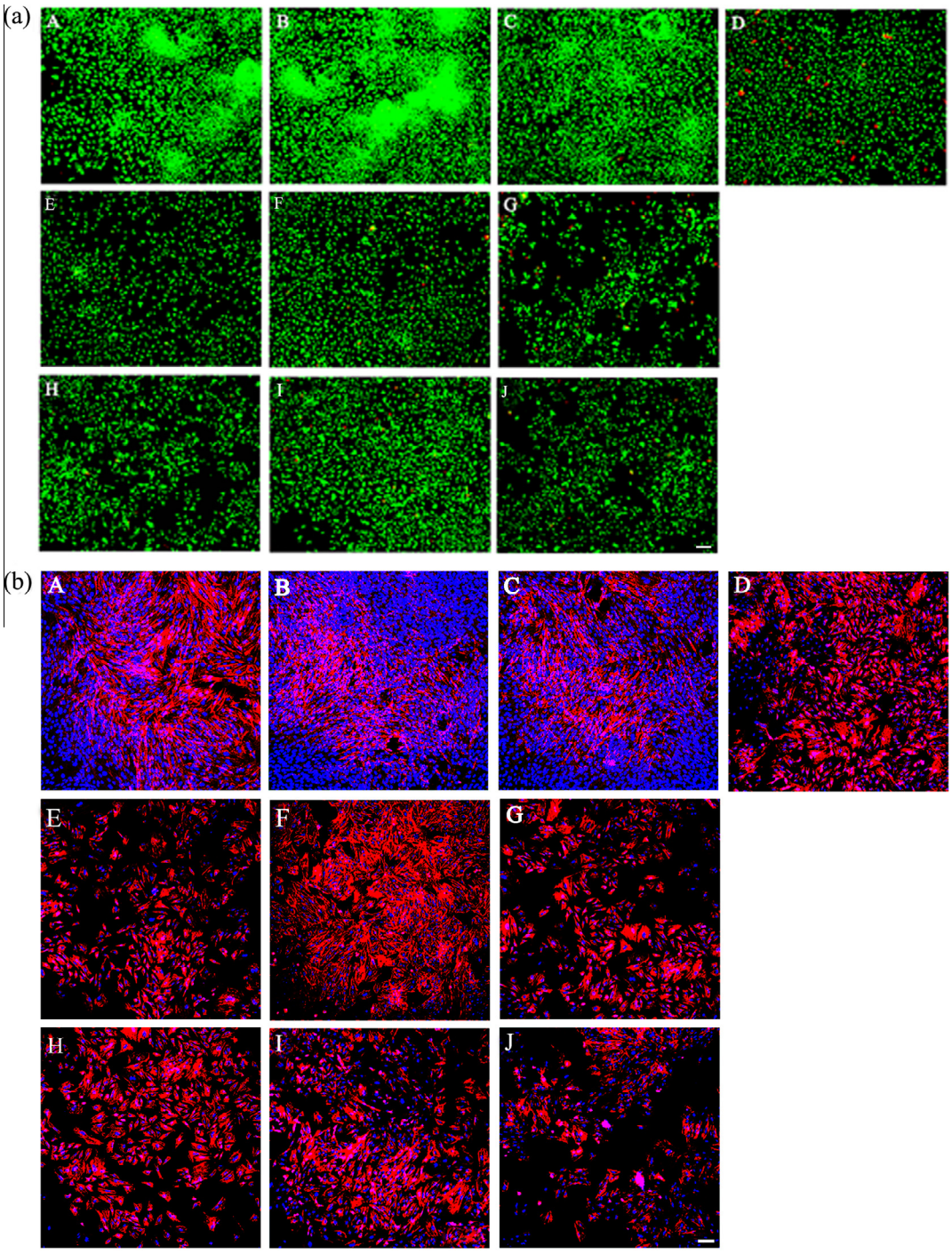

Fig. 7. Confocal laser scanning microscopy images showing the viability (a) and actin filaments (b) of chondrocytes cultured in vitro with different concentrations of SZNTC, GA and ST-Na for 6 d: (A) 3.91 μg/ml; (B) 7.82 μg/ml; (C) 15.64 μg/ml; (D) control (0 μg/ml); (E) 1.95 μg/ml; (F) 3.9 μg/ml; (G) 7.8 μg/ml; (H) 1.95 μg/ml; (I) 3.9 μg/ml; (J) 7.8 μg/ml; cell seeding density: 2×10^4/mL (original magnification × 100). Scale bar = 200 μm.

