Sinomenine inhibited Interleukin-1β-induced matrix metalloproteinases levels via SOCS3 up-regulation in SW1353 cells

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Matrix metalloproteinases (MMPs) are required for collagen degradation which play a key pathological role in arthritis progression. Herein, the effect of sinomenine (SN) on Interleukin 1 beta (IL-1β)-induced MMPs production and its underlying mechanism were explored in SW1353 cells. The MTT assay showed that 200 and 400 µM SN significantly inhibited SW1353 cell proliferation, thus the lower dose of SN (25-100 µM) were used in the subsequent experiments. Notably, the increased mRNA and protein levels of suppressor of cytokine signaling (SOCS) 3 were dose-dependently induced by SN. SN significantly suppressed mRNA and protein levels of MMPs in IL-1β-induced SW1353 cells. Through western blot analysis, SN showed inhibitory effect on IL-1β-induced TAK1 and p65 phosphorylation. Moreover, SN blocked the interaction of TRAF6 and TAK1 resulting in inactivation of IL-1β pathway. Mechanistically, the inhibitory effect of SN on MMPs levels alongside TRAF6 and TAK1 interactions was abrogated by silencing SOCS3. Moreover, SN didn’t inhibit TAK1 kinase activity. In TAK1 silencing cells, the levels of MMPs and p65 phosphorylation of SN-treated cells were lower than DMSO-treated cells, indicating that blocking interaction was not a unique way for SN to inhibit MMPs levels. Finally, SN significantly inhibited IL-6-induced JAK2 and STAT3 phosphorylation in SW1353 cells. The levels of JAK2 phosphorylation and MMPs didn’t show a significant difference between IL-6+SOCS3-siRNA+SN group and IL-6+SOCS3-siRNA+DMSO group. These findings demonstrated that SOCS3 expression was increased by SN blocked IL-1β-induced
interaction between TRAF6 and TAK1 as well as IL-6 pathway activation, thereby culminating in the inhibition of MMPs levels.

**Key words** Sinomenine; suppressor of cytokine signaling 3; Interleukin-1β; Matrix metalloproteinase; Interleukin-6
INTRODUCTION

As a family of proteolytic enzymes, matrix metalloproteinases (MMPs) are considered as the principal enzymes capable of degrading collagen in the extracellular matrix (ECM). Notably, MMPs secreted from inflammatory cytokines-activated chondrocytes and synoviocytes can break down all cartilage components, thereby contributing to cartilage destruction \(^1\). Many cytokines which have been found to play a crucial role in arthritis pathogenesis can be detected in synovial fluid of patients such as tumor necrosis factor alpha (TNF-\(\alpha\)), interleukin 1 beta (IL-1\(\beta\)), IL-6, IL-4 and IL-10 \(^2\). As an IL-1 cytokine family member that is produced via macrophage activation, IL-1\(\beta\) is regarded as a substantial inflammatory mediator capable of inducing MMPs production \(^3\). Metabolically, IL-1\(\beta\) can accelerate cartilage catabolism, inhibit the synthesis and metabolism of chondrocytes, create articular milieu pro-destruction, block the anabolic pathway and diminish cartilage ECM synthesis \(^4\). Moreover, the relevant clinical studies have shown that synovial fluid of arthritis patients contains abundant MMP-3, which also up-regulates the levels of other MMPs to induce cartilage degradation \(^1\).

Actually, the proteins of suppressor of cytokine signaling (SOCS) function to negatively regulate Janus tyrosine kinase (JAK)/STAT pathway in a feedback manner \(^5\). A wide range of stimuli induce production of SOCS proteins such as lipopolysaccharide (LPS), transforming growth factor beta (TGF-\(\beta\)), IL-1\(\beta\) and TNF-\(\alpha\) \(^6\). Negative regulation of innate and adaptive immune systems by SOCS-3 has been reported in IL-1-dependent acute arthritis \(^7\). Meanwhile, low SOCS3 expression has been shown to promote the expression and production of MMPs, cyclooxygenase-2 (COX-2), IL-6 and
inducible nitric oxide synthase (iNOS) in the samples of leptin-induced cartilage obtained from osteoarthritic (OA) patients \(^8\). It has also been reported that, in M2 macrophages, SOCS3 suppressed MMP-12 production and attenuated contact hypersensitivity in mice \(^9\). Mechanistically, IL-1β activated NF-κB pathway was inhibited by SOCS-3 through TRAF6 ubiquitination inhibition and TAK1 association prevention \(^10\).

