Gentiopicrin exerts anti-rheumatic effect in human fibroblast-like synoviocytes via inhibition of p38MAPK/NF-κB pathway

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Abstract: To investigate the effect of gentiopicrin on the expressions of inflammatory factors in human fibroblast-like synoviocytes (HFLS) and the underlying mechanism. Human fibroblast-like synoviocytes (HFLS) were cultured in vitro at 37 °C in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5 % fetal bovine serum (FBS) in a humidified incubator containing 5 % CO\textsubscript{2}. Cell viability was determined using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-tetrazolium bromide (MTT) assay, while real-time quantitative polymerase chain reaction (qRT-PCR) was used to determine the expressions of interleukin 1β (IL-1β) and interleukin 6 (IL-6) mRNAs. The expressions of p38 mitogen-activated protein kinase (p38 MAPK) and nuclear factor kappa light chain enhancer of activated B cells (NF-κB) were determined using Western blotting. Enzyme-linked immunosorbent assay (ELISA) was used to determine the levels of IL-1β and IL-6 in cell lysate. Treatment with 5-25 μM gentiopicrin did not significantly affect the number of viable cells, when compared with control group (p > 0.05). However, at 50 and 100 μM gentiopicrin, the number of viable cells were significantly increased, relative to control group (p < 0.05). Results of qRT-PCR showed that the expression levels of IL-1β and IL-6 mRNAs were significantly higher in TNF-α group than in control group (p < 0.05). However, treatment with gentiopicrin significantly and dose-dependently decreased their expression levels compared with TNF-α group (p < 0.05). Western blotting results showed that the expressions of p-p38MAPK and NF-κB-p65 proteins were significantly upregulated in TNF-α group, when compared with control group (p < 0.05). However, treatment with gentiopicrin significantly and dose-dependently down-regulated the expression of these proteins compared with TNF-α group (p < 0.05). The levels of IL-1β and IL-6 in cell lysate were significantly higher in TNF-α group than in control group (p < 0.05). However, treatment with gentiopicrin, and p38MAPK/NF-κB pathway inhibitors (SB203580 and BAY11-7082) significantly reduced the levels of these inflammatory factors compared with TNF-α group (p < 0.05). Gentiopicrin has therapeutic potential for Rheumatoid arthritis (RA) through a mechanism involving the inhibition of p38MAPK/NF-κB pathway.

Key words: Rheumatoid arthritis; Human fibroblast-like synoviocytes; Gentiopicrin; Inflammatory factors; Expression.

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease of the joint. It occurs mostly in middle-aged women, with 80 % occurring in women aged 35 to 50 years (1, 2). This disease is characterized mainly by inflammatory hyperplasia of synovial tissue, progressive destruction of articular cartilage, repeated attacks, severe joint deformity and dysfunction. Its pathogenesis is complex and has not been fully elucidated. Studies have implicated fibroblast-like synoviocytes (FLS) in the pathogenesis of RA (3-5). Abnormal proliferation of synovial cells and infiltration of inflammatory cells are the main pathological features of RA. In RA patients, there is usually increased proliferation of synovial cells, and this is responsible for the huge amount of FLS, which in turn stimulate the release of inflammatory factors such as IL-6, IL-1β, and tumor necrosis factor α (TNF-α). These inflammatory factors promote the proliferation and release of other cytokines, thus forming a vicious circle, and promoting the occurrence and development of synovitis in RA patients (6-9). Therefore, inhibition of the secretion of FLS inflammatory factors is key to the treatment of RA. Synovitis in RA patients is accompanied by the activation of signal transduction pathways such as p38MAPK and NF-κB. The p38MAPK, as a ubiquitous serine/threonine protein kinase, is an important member of mitogen-activated protein kinase (MAPK) family, which plays a key role in inflammatory response (10). The various subtypes of p38MAPK are activated by different stimuli, producing responses such as protein phosphorylation, complex third-order kinase cascade reactions, signal transduction, and other cellular effects (11). Studies have shown that p38MAPK and NF-κB signaling pathways together constitute an important mechanism of synovitis in RA patients. Inflammatory factors such as IL-1β and TNF-α stimulate the phosphorylation of p38MAPK, promote NF-κB transcription, and increase the expression levels of other inflammatory factors (12). Increased levels of inflammatory factors in turn promote inflammation, which leads ultimately to persistent synovitis.

