

# Sulforaphane suppresses polyinosinic-polycytidylic acid-stimulated release of cytokines, chemokines and MMPs by human corneal fibroblasts

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**Abstract.** Viral corneal infection is a common cause of visual impairment and blindness. Polyinosinic-polycytidylic acid, or poly(I:C), is similar to viral double-stranded RNA in structure and has been implicated in the release of a variety of cytokines, chemokines and matrix metalloproteinases (MMPs) by corneal fibroblasts. Sulforaphane (SFN) is an isothiocyanate compound found in cruciferous vegetables. The present study investigated the potential effect of SFN on the poly(I:C)-stimulated release of cytokines, chemokines and MMPs in human corneal fibroblasts (HCFs). ELISA showed that SFN was associated with a time- and dose-dependent reduction in poly(I:C)-stimulated production of interleukin (IL)-8, chemoattractant protein-1, IL-6, MMP-1 and MMP-3 by HCFs. Western blot analysis indicated that SFN suppressed the function of poly(I:C) by modulating mitogen-activated protein kinases (MAPKs), including p38 and extracellular signal-regulated kinase (ERK), activator protein-1 (AP-1) component c-Jun and the kinase, Akt, and the phosphorylation and degradation of the nuclear factor (NF)- $\kappa$ B inhibitor I $\kappa$ B- $\alpha$ . Immunofluorescence analysis revealed that SFN attenuated the production of poly(I:C)-induced nuclear translocation of the NF- $\kappa$ B p65 subunit. Reverse transcription-quantitative PCR analysis revealed that SFN prevented the poly(I:C)-induced upregulation of Toll-like receptor 3 (TLR3) mRNA expression in HCFs. No significant cytotoxic effect of SFN on HCFs was observed. In summary, SFN attenuated the poly(I:C)-induced production of proinflammatory chemokines, cytokines and MMPs by HCFs, by inhibiting TLR3, MAPK (p38 and

ERK), AP-1, Akt and NF- $\kappa$ B signaling. SFN may therefore be a potential novel treatment for viral corneal infection by limiting immune cell infiltration.

## Introduction

Viral stromal keratitis is an inflammatory disease of the corneal stroma caused by viruses, of which herpes simplex virus type 1 (HSV-1) is the leading cause (1). HSV-1 keratitis can lead to visual impairment and blindness, as a result of corneal scarring, thinning, opacity and neovascularization (1). An epidemiological study in 2012 revealed that the global prevalence of HSV-1 keratitis was estimated to be 1.5 million cases, of which nearly 40,000 new cases of severe monocular visual impairment or blindness occur every year (2). Viral stromal keratitis is not only a simple infectious disease, but a chronic immune disorder, that adversely affects quality of life, even when the infection is not active (3). It is characterized by complex interactions between infiltrating immune cells and corneal intrinsic cells. These interactions induce and sustain an inflammatory response that ultimately lead to corneal damage (4). Current treatment for viral stromal keratitis predominantly consists of the administration of antiviral drugs and topical steroids to control inflammation (5). However, the choice of anti-inflammatory agents is relatively limited. Furthermore, topical glucocorticoids can potentially lead to various complications, such as glaucoma, cataracts and delayed healing (6). The development of reliable anti-inflammatory drugs with fewer side effects is thus, a clinical priority.

The stroma accounts for over 90% of the corneal thickness, and largely consists of resident keratocytes embedded in a matrix of type I collagen (7). The parallel arrangement of collagen fibers in the extracellular matrix of the corneal stroma is a key determinant of corneal transparency (7). These collagen fibers are produced largely by keratocytes (7). Activated keratocytes, also called corneal fibroblasts, are highly involved in regulating local inflammatory and immune responses to viral infections, through the production of various inflammatory factors, such as IL-6, IL-8 and chemoattractant protein-1 (MCP-1) (8,9). Polyinosinic-polycytidylic acid, or poly(I:C), is structurally similar to viral double-stranded RNA (dsRNA) and has been