Sinomenine (SN) is an alkaloid that is isolated from the herbal plant (Sinomenium acutum), and has been used therapeutically for arthritis patients in China over many years \(^11\). Increasing studies have shown several pharmacological effects of SN such as anti-rheumatism, anti-inflammation, anti-angiogenesis, anti-cancer and immuno-suppression \(^12\). Besides, SN could prevent cardiomyopathy development via blockade of NF-κB activation in diabetic rats \(^13\). Moreover, SN inhibited inflammatory cytokines levels induced by IL-1β through the signaling pathway of toll-like receptor (TLR) 4-MyD88-NF-κB in human fibroblast like synoviocytes (HFLS) \(^14\). Furthermore, an in-vivo study showed the reduction of protein levels and activities of MMPs as well as the elevation of protein levels and activities of TIMP1 and 3 by SN in the paw tissues of rats \(^15\). It has been demonstrated that the invasion and migration capability in activated human monocytes is influenced by SN mainly through the inhibition of MMPs expressions \(^16\). Also, SN was reported to inhibit human glioblastoma (GBM) cells metastasis via MMPs suppression \(^17\).

So far, little information regarding the effect of SN on MMPs in chondrocytes and the underlying mechanism has been reported. Herein, we postulated that SN may inhibit IL-1β-induced MMPs levels via up-regulation of SOCS3 in the SW1353 cells.
MATERIALS AND METHODS

**Cell culture and reagents** The American Type Culture Collection (Manassas, VA) supplied SW1353 cells (human chondrosarcoma cell line), which were cultured in Dulbecco's modified eagle's medium (DMEM, Thermo Fisher Scientific, Waltham, MA) containing fetal bovine serum (FBS, 10%), penicillin (100 U/ml) and streptomycin (100 U/ml, Sigma, St. Louis, MO) in 5% CO₂ humidified atmosphere at 37 °C. TRIzol™ reagent and RIPA lysis buffer were obtained from Life Technologies (Carlsbad, CA). Fisher Scientific (Waltham, MA) provided protease and phosphatase inhibitor cocktails, while Sigma-Aldrich (Saint Louis, MO) supplied all the other chemicals and reagents that were not mentioned in this study.

**Measurement of cytotoxicity** The culturing of SW1353 cells was performed in a 96-well plate prior to treatment with different concentrations of SN for 24 h in the absence or presence of IL-1β (40 ng/ml). In each well, a solution (50 µg) of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) was added for 4 h at 37 °C. In order to dissolve the formazan, DMSO (200 µl) was used to replace the supernatant. Next, microplate reader (BioTek, Winooski, VT) was applied to measure the optical density at 490 nm wavelength.

**siRNA transfection** TAK1 siRNA (#6317) was purchased from Cell Signaling Technology (Danvers, MA). Santa Cruz Biotechnology (Santa Cruz, CA) provided the small interfering RNA (siRNA) of specific SOCS3 (sc-41000). Transfection of SOCS3, TAK1 and a negative control (NC, sc-37007) siRNA were performed using Lipofectamine™ RNAiMAX reagent in accordance with the instructions of the manufacturer (Thermo Fisher Scientific, Waltham,
Prior to incubation for 5 min at room temperature, the transfect reagent and siRNA were gently mixed in Opti-MEM medium. Afterwards, the reagent/siRNA mixture was placed into the 6-well plate. After 4 h incubation, DMEM medium supplemented with 10% FBS was subsequently added, while culturing of the cells were performed for 24 h before the following experiments.

**Quantitative polymerase chain reaction (qPCR)** Based on the specifications of the manufacturer, the total RNA obtained from SW1353 cells was prepared with Trizol™ reagent. Next, equal amounts of RNA was employed to synthesize cDNA via reverse transcriptase (Thermo Fisher Scientific, MA). Afterwards, the SYBR dye was applied for qPCR. The primers used in this study were as follows: GAPDH: F, 5'-AACAGCCTCAAGATCATCAGC-3', R, 5'-GGATGATGTCTGGAGAGCC-3'; SOCS3: F, 5'-AGCCTATTACATCTACTCCGGG-3', R, 5'-GCTGGGTGACTTTCTCATAGG-3'; MMP-1: F, 5'-CGGTTTTTCAAAGGGAATAAGTACT-3', R, 5'-TCAGAAAGAGCAGCATGATATG-3'; MMP-3: F, 5'-TGGCATTGCATCCCTCTATGG-3', R, 5'-AGGACAAAGCAGGAGTCTATTTTCTCATTAGG-3'; and MMP-13: F 5'-AAGGAGCATGGCGACTTCT-3', R 5'-TGGCCCA GGAGAAAAGC-3'.