Anti-arthritic drugs in current use include non-steroidal anti-inflammatory drugs (NSAIDs), immune-suppressants, biological agents and glucocorticoids (13-15). These drugs are used primarily for the control
and relief of symptoms in patients, and they produce serious side effects. Gentiopicrin (Figure 1), an iridoid glycoside, is a biologically active compound isolated from \textit{Gentiana macrophylla} and \textit{Gentiana gentian}. It is the bitter principle in \textit{Swertia pseudochinensis} and \textit{Swertia bimaculata}. It belongs to the cleft cyclic iridoid glycosides, and possesses hepatoprotective, antioxidant, anti-inflammatory, analgesic and antitumor effects (16-20). It was only recently that the anti-inflammatory and analgesic effects of gentiopicrin and its derivatives were established. It inhibits foot swelling and release of inflammatory factors in arthritic rats (19). The present study investigated the effect of gentiopicrin on the levels of expression of inflammatory factors in HFLS and the underlying mechanism.

Materials and Methods

Materials and reagents

Human fibroblast-like synovial cells (HFLS) were purchased from Beijing Qingyuanhao Biotechnology Co., Ltd. Gentiopicrin was purchased from Chengdu Angsaisi Biotechnology Co., Ltd.; recombinant human TNF-α was product of Peprotech Company (USA), while FBS and DMEM were obtained from Gibico Co., Ltd. (USA). Trypsin, SB203580, MTT assay kit, BAY11-7082, radioimmunoprecipitation assay (RIPA) buffer, sample buffer, bicinchoninic acid (BCA) protein concentration kit and all the secondary antibodies were purchased from Beyotime Biotechnology Co., Ltd. Phosphate-buffered saline and dimethyl sulfoxide (DMSO) were products of Chongqing Haiyun Biotechnology Co., Ltd.; IL-6 and IL-1β ELISA kits were obtained from Wuhan Boshide Bioengineering Co., Ltd., while total RNA extraction kit was purchased from Kangwei Century Biotech Co., Ltd. Polymerase chain reaction (PCR) reverse transcription kit was purchased from Toyobo Biotechnology Co., Ltd. (Shanghai); sodium dodecyl sulfate (SDS), concentrated hydrochloric acid, Tween 20, sodium chloride, Tris buffer, acrylamide and ammonium persulfate were products of Sangon Co., Ltd. (Shanghai). Luminous solution was a product of Beijing Yinggeen Biotechnology Co., Ltd.; skimmed milk powder was purchased from Inner Mongolia Yili Industrial Group Co., Ltd., while p-p38MAPK and p38MAPK antibodies were obtained from Nanjing Baoode Biotechnology Co., Ltd. Histone antibody and NF-κB-p65 were purchased from Cell Signaling Technology Company.

Equipment

Gel imager was a product of Bio-Rad Co., Ltd. (USA); purification workbench was obtained from Suzhou Purification Equipment Co., Ltd., while inverted phase contrast microscope was purchased from Olympus Ltd. Low temperature refrigerator was obtained from Midea Co., Ltd. Microbalance was purchased from Sedorus Group (Germany); Cell incubator and ELISA kits were products of Thermo Fisher Scientific Company. Constant temperature water bath box and oscillator were obtained from Shanghai Yiheng Scientific Instruments Co., Ltd., while high speed freezing centrifuge was a product of Sigma-Aldrich (Germany).