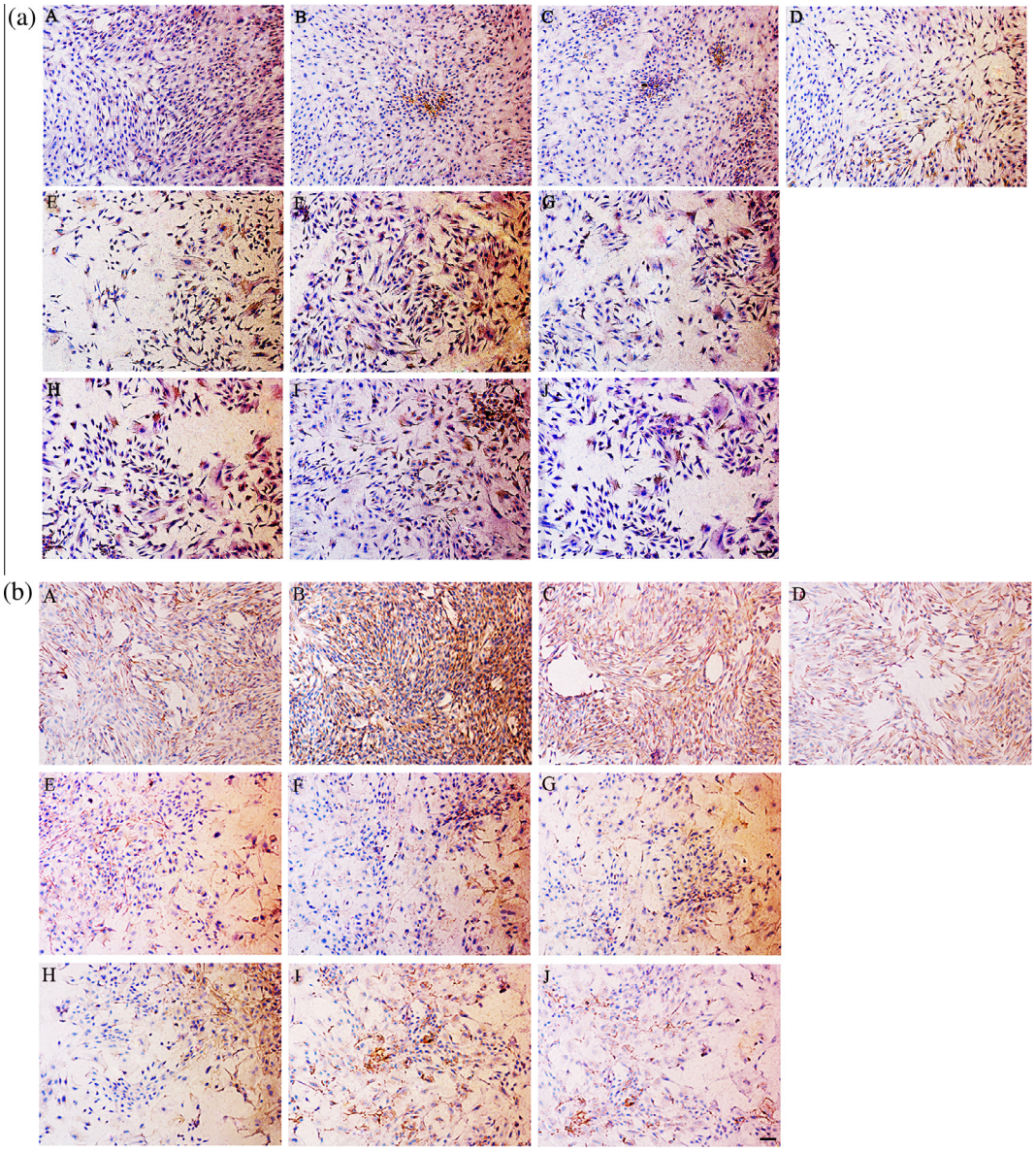

Fig. 8. Immunohistochemical staining images revealed the presence of type I (a) and type II (b) collagen. Chondrocytes cultured in vitro with different concentrations of SZNTC, GA and ST-Na for 6 d: (A) 3.91 μg/ml; (B) 7.82 μg/ml; (C) 15.64 μg/ml; (D) Control (0 μg/ml); (E) 1.95 μg/ml; (F) 3.9 μg/ml; (G) 7.8 μg/ml; (H) 1.95 μg/ml; (I) 3.9 μg/ml; (J) 7.8 μg/ml; cell seeding density: 2×10^4/ml (original magnification × 100). Scale bar = 200 μm.

Table 3
Type I collagen and type II collagen expression.

	Type I collagen expression Positive area	Type II collagen expression Positive area
A (3.91 μg/ml)	18.9%	90.9%
B (7.82 μg/ml)	10.2%	94.5%
C (15.64 μg/ml)	5.1%	93.5%
D (0 μg/ml)	7.7%	79.4%
E (1.95 μg/ml)	23.9%	36.8%
F (3.9 μg/ml)	20.1%	59.5%
G (7.8 μg/ml)	22.1%	48.7%
H (1.95 μg/ml)	25.8%	31.2%
I (3.9 μg/ml)	21.0%	67.3%
J (7.8 μg/ml)	24.1%	37.7%

the concentration of 7.82 μg/ml demonstrated the strongest aggrecan and collagen II expression, which was in agreement with the results of GAG production (Fig. 5B, Fig. 6b).

4. Discussion

GA was reported to have an effect on OA. However, GA has much weaker antioxidant effects than its esters and inferior bioactivity, which may be due to its hydrophilicity. Based on the hypothesis that synthetic compounds of gallates and sulfonamides may enhance its chondro-protective and pharmacological effects, we synthesized SZNTC and examined its effects.

Many studies have demonstrated that IL-1 inhibits chondrocyte compensatory biosynthesis pathways, which can further compromise cartilage repair [16]. Chondrocytes stimulated with IL-1β in vitro have been exploited to imitate the microenvironment that occurs in osteoarthritis (OA) [17]. IL-1β is known to exert a key role in cartilage degradation, through the induction of MMPs secreted by chondrocytes. Targeting MMPs is a promising approach to the treatment of OA because the MMPs, particularly MMP-1 and MMP-13, are interstitial collagenases that degrade type II collagen in the cartilage, which is a critical step in the progression of OA. In