**Enzyme linked immunosorbent assay (ELISA)** The culturing and treatment of SW1353 cells with sinomenine for 24 h took place in a 6-well plate. Next, harvesting of supernatants was performed, while the determination of MMPs levels was conducted with ELISA commercial kits (R&D Systems, Inc., Minneapolis, MN) concordance with the instructions of the manufacturer.
Western blot analysis The RIPA buffer containing protease and phosphatase inhibitor cocktails was used to lyse the SW1353 cells after harvesting. Next, determination of concentration of protein was carried out with a Bio-Rad protein assay kit (Bio-Rad Labs, Hercules, CA). SDS polyacrylamide gel electrophoresis (PAGE) was used to separate the total protein mixed with 6 fold SDS sample buffer before transferring the resultant product to a PVDF membrane (Millipore, Billerica, MA, USA). Subsequently, the membrane was subjected to overnight incubation with primary antibodies at 4°C after blocking at room temperature with non-fat dry milk (5%) for 1 h. Next, the incubation of membrane with Horseradish peroxidase (HRP)-conjugated secondary antibody (Cell Signaling Technology, Danvers, MA) was carried out at similar conditions as described above. Tris-buffered saline containing 0.1% Tween-20 was used to wash the membranes prior to their visualization with an enhanced chemiluminescence (ECL) solution based on specifications of the manufacturer. Cell Signaling Technology (Danvers, MA) supplied the following; anti-SOCS3 (#52113), anti-TRAF6 (#8028), anti-TAK1 (#5206), anti-p-STAT3 (#9145), anti-p-TAK1 (#4508), anti-p65 (#8242), anti-JAK2 (#3230), anti-p-p65 (#3033),anti-p-JAK2 (#3771), anti-STAT3 (#9139) and anti-GAPDH (#5174) antibodies.

Co-Immunoprecipitation (Co-IP) assay SW1353 cells were harvested before being subjected to lysis with a Triton X-100 buffer. After 10 min centrifugation at 12,000 g, the incubation of the supernatants with TRAF6 antibody or IgG was performed prior to immunoprecipitation with protein A/G-Sepharose. Next, the washing of antibody-protein A/G-Sepharose complex
was carried out before separation on SDS-PAGE gel. Afterwards, the western blot detection system was applied to visualize the protein bands. 

**In vitro kinase assay** The kinase assay for TAK1 was performed using the cell free TAK1 Kinase Enzyme System (Promega, Madison, WI, V4088) according to the manufacturer's instructions. TAK1 inhibitor Takinib (Monmouth Junction, NJ; HY-103490) was purchased from Med Chem Express LLC as a positive control. In brief, SN at different concentrations were added to kinase buffer containing enzyme, substrate and ATP. After incubation for 1 h, ADP-Glo™ and kinase detection reagents were added to the reaction buffer and the activity of TAK1 kinase was determined using a luminescence reader.

**Statistical analysis** All statistical analyses were performed using Statistical Analysis System (SAS, version 9.4, Institute Inc., Cary, NC). One-way analysis of variance (ANOVA) was applied to analyze data with multiple groups, followed by Dunnett test for multiple comparisons. The p value < 0.05 was considered statistically significant. For some experiments, a 2×2 factorial ANOVA was applied to analyze data with four groups. When interaction effect between two factors is significant (p<0.05), a CONTRAST statement (computer programming) in Statistical Analysis System was used to analyze the difference of simple effects of the respective factors. The detail of the analysis programming was shown in supplementary materials.

**RESULTS**

**SN increased the expression of SOCS3 in the SW1353 cells** Based on previous study\(^{18,19}\), the effect of SN on MMPs production in chondrocytes, a human chondrosarcoma cell line SW1353 was ascertained. We first
examined the cytotoxicity of SN on the SW1353 cells. The MTT assay results showed that SN treatment (25, 50 and 100 µM) for 24 h did not inhibit the proliferation of SW1353 cells (Fig. 1A). However, the dose at 200 and 400 µM exhibited a reducing trend compared with the DMSO treatment (p=0.0005 and p<0.0001). Moreover, in the presence of IL-1β (40 ng/ml), SN (25-100 µM) treatment did not affect the growth of SW1353 cells (Fig. 1B). Therefore, the concentrations (25-100 µM) were used in the subsequent experiments. We further investigated SOCS3 expression level in SW1353 cells. The qPCR result depicted that SN dose-dependently upregulated mRNA expression of SOCS3 in SW1353 cells (Fig. 1C). Also, in a dose-dependent fashion, SN was shown to upregulate SOCS3 protein level in SW1353 cells through western blot assay (Fig. 1D).