Cell culture

Cell recovery and subculture were performed according to standard methods (21). After thawing the synovial cells in a water bath for 1 min, they were sucked out of the frozen tube and put in a prepared culture dish mixed with FBS-supplemented DMEM and incubated at 37 °C overnight. The culture medium was replaced with fresh medium after 24 h and the cells were trypsinized after attaining 80-90 % confluency.

Determination of cell viability (MTT assay)

The cells (1 \times 10^6 cells/well) were seeded into a 96-well plate by adding DMEM and were incubated for 24 h. Varied concentrations of gentiopicrin (5 - 100 μM) were added to the cells and they were further incubated for 3 days. At the end of the third day, 20 μl of MTT solution was added to the wells, followed by incubation for another 4 h. The medium was finally replaced with 150 μl of DMSO solution, agitated at 50 oscillations/min for 10 min, and absorbance of the samples were read using a microplate reader at 490 nm. The procedure was performed in triplicates and cell proliferation was calculated thus:

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\text{Cell proliferation (%)} = (1 - \text{Abs})/\text{Abs} \times 100 \%
\]

where \text{Abs = absorbance of sample well; and } \text{Abs = absorbance of control well}.

qRT-PCR

This was carried out according to standard procedure (22). Total RNAs were isolated from the treated and control cells using Trizol reagent. The purity of the isolated RNAs was determined spectrophotometrically at 260 nm and 280 nm. The RNAs were reverse-transcribed to cDNAs using random primers at 45 °C for 2 h. The samples were heated at 95 °C for 10 min. The PCR amplification of the reverse transcribed reaction mixture was carried out using 20 μl reaction mixture and equal volume of SYBR Premix Ex TaqTM II. The reaction mixture also contained reverse transcribed cDNA (2 μl), mixture of forward and reverse primers (0.8 μl) and double-distilled H2O (6 μl). The PCR conditions were: pre-denaturation at 95 °C for 30 sec, denaturation at 95 °C for 3 sec, annealing at 60 °C for 34 sec, and 40 cycles. The procedure was performed in triplicates and the mean taken. Relative expression was quantified using \text{2}^{-\Delta \Delta Cq} \text{ method, and } \beta\text{-actin gene was used as internal reference. The specific primer sequences of } \text{IL-1β} (391bp) \text{ and } \text{IL-6} (496 bp) \text{ were:}

\begin{align*}
\text{IL-1β forward: } & 5’-AAACATGAGTGCTCTTCCAGG-3’ \\
\text{IL-1β reverse: } & 5’-TGGAGAACACCTGTTGCCA-3’ \\
\text{IL-6 forward: } & 5’-CCTTGGTCCAGTTGCCCTTCT-3’ \\
\text{IL-6 backward: } & 5’-GCCATTGTGTTGGGTCA-3’
\end{align*}
Western blotting

The expressions of p38MAPK and NF-κB-p65 were determined using Western blotting. The cells were lysed using ice-cold RIPA buffer for 2.5 h. The cell lysate was washed twice with phosphate-buffered saline (PBS) and then treated with Nonidet P-40 (1 %), Triton X-100 (0.1 %), Na₂PO₄ (30 mM) mixed with sodium orthovanadate (1 mM), Tris-HCl (2.5 mM), sodium chloride (100 mM), leupeptin (10 μg/ml) and aprotinin for 45 min at 4 °C. The cell suspension was then centrifuged at 12,000 g for 10 min at 4 °C. The protein concentration of the supernatant was determined using BCA assay kit. A portion of total cell protein (35 μg) from each sample was separated on a 10 % SDS-polyacrylamide gel electrophoresis and transferred to a fixed polyvinylidene fluoride membrane at 110 V and 90 °C for 120 min. Subsequently, non-fat milk powder (0.05 %) in Tris-buffered saline containing 0.2 % Tween-20 (TBS-T) was added with gentle shaking at 37 °C and incubated to block non-specific binding of the blot. Thereafter, the blot was incubated overnight with primary antibodies of p38MAPK, NF-κB-p65 and histone at a dilution of 1 to 1000 at 4 °C. Then, the membrane was washed thrice with TBS-T and further incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody for 1 h at room temperature. The blot was developed using an X-ray film. Grayscale analysis of the bands was performed using ImageJ analysis software (4.6.2). Respective protein expression levels were normalized to that of histone which was used as a standard reference.