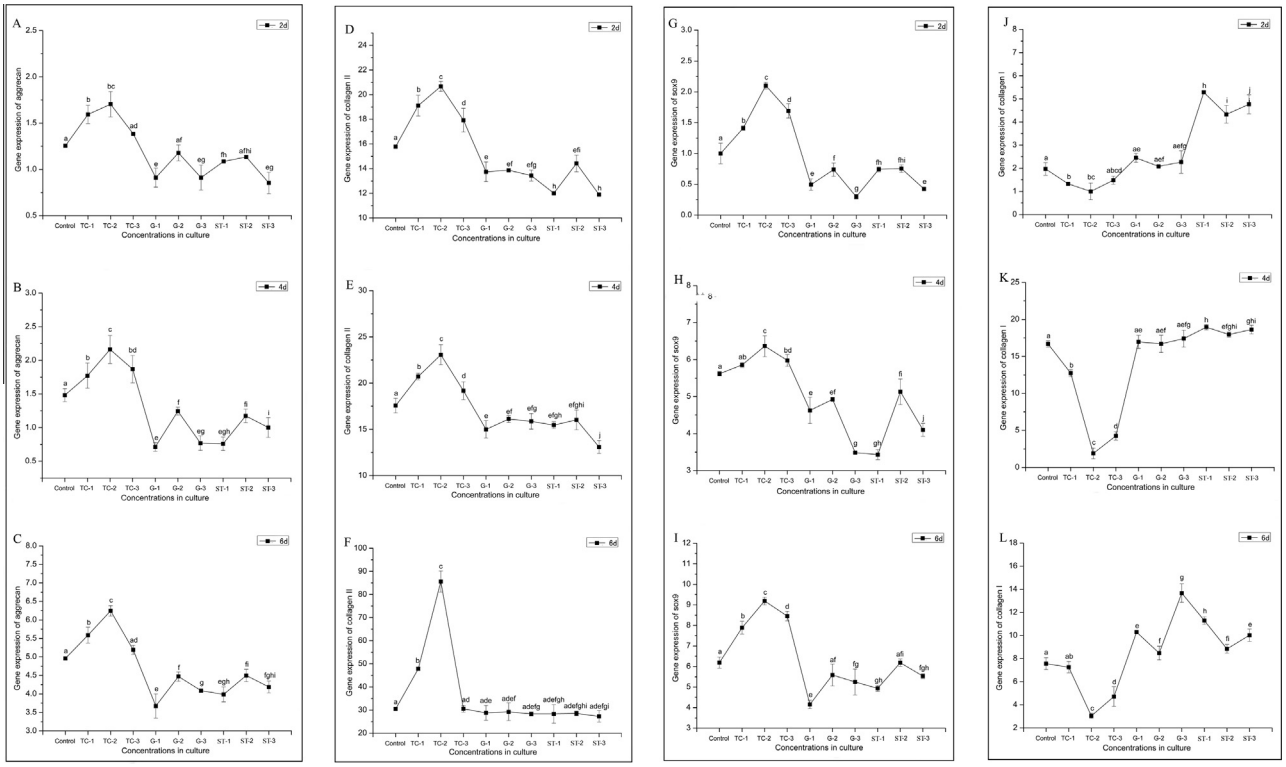

Fig. 9. Quantitative comparison of ECM-related gene expression of aggrecan (A, B, C), collagen II (D, E, F), Sox9 (G, H, I) and collagen I (J, K, L) by qRT-PCR. The chondrocytes were cultured with different concentrations of SZNTC, GA and ST-Na for 2, 4 and 6 d (Control: 0 μg/ml; TC-1: 3.91 μg/ml; TC-2: 7.82 μg/ml; TC-3: 15.64 μg/ml; G-1: 1.95 μg/ml; G-2: 3.9 μg/ml; G-3: 7.8 μg/ml; ST-1: 1.95 μg/ml; ST-2: 3.9 μg/ml; ST-3: 7.8 μg/ml) (n = 3 for each experiment). The gene expression levels in SZNTC, GA and ST-Na media relative to the control group were analyzed by the $2^{-\Delta\Delta CT}$ method using GAPDH as the internal control. The data represent the means ± SD of three independent culture experiments. Bars with different letters are significantly different from each other at $P < 0.05$.

the present study, we utilized IL-1β to induce MMP gene expression, and then assessed the effects of SZNTC, ST-Na and GA on MMP induction in rabbit articular chondrocytes. We observed that SZNTC inhibited the IL-1β-mediated induction of MMP-1 and MMP-3 and induced the expression of TIMP-1, a metalloproteinase inhibitor, in rabbit articular chondrocytes (Fig. 3). However, its substrates, including GA and ST-Na, exhibit weaker effects. These results demonstrated that SZNTC has the potential to be developed as potent anti-inflammatory agent in the treatment of OA.