**SN inhibited IL-1β-induced MMPs levels in the SW1353 cells** In the articular joints, the MMPs are mainly secreted by chondrocytes. Also, in the absence or presence of IL-1β (40 ng/ml), the treatment of the SW1353 cells with SN (at chosen concentrations) was carried out for 6 h. As indicated in Fig. 2A, 100 µM SN alone treatment did not increase the mRNA expression of MMPs. However, 50 and 100 µM SN doses significantly inhibited mRNA expressions of MMP-1, 3 and 13 induced by IL-1β. Consistent with the results of qPCR, the ELISA assay demonstrated that the levels of MMP-1, 3 and 13 in the supernatants were significantly inhibited by the 50 and 100 µM doses of SN in the IL-1β-treated SW1353 cells.

**SN blocked the interaction of TRAF6 and TAK1 and inactivated IL-1β pathway in SW1353 cells** It has been demonstrated in a previous study that the signaling of IL-1 was inhibited by SOCS3 through TRAF6/TAK1 complex
targeting IL-1β. The results of western blot showed that IL-1β could induce increase in SOCS3 protein level, while SN treatment also increased SOCS3 expression in IL-1β presence (Fig. 3A). However, SN treatment did not have any effect on TRAF6 protein expression, while the phosphorylation level of TAK1 was highly inhibited by SN treatment in the cells induced by IL-1β compared to the IL-1β alone treatment. Moreover, SN treatment inhibited IL-1β-induced p65 phosphorylation level indicating that MMPs gene expression regulated by p65 would be suppressed by SN. Next, the interaction of TRAF6 and TAK1 was investigated in SN-treated cells. The IL-1β induced the interaction of TRAF6 and TAK1 (p=0.0001), which contributed to the downstream signaling activation such as p65 phosphorylation, albeit SN treatment blocking their interaction (p<0.0001) (Fig. 3B).

**SN inhibited IL-1β-induced the interaction of TRAF6 and TAK1 by up-regulation of SOCS3 expression in SW1353 cells** In the TRAF6/TAK1 complex, the increasing role of SOCS3 after SN treatment (with 100 µM SN in 40 ng/ml IL-1β presence for 30 min) was investigated through the transfection of the cells for 24 h with the siRNA which consisted of a scrambled sequence as a negative control (NC siRNA) or SOCS3 siRNA. Co-immunoprecipitated (Co-IP) assay showed that, after IL-1β stimulation, TAK1 interacted well with TRAF6 (p=0.048), while SN treatment suppressed their interaction (p=0.0002). However, silencing SOCS3 abrogated the suppression of the interaction in SN-treated cells, indicating that SN might have blocked IL-1β-induced interaction of TRAF6 and TAK1 by up-regulating SOCS3 expression (Fig. 4A). Meanwhile, we tested the MMPs levels in the supernatants. In line with Co-IP results, ELISA assay results showed that, after IL-1β stimulation, MMPs levels
of SN+SOCS3 siRNA group were significantly higher than that of SN+NC-siRNA group. However, SN failed to inhibit the levels of MMPs in SOCS3 siRNA-treated cells in comparison with the no treatment+SOCS3 siRNA group (Fig. 4B).

**SN failed to directly inhibit TAK1 kinase activity to reduce IL-1β-induced MMPs production** To confirm the role of TAK1 in the inhibitory effect of SN, the activity of TAK1 kinase was determined by a cell free enzyme system. As shown in Fig. 5A, SN at different concentrations did not inhibit the activity of TAK1 kinase. Takinib (a TAK1 inhibitor) as a positive control at 10 μM significantly inhibited the activity of TAK1 kinase (p=0.0014). Next, we aimed to confirm these results and further wondered if the blocking of TRAF6 and TAK1 interaction was a unique way to trigger the inhibition of IL-1β-induced MMPs levels caused by SN. The transfection of the cells for 24 h with NC siRNA (as negative control) or TAK1 siRNA were performed and treated with 100 μM SN for 30 min in IL-1β (40 ng/ml) presence. Western blot assay results showed that TAK1 silencing (p=0.0047) and SN treatment (p=0.0042) significantly inhibited IL-1β-induced p65 phosphorylation compared with the NC siRNA+IL-1β group (Fig. 5B). Surprisingly, the phosphorylation level of p65 in TAK1 siRNA+SN+IL-1β group was lower than that of the TAK1 siRNA+DMSO+IL-1β group (p=0.0049) (Fig. 5B). Moreover, the phosphorylation level of p65 in TAK1 siRNA+SN+IL-1β group was also lower than that of the NC siRNA+SN+IL-1β group (p=0.0056) (Fig. 5B). These data suggested that SN didn’t directly inhibit the activity of TAK1 and blocking the interaction was not a unique way for SN’s action. Consistent with western
blot assay, it was also observed that MMPs levels in TAK1 siRNA+SN+IL-1β group was lower than the NC siRNA+SN+IL-1β group (Fig. 5C).