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applied experimentally to investigate the effects of viral infection on various cell types, including microglia, human mucosal epithelial cells and airway epithelial cells (10-12). The interaction of poly(I:C) with Toll-like receptor 3 (TLR3) results in the activation of nuclear factor (NF)- $\kappa$ B and mitogen-activated protein kinases (MAPKs) (13). These mediate pivotal intracellular signaling pathways underlie the regulation of cell proliferation, differentiation, migration, senescence and apoptosis in response to a variety of extracellular stimuli, including viruses, lipopolysaccharide and certain proinflammatory cytokines, such as IL-1 and TNF- $\alpha$  (14,15). These effects of poly(I:C) on intracellular signaling trigger the release of various proinflammatory factors (13) and matrix metalloproteinases (MMPs) (16) in human corneal fibroblasts (HCFs). The proinflammatory factors, including IL-8, MCP-1 and IL-6, have vital roles in immune cell infiltration and the inflammatory response (17-19). An inflammatory response contributes to the removal of pathogens; however, an excessive infiltration of inflammatory cells and sustained release of cytokines and chemokines can lead to tissue damage, as evidenced by the destruction of corneal structure in viral stromal keratitis (20). The release of MMPs, particularly MMP-1 and MMP-3, has been significantly associated with degradation of the corneal stromal matrix and corneal ulceration in viral stromal keratitis (21).

Sulforaphane (1-isothiocyanate-4-methanesulfonyl butane; SFN) is a natural plant compound found in cruciferous plants, particularly in broccoli. SFN is derived from the hydrolysis of its precursor, glucosinolate (a reaction catalyzed by the enzyme myrosinase) (22). It is well-known that SFN is a natural antioxidant, and has anti-cancer (23) and anti-inflammatory (24,25) effects. It has been shown that SFN inhibits the proinflammatory action of lipopolysaccharides on microglia through the downregulation of the NF- $\kappa$ B and MAPK signaling pathways (26). Furthermore, *in vitro* and *in vivo* studies have shown that SFN can suppress the occurrence and progression of ocular diseases, such as cataracts (27), age-related macular degeneration (28) and Fuchs' corneal endothelial dystrophy (29), which were associated with its potential antioxidant effects. However, the possible effect of SFN on ocular inflammatory diseases is unclear. The present study investigated the potential anti-inflammatory effects of SFN on the release of cytokines, chemokines, and MMPs in HCFs stimulated by poly(I:C).

## Materials and methods

**Materials.** Fetal bovine serum (FBS), trypsin-EDTA and Eagle's minimum essential medium (MEM) were purchased from Gibco (Thermo Fisher Scientific, Inc.). Cell culture dishes, 24-well plates and flasks were obtained from Corning Inc. Sigma-Aldrich (Merck KGaA) supplied the SFN; and poly(I:C) was provided by InvivoGen. ELISA kits for IL-8 (cat. no. Q8000B), MCP-1 (cat. no. DCP00) and IL-6 (cat. no. Q6000B), were obtained from R&D Systems, Inc., while the kits for MMP-1 (cat. no. ab215083) and MMP-3 (cat. no. ab269371) were obtained from Abcam. The following antibodies were obtained from Cell Signaling Technology, Inc.: Anti-JNK (cat. no. 9252), phosphorylated (p)JNK (cat. no. 9251), I $\kappa$ B- $\alpha$  (cat. no. 9242), p-I $\kappa$ B- $\alpha$  (cat. no. 2859),

ERK (cat. no. 9102), p-ERK (cat. no. 9106), c-Jun (cat. no. 9165), p-c-Jun (cat. no. 9164), p38 MAPK (cat. no. 9212), p-p38 MAPK (cat. no. 9211), Akt (cat. no. 9272) and p-Akt (cat. no. 9271). The antibody against GAPDH (cat. no. 60004-1-Ig) was purchased from ProteinTech Group, Inc. Goat anti-mouse horseradish peroxidase (HRP)-conjugated antibody (cat. no. A0216), rabbit immunoglobulin G (cat. no. A0208), bovine serum albumin and enhanced chemiluminescence (ECL) reagents were purchased from Beyotime Institute of Biotechnology. The primary antibody against NF- $\kappa$ B p65 (cat. no. sc-8008) was purchased from Santa Cruz Biotechnology, Inc. Molecular Probes (Thermo Fisher Scientific, Inc.) supplied the 4',6-diamidino-2-phenylindole (DAPI) and Alexa Fluor 488-labeled donkey antibodies to mouse immunoglobulin G (cat. no. A-21202). The RNAprep pure kit (cat. no. DP430) was supplied by Tiangen Biotech Co., Ltd. A non-radioactive cytotoxicity assay kit for lactate dehydrogenase (LDH) was purchased from Promega Corporation. Endotoxin minimization was performed for all media and reagents used for cell culture.