The results indicated that SZNTC could observably promote chondrocytes proliferation (Fig. 5A). SZNTC also markedly promoted GAGs deposition in cultured chondrocytes, which was shown with a biochemical assay (Fig. 5B). Proteoglycans (PGs) are important components of extracellular matrices [18]. For all PGs, glycosaminoglycans (GAGs) constitute a major component of their molecular mass; moreover, GAG and a large number of water molecules generate the expansion pressure and make the cartilage flexible, which plays an important role in maintaining cartilage load-bearing capacity [19]. Consistent with the increase in GAG production, SZNTC could upregulate the gene expression of cartilage-specific aggrecan, collagen II and Sox9 (Fig. 9). Chondrogenic transcription factor Sox9 played a major role in an increased level of chondrogenesis [20,21], in particular activating co-expression with collagen type II [22–24]. In addition, extensive gene therapy approaches using viral methods to over-express Sox9 resulted in marked improvements in the secretion of cartilaginous matrix by articular chondrocytes, bone marrow–derived stem cells and nucleus pulposus cells [25–27]. These data hinted that SZNTC could facilitate chondrocytes proliferation and stimulate exuberant cartilage matrix secretion.

In addition, the expression of collagen type I, which marks dedifferentiation of chondrocytes, was effectively inhibited by SZNTC. Dedifferentiation happens when the differentiated phenotype of chondrocytes, primarily composed of type II collagen and cartilage-specific proteoglycans, is bereaved and replaced by a complex collagen phenotype consisting of the vast majority of type I collagen and a low level of proteoglycan synthesis [28–30]. Furthermore, collagen type X, which is specifically associated with hypertrophic chondrocytes and precedes the onset of endochondral ossification [31], was nearly undetectable in SZNTC groups, implying that the hypertrophy of chondrocytes would not be induced by SZNTC. As a consequence, the decreasing collagen I expressions and the inconspicuous expressions of collagen X might suggest that SZNTC may be preventing the dedifferentiation and hypertrophy of chondrocytes.

Because GA possesses inferior pharmacological effects and biological properties, modification of GA may be meaningful. Epigallocatechin-3-gallate (EGCG), the ester of epigallocatechin and gallic acid, was found to repress the degradation of human cartilage proteoglycan and type II collagen and selectively inhibit ADAMTS-1, ADAMTS-4 and ADAMTS-5 [32,33]. Another study revealed that EGCG ameliorates IL-1β-mediated suppression of TGF-β synthesis, and enhances type II collagen and aggrecan core protein synthesis in human articular chondrocytes [34]. A study reported that sulfonamides could also inhibit cell wall synthesis [35]. At the same time, another study showed that sulfonamides were slightly cytotoxic in human keratinocytes and rat hepatocytes [36]. In agreement with this work, Lin et al. showed the enhanced bioactivity of GA derivatives, but comparison with substrates and effect on arthritis have not been performed [15]. In this study, SZNTC, as a novel derivative of GA, can also support the chondrocyte growth and maintain their phenotype. This implied that suitable modification of GA may lead to the improvement of its pharmacological effects.

Our results demonstrated that the concentration of SZNTC with respect to enhancing chondrocytes proliferation ranged from 1.95 to 18.75 μg/ml (Fig. 2). DNA production of rabbit articular chondrocytes was enhanced in a dose-dependent manner when chondrocytes were cultured in the medium containing SZNTC at concentration of 3.91–15.64 μg/ml, and the 7.82 μg/ml group supported the strongest cell proliferation and stimulated the greatest matrix secretion.

This study is a preliminary exploration of the effect of derivatives of GA on chondrocytes metabolism. Further studies are needed to elucidate the underlying mechanism of chondrogenesis induced by SZNTC. Besides, the application of SZNTC on arthritis should also been carried out.

5. Conclusion

In conclusion, sulfonamido-based gallate SZNTC may relieve IL-1 destruction as a means to reduce the progression of OA. This novel compound also exhibits a chondro-protective effect by promoting cell proliferation and maintaining the phenotype of articular chondrocytes. In contrast, its source products of GA and ST-Na have weak effects, not only in the inhibition of OA but also in the bioactivity of chondrocytes, which indicated the significance of this modification. SZNTC as a novel agent is promising in the treatment of chondral and osteochondral lesions.

Conflict of Interest

The authors confirm that this article content has no conflicts of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

Acknowledgments

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