**SN suppressed IL-6-induced activation of JAK2/STAT3 pathway**

Based on the previous results in this study, SN may exhibit multiple functions to inhibit MMPs production through inducing SOCS3 expression in a TAK1-independent manner. Numerous studies have reported that IL-1β stimulated IL-6 production and SOCS3 blocked IL-6 signaling pathway \(^{20}\). In this regard, the effect of SN-induced SOCS3 up-regulation on JAK2/STAT3 pathway was investigated in SW1353 cells. As shown in Fig. 6, IL-6 treatment could increasingly induce SOCS3 expression (p=0.0011). Also, SN treatment increased SOCS3 expression in IL-6 presence. Moreover, through western blot analysis, an increased JAK2 and STAT3 phosphorylation levels in SW1353 cells were observed after IL-6 treatment but SN treatment highly inhibited their phosphorylation levels. Altogether, these results indicated that SN might suppress JAK2/STAT3 pathway activated by IL-6 via SOCS3 up-regulation.

**Silencing SOCS3 abrogated IL-6-induced JAK2 phosphorylation and MMPs production decreasing in SN-treated cells**

To determine the effect of SOCS3 on IL-6-induced JAK2 phosphorylation and MMPs production in SN-treated cells, SW1353 cells transfected for 24 h with NC siRNA or SOCS3 siRNA were treated for 30 min with SN (100 µM) in the presence of 100 ng/ml IL-6. As shown in Fig. 7, JAK2 phosphorylation in IL-6+SOCS3 siRNA group was significantly increased than that of IL-6+NC siRNA group (p=0.0001). However, levels of JAK2 phosphorylation and MMPs did not show a significant difference between IL-6+SOCS3 siRNA+SN group and IL-
6+SOCS3 siRNA+DMSO group, suggesting that SN inhibited IL-6-induced JAK2 phosphorylation and MMPs production via inducing SOCS3 expression.

DISCUSSION

The SN effect on MMPs production and its concomitant underlying molecular mechanism was investigated in the current study. Over many years, a bioactive alkaloid SN, which is isolated from Chinese herbal plant S. acutum, has widely been applied as a therapeutic option for arthritis in China. It was shown therein that SN below 100 µM did not affect the proliferation of SW1353 cells, indicating its low cytotoxicity and safety. The MTT assay results also showed that the viability of cell stimulated by 40 ng/ml IL-1β coupled with 100 µM SN did not decrease (Fig. 1B). In line with the previous study \( ^{21} \), SN exhibited low cytotoxicity under inflammatory cytokine stimulation. It has been reported that SN suppressed invasion and metastasis of cancer cells by blocking IL-4/miR-324-5p/CUEDC2 axis mediation of NF-κB activation \( ^{22} \). Also, the human hepato-cellular carcinoma and lung cancer cell proliferation inhibited by SN has been reported \( ^{23, 24} \). Notably, many studies have reported the cytoprotective effect of SN \( ^{25-27} \). In general, anticancer drugs usually inhibit cancer cell proliferation through their toxicity. Although SN exhibited an anti-cancer activity, our study suggested that SN dose less than 100 µM might have cytoprotective effect on chondrocytes, which is in line with previous studies \( ^{25, 28} \). Therefore, further investigation of the pharmacological activities and molecular mechanism of SN may accelerate its development in the treatment of arthritis.