Determination of IL-1β and IL-6 levels

This was performed using ELISA. Standard and cell lysates were seeded into 96-well plates pretreated with antibodies. After thorough mixing, the membrane was sealed. After 90 min of incubation at 37 °C, biotin-labeled IL-6 or IL-1β antibody working solution was added. Following incubation for 60 min at 37 °C, the reaction mixture was rinsed thrice with washing buffer. Subsequently, ABC working solution was added to each well and incubated for another 30 min at 37 °C, then rinsed 5 times with washing buffer solution. This was followed by the addition of TMB chromogenic solution to each well and further incubation at 37 °C for 20 min. Finally, 100 μl of TMB termination solution was added to terminate the reaction, and absorbance of each well was measured at 450 nm within 20 min.

Statistical analysis

Data are expressed as mean ± SD, and statistical analysis was performed using SPSS (13.0). Groups were compared using Student t-test and values of p < 0.05 were considered statistically significant.

Results

Effect of gentiopicrin on the viability of HFLS

As shown in Figure 2, treatment with gentiopicrin at doses of 5-25 μM did not significantly increase the number of viable cells, when compared with control group (p > 0.05). However, gentiopicrin at 50 and 100 μM significantly increased the number of viable cells, relative to control group (p < 0.05).

Level of expression of IL-1β mRNA in HFLS as induced by TNF-α

Results of qRT-PCR showed that the level of expression of IL-1β mRNA was significantly higher in TNF-α group (1.97 ± 0.14) than in control group (0.92 ± 0.06) (p < 0.05). However, treatment with gentiopicrin significantly and dose-dependently decreased the IL-1β mRNA expression (p < 0.05). These results are shown in Figure 3.

Level of expression of IL-6 mRNA as induced by TNF-α

As shown in Figure 4, the expression level of IL-6 mRNA was significantly higher in TNF-α group (1.36 ± 0.09) than in control group (0.77 ± 0.05) (p < 0.05). However, treatment with gentiopicrin significantly and dose-dependently decreased the expression of IL-6

![Figure 2](image-url). Effect of gentiopicrin on the viability of HFLS (n = 6). *p < 0.05 when compared with control group.

![Figure 3](image-url). Effect of gentiopicrin on the expressions of IL-1β mRNA in HFLS (n = 3). *p < 0.05, **p < 0.01, compared with TNF-α group.

![Figure 4](image-url). Effect of gentiopicrin on the expression of IL-6 mRNA in HFLS (n = 3). *p < 0.05, **p < 0.01, compared with TNF-α group.
mRNA ($p < 0.05$).

Expression of p-p38MAPK in HFLS
Western blotting results showed that the expression of p-p38MAPK was significantly upregulated in TNF-α group (1.47 ± 0.09), relative to control group (0.83 ± 0.07) ($p < 0.05$). However, treatment with gentiopicrin significantly and dose-dependently down-regulated the expression of p-p38MAPK stimulated by TNF-α ($p < 0.05$; Figure 5).

Expression of NF-κB-p65 protein in HFLS
As shown in Figure 6, the expression of NF-κB-p65 protein was significantly upregulated in TNF-α group (1.42 ± 0.08), when compared with control group (1.00 ± 0.04) ($p < 0.05$). However, treatment with gentiopicrin significantly and dose-dependently down-regulated NF-κB-p65 protein expression ($p < 0.05$).