**Cell culture and treatment with SFN.** Human keratocytes (cat. no. 6520) were purchased from ScienCell Research Laboratories, Inc. The cultured keratocytes (activated keratocytes), also called corneal fibroblasts, were maintained in MEM supplemented with 10% FBS at 37°C in a humidified incubator with 5% CO<sub>2</sub>. After four to seven passages, HCFs used for subsequent experimentation were harvested at the subconfluent stage, and further seeded into 24-well plates (3x10<sup>4</sup> cells/well) or 60-mm culture dishes (5x10<sup>5</sup> cells/dish). When the HCFs obtained confluence, the previous media was discarded, and serum-free culture media was added for an additional day. Poly(I:C) (3  $\mu$ g/ml) was added to the medium as a mimic of viral dsRNA, alongside various concentrations of SFN (1, 2, 5 or 10  $\mu$ M) as interventions.

**Assays of IL-8, MCP-1, IL-6, MMP-1 and MMP-3.** Serum-deprived corneal fibroblasts were incubated in the serum-free MEM with or without SFN (10  $\mu$ M) for 24 h, and then maintained in the same buffer containing poly(I:C) (3  $\mu$ g/ml) for another 12, 24, 36 and 48 h. Cell supernatant fluid was obtained following centrifugation at 120 x g for 5 min at 4°C, and frozen at -80°C for subsequent assessments of IL-8, MCP-1, IL-6, MMP-1 and MMP-3 with ELISA kits. Following exposure to trypsin-EDTA, the cells were isolated from the culture plates, stained with trypan blue for 3 min at room temperature and further counted using a hemocytometer. As counting of the cells and morphology were not influenced by exposure to SFN or poly(I:C), the measurements of these proteins in the culture supernatants were normalized by cell number.

**Western blot analysis.** The protein expression levels of Akt, c-Jun, MAPKs and I $\kappa$ B- $\alpha$  in the HCFs were detected using western blot analysis. During the first 24 h, cells were maintained in MEM with 0.5% FBS, and then cultured in serum-free medium for another 24 h. The serum-deprived cells were further incubated in serum-free MEM with or without SFN (10  $\mu$ M) for 24 h, and were incubated in the same solution with or without poly(I:C) (3  $\mu$ g/ml), as previously described (16) for

an additional 30 min (for Akt) or 90 min (for MAPKs, I $\kappa$ B- $\alpha$  and c-Jun). The cells were washed twice with ice-cold PBS and lysed in a solution containing the following: 1% protease inhibitor cocktail; 100 mM NaCl; 1% Nonidet P-40; 10 mM MgCl<sub>2</sub>; 1 mM dithiothreitol; 50 mM Tris-HCl (pH 7.4); and 1 mM phenylmethylsulfonyl fluoride. Following centrifugation (120 x g, for 10 min at 4°C), the cell lysates were collected to measure the protein concentration using the Bradford method. The total protein (10  $\mu$ g) was first stacked with 10% SDS-PAGE gels and then resolved with 6% gels. Subsequently, the proteins were transferred onto polyvinylidene difluoride membranes (0.45  $\mu$ m), then blocked in a mixture containing 5% skimmed milk in TBS-Tween-20, following which the membranes were incubated overnight at 4°C, with the primary antibodies (all at 1:1,000) diluted in the blocking buffer. The following day, the membranes were washed using a mixture of 20 mM Tris-HCl (pH 7.4) and 0.1% Tween-20, and incubated with the secondary antibody conjugated to HRP (dilution 1:3,000) at room temperature for 1 h. The proteins were imaged immediately with a Tanon-5200 Multi-imaging System following incubation with ECL solutions (Tanon Science and Technology Co., Ltd.).

**Immunofluorescence staining.** HCFs were maintained for 24 h in MEM containing 0.5% FBS, then serum-free media for an additional 24 h. This was followed by another 24 h with or without SFN (10  $\mu$ M) in serum-free MEM, and an additional 90 min in the same solution with or without poly(I:C) (3  $\mu$ g/ml). The cells were fixed with 4% paraformaldehyde at room temperature for 15 min before being permeabilized with 0.2% Triton X-100 for 15 min at room temperature. Between each step, the cells were washed with PBS. Non-specific adsorption of antibodies was blocked by adding 3% bovine serum albumin for 15 min at room temperature. The cells were then incubated with a mouse monoclonal antibody (anti-NF- $\kappa$ B; 1:50) at room temperature for 1 h. Subsequently, the cells were further incubated for 1 h at room temperature with Alexa Fluor 488-conjugated secondary antibodies (diluted 1:500) and DAPI. Finally, the images were obtained using a fluorescence microscope (Zeiss AG).