It has been reported that MMPs were the most important catabolic factors that increase the catabolism of collagen \( ^{1} \). Existing clinical studies have
shown the serum levels of MMP-3 increasing in rheumatic disorders reflected synovial inflammation \(^{29}\). In rheumatoid arthritis (RA), serum pro-MMP-3 also has been proven to be a specific indicator \(^{30}\). It was shown that SN inhibited IL-1β-induced mRNA and protein levels of MMPs in SW1353 cells in this study. Although the previous two studies have reported substantial inhibitory activities of SN on mRNA expression of MMP-9 in macrophages \(^{31, 32}\), the mechanisms underlying SN effect on MMPs production remains unclear. Herein, we firstly attempted to address that SN could increasingly induce SOCS3 expression in SW1353 cells. Indeed, abnormal expression of SOCS3 is specifically associated with joint disease as shown previously that SOCS3 knock-out mice developed a particularly severe joint inflammation in acute inflammatory arthritis which is dependent on IL-1 pathway \(^{7}\). Meanwhile, the abundant expression of SOCS3 mRNA was found in RA patients. Therefore, the blocking of JAK/STAT3 signaling pathway induced by IL-6 might promote RA treatment \(^{33}\). Herein, we demonstrated significant up-regulation of SOCS3 mRNA and protein level by SN (Fig. 1). Importantly, TRAF6 ubiquitination has been reported as the requirement for TAK1 and SOCS3 activation, which in turn inhibited the modification of TRAF6 by ubiquitin \(^{10}\). Our findings suggested that SN treatment effectively blocked the IL-1β-induced TRAF6/TAK1 complex formation. It has been reported that SN decreased expression of TLR4 and TRAF6 during osteoclast differentiation, and reduced expression of TLR4 (but not TRAF6) in osteoclast survival \(^{32}\). SN also was shown to reduced TRAF6 and Myd88 expression in rheumatoid arthritis fibroblast-like synoviocytes \(^{34}\). However, in our study, SN did not inhibit TRAF6 level in SW1353 cells, suggesting that SN exhibited multiple
functions in different cells. Intriguingly, our results showed that SN failed to inhibition of the activity of TAK1 kinase and TAK1 silencing amplified the inhibitory effect of SN compared with the NC siRNA+SN treated group (Fig. 5), indicating that TAK1 was not a direct target of SN to inhibit MMPs levels induced by IL-1β. Importantly, these results triggered a further investigation, which is independent of blocking the interaction caused by SN. As a natural medicine, SN plays multiple roles in the diverse signal pathways. Therefore, development of the application of natural medicines could serve as a significant therapeutic strategy for arthritis treatment.

As noted in several studies, NF-κB signaling pathway plays a crucial role in the regulation of expressions of MMPs, cytokines and inflammatory mediators. In addition, various intracellular signaling systems such as IL-6-induced STAT3 translocation are also involved in the MMPs production, which result in the arthritis development and progression. Indeed, IL-6 is a multi-functional cytokine which activates the down-stream signaling and plays a significant inflammatory and anti-inflammatory effects. As a negative regulator, SOCS3 simultaneously binds to JAKs and gp130 to inhibit the activity of JAK independent of ATP. Our findings indicated that SN might suppress IL 6-induced JAK2/STAT3 pathway activation to reduce MMPs production by inducing the up-regulation of SOCS3 (Fig. 6 and 7). Indeed, it has been reported that many factors are involved in STAT3-regulated production of MMPs. p21 deficiency increased mRNA expression of MMP-13, which is susceptible to osteoarthritis through STAT3 phosphorylation. microRNA-mediated IL-6/STAT3 signaling regulate MMPs levels. In SN-treated cells, silencing SOCS3 abrogated IL-6-induced JAK2 phosphorylation and MMPs
production decreasing. Consistent with our study, it has been reported that SN inhibits the progression of plasma cell mastitis by suppressing IL-6/JAK2/STAT3 cascades \(^{39}\). Our results suggested that an increased SOCS3 expression by SN treatment might not only inhibit IL-1β but also activate IL-6 pathway to induce production of MMPs.

This study demonstrated that SN potentially inhibited IL-1β-induced production of MMPs in chondrosarcoma cells, and its underlying mechanism is associated with the up-regulation of SOCS3, which in turn blocked the interaction of TRAF6 and TAK1. Moreover, up-regulation of SOCS3 expression by SN treatment suppressed IL-6-induced activation of JAK2/STAT3 pathway, which contributed to MMPs production. Our findings provided a molecular basis for the potential application of SN and suggested that SN exhibited a cartilage protective property for the treatment of arthritis.

**Conflict of Interest**

The authors declare no conflict of interest.