Effects of gentiopicrin and p38MAPK/NF-κB pathway inhibitors on secretion of IL-1β
Results of ELISA showed that the level of IL-1β in cell lysate was significantly higher in TNF-α group than in control group ($p < 0.05$). However, treatment with gentiopicrin and p38MAPK/NF-κB pathway inhibitors (SB203580 and BAY11-7082) significantly reduced IL-1β levels ($p < 0.05$). These results are shown in Figure 7.

Effects of gentiopicrin and p38MAPK/NF-κB pathway inhibitors on secretion of IL-6
The level of IL-6 was significantly higher in TNF-α group than in control group ($p < 0.05$). However, treatment with gentiopicrin or p38MAPK/NF-κB pathway inhibitors (SB203580 and BAY11-7082) significantly reduced the expression levels of IL-6 compared with TNF-α group ($p < 0.05$; Figure 8).

Discussion
Rheumatoid arthritis (RA), a chronic autoimmune disease, is characterized by inflammatory hyperplasia of synovial membrane and destruction of articular cartilage. Repeated attacks result in joint deformity and dysfunction in the late stage of the disease (23). The pathogenesis of RA has not been fully elucidated. Abnormal proliferation of synovial cells and infiltration of
inflammatory cells are the major pathological features of RA (24).

Inflammatory factors have been implicated in the occurrence and development of synovitis in patients with RA (25). Therefore, inhibition of the secretion of these factors is key to the treatment of RA, and p38MAPK and NF-κB pathways are the major signaling pathways that mediate the secretion of inflammatory factors. Inflammatory factors such as IL-1β and TNF-α stimulate phosphorylation of p38MAPK and transcription of NF-κB, and promote the expression of other inflammatory cytokines (26, 27). Elevated amounts of inflammatory factors promote the activation of signal pathways, thus aggravating the inflammatory response, leading to prolonged and non-healing synovitis.

Gentiopicrin is a pentacyclic triterpenoid isolated from plants, and it possesses anti-inflammatory, antioxidant and antitumor effects. Studies have shown that gentiopicrin and its derivatives significantly inhibit the release of inflammatory cytokines and paw swelling in RA rats (21, 22). In the present study, treatment with gentiopicrin at 5 - 25 μM did not significantly increase the number of viable cells, when compared with control group. However, at 50 and 100 μM, the number of viable cells were significantly increased, relative to control group. Results of qRT-PCR showed that the levels of expression of IL-1β and IL-6 mRNAs were significantly higher in TNF-α group than in control group. However, after treatment with gentiopicrin their expression levels decreased significantly and dose-dependently compared with TNF-α group. Western blotting results showed that the expressions of p-p38MAPK and NF-κB-p65 proteins were significantly upregulated in TNF-α group, when compared with control group. However, treatment with gentiopicrin significantly and dose-dependently decreased the expression of these proteins compared with TNF-α group. The levels of IL-1β and IL-6 in cell lysate were significantly higher in TNF-α group than in control group. However, treatment with gentiopicrin, and p38MAPK/NF-κB pathway inhibitors (SB203580 and BAY11-7082) significantly reduced the levels of these inflammatory factors compared with TNF-α group.

These results suggest that TNF-α may promote the expression and secretion of IL-6 and IL-1β, phosphorylation of p38MAPK and nuclear translocation of NF-κB in HFLS. It is likely that gentiopicrin inhibits the TNF-α-induced expressions of IL-6 and IL-1β, as well as phosphorylation of p38MAPK and nuclear translocation of NF-κB.

Gentiopicrin has therapeutic potential for RA through a mechanism involving the inhibition of p38MAPK/NF-κB pathway.

Acknowledgements
None.

Conflict of Interest
There are no conflict of interest in this study.

Author’s contribution
All work was done by the author s named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Fangxiang Mu; Nian Zhang, Yi Jiang, Fangxiang Mu, Hong Wu and Qingxia You collected and analysed the data; Nian Zhang and Yi Jiang wrote the text and all authors have read and approved the text prior to publication. Nian Zhang and Yi Jiang contributed equally to this work and should be considered as co-first authors.

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