**Supplementary Materials**

The online version of this article contains supplementary materials.
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Fig. 1. Sinomenine (SN) increased SOCS3 expression in SW1353 cells. (A) SW1353 cells were treated for 24 h with SN (25-400 µM). Determination of the cell viability was via MTT assay. (B) Treatment of SW1353 cells for 24 h with SN (25-100 µM) in the absence or presence of IL-1β, MTT assay was used to determine cell viability. (C) The cells were treated for 6 h with SN (25-
100 µM). The isolation of total RNA was carried out and the SOCS3 mRNA level was determined via qPCR and GAPDH as the control. (D) The cells were treated for 12 h with SN (25-100 µM). Western blot analysis was used to determine the SOCS3 protein level and GAPDH as the control. The density of band was analyzed with ImageJ software. The entire experiments were conducted in triplicate, while all data were analyzed with one-way ANOVA followed by Dunnett test and presented as means ± SEM (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 vs. Control).
Fig. 2. Sinomenine (SN) inhibited IL-1β-induced matrix metalloproteinases (MMPs) levels in SW1353 cells. (A) SW1353 cells were treated for 6 h with SN (25-100 µM) in the absence or presence of IL-1β (40 ng/ml). The isolation of total RNA was performed, while qPCR was applied to determine mRNA levels of MMPs. GAPDH was shown as the control. (B) SW1353 cells were
treated for 24 h with SN (25-100 µM) in the absence or presence IL-1β (40 ng/ml). The MMPs protein levels were determined by ELISA assay. The entire experiments were conducted in triplicate, while all data were analyzed with one-way ANOVA followed by Dunnett test and presented as means ± SEM (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 vs. 40 ng/ml IL-1β alone treatment).
Fig. 3. Sinomenine (SN) blocked the interaction of TRAF6 and TAK1 and inactivated IL-1β signaling in SW1353 cells. (A) SW1353 cells were treated for 30 min with SN at 25-100 µM in the absence or presence of 40 ng/ml IL-1β. The indicated protein levels were measured with western blot analysis. GAPDH was presented as the control. The density of band was analyzed with...
ImageJ software. The entire experiments were conducted in triplicate, while the data were analyzed with one-way ANOVA followed by Dunnett test and presented as means ± SEM (*p <0.05, **p <0.01 vs. IL-1β+DMSO treatment). (B) SW1353 cells were treated for 30 min with 100 µM SN in the absence or presence of 40 ng/ml IL-1β. TRAF6 was immunoprecipitated by antibody and the indicated protein levels were ascertained via western blot analysis. GAPDH was shown as the control. The entire experiments were conducted in triplicate, while data were analyzed with 2×2 factorial ANOVA (IB:TAK1 interaction effect: f=25.28, p=0.01) and simple effect was performed to analyze the difference between two groups. All data were presented as means ± SEM (**p <0.01 vs.Control; ####p <0.0001 vs. 40 ng/ml IL-1β alone treatment).
Fig. 4. Sinomenine (SN) inhibited IL-1β-induced the interaction of TRAF6 and TAK1 by up-regulation of SOCS3 in SW1353 cells. (A) SW1353 cells transfected for 24 h with NC siRNA (as a negative control) or SOCS3 siRNA were treated for 30 min with SN (100 µM) in the presence of IL-1β (40 ng/ml). TRAF6 was immunoprecipitated by antibody and the indicated protein levels
were measured with western blot analysis. GAPDH was shown as the control. Data were analyzed with 2×2 factorial ANOVA (IB:TAK1 interaction effect: f=16.51, p=0.0036) and simple effect was performed to analyze the difference between two groups. (B) SW1353 cells transfected for 24 h with siRNA (as negative control) or SOCS3 siRNA were treated for 24 h with SN (100 µM) in the presence of IL-1β (40 ng/ml). The MMPs protein levels were measured with ELISA assay. The entire experiments were conducted in triplicate, while data were analyzed with 2×2 factorial ANOVA (MMP-1 interaction effect f=4.48, p=0.0671; SOCS3 siRNA main effect f=73.87, p<0.0001; SN main effect f=20.27, p=0.002; MMP-3 interaction effect f=5.2224, p=0.0516; SOCS3 siRNA main effect f=76.99, p<0.0001; SN main effect f=15.93, p=0.004; MMP-13 interaction effect f=2.23, p=0.1739; SOCS3 siRNA main effect f=64.66, p<0.0001; SN main effect f=14.07, p=0.0056) and simple effect was performed to analyze the difference between two groups. All data were presented as means ± SEM (*p <0.05, **p <0.01, ***p <0.001 vs. IL-1β+NC-siRNA; ###p <0.001, ####p <0.0001 vs. IL-1β+NC-siRNA+SN).
Fig. 5. SN failed to directly inhibit TAK1 kinase activity to reduce IL-1β-induced MMPs production. (A) SN at different concentrations (25-200 μM) were added to the kinase buffer containing enzyme, substrate and ATP. After incubation for 1 h, ADP-Glo™ and kinase detection reagents were added to the reaction buffer and the activity of TAK1 kinase was determined using a

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luminescence reader. Tak1 inhibitor Takinib (10 μM) was used as a positive control. The entire experiments were conducted in triplicate, while all data were analyzed with one-way ANOVA followed by Dunnett test and presented as means ± SEM (**p <0.01 vs.Ctrl). (B) SW1353 cells transfected for 24 h with NC siRNA (as a negative control) or TAK1 siRNA were treated for 30 min with SN (100 μM) in the presence of 40 ng/ml IL-1β. The indicated protein levels were measured with western blot analysis. GAPDH was shown as the control. The density of band was analyzed with ImageJ software. Data were analyzed with 2×2 factorial ANOVA (p-p65 interaction effect f=0.0073, p=0.9342; TAK1 siRNA main effect f=29.05, p=0.0007; SN main effect f=30.42, p=0.0006 ) and simple effect was performed to analyze the difference between two groups. (C) SW1353 cells transfected for 24 h with siRNA (as negative control) or TAK1 siRNA were treated for 24 h with SN (100 μM) in the presence of 40 ng/ml IL-1β. ELISA assay was used to measure the MMPs protein levels. The entire experiments were conducted in triplicate, while data were analyzed with 2×2 factorial ANOVA (MMP-1 interaction effect f=2.02, p=0.1929; TAK1 siRNA main effect f=33.61, p=0.0004; SN main effect f=67.88, p<0.0001; MMP-3 interaction effect f=0.68, p=0.4345; TAK1 siRNA main effect f=28.75, p=0.0007; SN main effect f=42.28, p=0.0002; MMP-13 interaction effect f=7.84, p=0.0232; TAK1 siRNA main effect f=92.22, p<0.0001; SN main effect f=66.82, p<0.0001) and simple effect was performed to analyze the difference between two groups. All data were presented as means ± SEM (**p <0.01, **p <0.001, ****p <0.0001 vs. IL-1β+NC-siRNA; #p <0.05, ##p <0.01, vs. IL-1β+TAK1-siRNA; $p <0.05, $$p <0.01, vs. IL-1β + NC-siRNA+SN).
Fig. 6. Sinomenine (SN) suppressed IL-6-induced activation of JAK2/Stat3 pathway in the SW1353 cells. SW1353 cells were treated for 30 min with SN (25-100 µM) in the absence or presence of IL-6 (100 ng/ml). The indicated protein levels were identified by western blot. GAPDH was shown as a control. The density of band was analyzed with ImageJ software. The entire
experiments were conducted in triplicate, while all data were analyzed with one-way ANOVA followed by Dunnett test and presented as means ± SEM (*p <0.05, **p <0.01, ***p <0.001 vs. 100 ng/ml IL-6 alone treatment).
Fig. 7. Silencing SOCS3 abrogated IL-6-induced JAK2 phosphorylation and MMPs production decreasing in SN-treated cells. (A) SW1353 cells transfected for 24 h with NC siRNA (as a negative control) or SOCS3 siRNA were treated for 30 min with SN (100 µM) in the presence of 100 ng/ml IL-6. The indicated protein levels were measured with western blot analysis.
GAPDH was shown as the control. The density of band was analyzed with ImageJ software. Data were analyzed with 2×2 factorial ANOVA (interaction effect: p-JAK2, f=7.34, p=0.0267) and simple effect was performed to analyze the difference between two groups. (B) SW1353 cells transfected for 24 h with siRNA (as negative control) or SOCS3 siRNA were treated for 24 h with SN (100 µM) in the presence of 100 ng/ml IL-6. ELISA assay was used to measure the MMPs protein levels. The entire experiments were conducted in triplicate, while data were analyzed with 2×2 factorial ANOVA (interaction effect: MMP-1, f=5.77, p=0.0431; MMP-3, f=9.25, p=0.016; MMP-13, f=5.34, p=0.0496) and simple effect was performed to analyze the difference between two groups. and presented as means ± SEM (**p <0.01, ***p <0.001 vs. IL-6+NC-siRNA; ####p <0.0001 vs. IL-6 + NC-siRNA+SN).