**Reverse transcription-quantitative PCR (RT-qPCR) analysis.** Serum-deprived HCFs were incubated first for 24 h with or without SFN (10  $\mu$ M) in serum-free MEM, and then for an additional 4 h with or without poly(I:C) (3  $\mu$ g/ml) in the same medium. Total RNA was isolated from the cells using the RNeasy pure kit and subjected to RT-qPCR analysis, as previously described (30). The sequences of the PCR primers were as follows (30): TLR3 sense, 5'-CGCCAACTTCAC AAGGTA-3' and antisense, 5'-GGAAGCCAAGCAAAG GAA-3'; hypoxanthine phosphoribosyltransferase 1 (HPRT1) sense, 5'-AGATGGTCAAGGTCGCAAGC-3'; and antisense, 5'-CATATCCTACAACAACTTGTCTGGAA-3'. PCR was performed with an ABI Prism 7900 Sequence Detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The following thermocycling conditions were used: Denaturation at 95°C for 15 sec, annealing at 60°C for 15 sec followed by elongation at 60°C for 15 sec. The qPCR results, recorded as threshold cycle numbers (Cq), were calculated using the 2<sup>- $\Delta\Delta$ Cq</sup> method (31) with normalization against HPRT1 mRNA as an internal control.

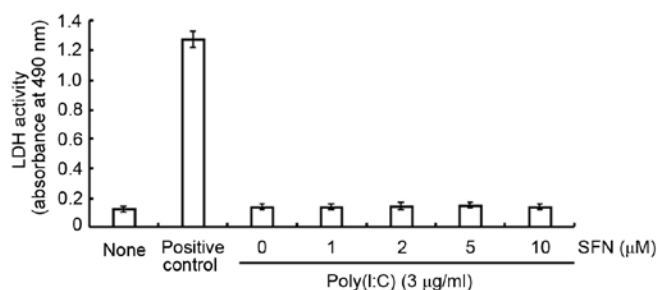


Figure 1. Cytotoxic effect of SFN in the HCFs. Serum-deprived HCFs were incubated with the corresponding doses of SFN for 24 h, then with poly(I:C) (3  $\mu$ g/ml) for another 24 h. The release of LDH was detected using an assay. Data are shown as the mean  $\pm$  standard deviation, averaged from triplicates and was repeated three times with similar results. SFN, sulforaphane; HCF, human corneal fibroblasts; poly(I:C), polyinosinic-polycytidylic acid; LDH, lactate dehydrogenase.

**LDH (cytotoxicity) assay.** A non-radioactive cytotoxicity assay was used to assess the production of LDH by cultured corneal fibroblasts. Portions (50  $\mu$ l) of the same culture supernatants, used for the assessment of IL-8, MCP-1 and IL-8, were transferred to a 96-well flat-bottom plate, then mixed with 50  $\mu$ l CytoTox reagent. The plate was covered with an opaque box to prevent light exposure for 30 min at room temperature, then stop solution was added to each well (50  $\mu$ l). The LDH assay was performed at an optical density of 490 nm using a microplate reader (Bio-Rad Laboratories, Inc.). As a positive control, cells were lysed prior to the assay with a lysis solution containing detergent provided with the assay kit.

**Statistical analysis.** Data for each group were derived from at least three independent samples, and all sampling was repeated three times in the experimental groups. All statistical analyses were performed using SPSS software (version 20.0; IBM Corp.). Descriptive results are expressed as the mean  $\pm$  standard deviation and were compared with an unpaired two-tailed t-test or one-way analysis of variance, followed by a Tukey's post hoc test.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Cytotoxicity of SFN in HCFs.** To examine whether SFN is cytotoxic to HCFs, the effect of SFN on the release of LDH was investigated. SFN at concentrations of 1, 2, 5 or 10  $\mu$ M had no significant effect on LDH release in the presence of poly(I:C) at 3  $\mu$ g/ml (Fig. 1), which suggests a lack of cytotoxicity.

**Effects of SFN on the poly(I:C)-induced release of IL-8, MCP-1 and IL-6 by HCFs.** Exposure of HCFs to various concentrations of SFN for 24 h prior to incubation with poly(I:C) (3  $\mu$ g/ml) for 24 h attenuated the secretion of IL-6 and IL-8 in a dose-dependent manner and that of MCP-1 at 5 and 10  $\mu$ M SFN (Fig. 2A). The inhibitory effects of SFN were statistically significant at concentrations of  $\geq 2$   $\mu$ M for IL-6 and IL-8 and at  $\geq 5$   $\mu$ M for MCP-1 compared with 0  $\mu$ M SFN. Furthermore, SFN (10  $\mu$ M) attenuated the poly(I:C)-induced release of these inflammatory mediators in a time-dependent manner (Fig. 2B), with each inhibitory effect being statistically

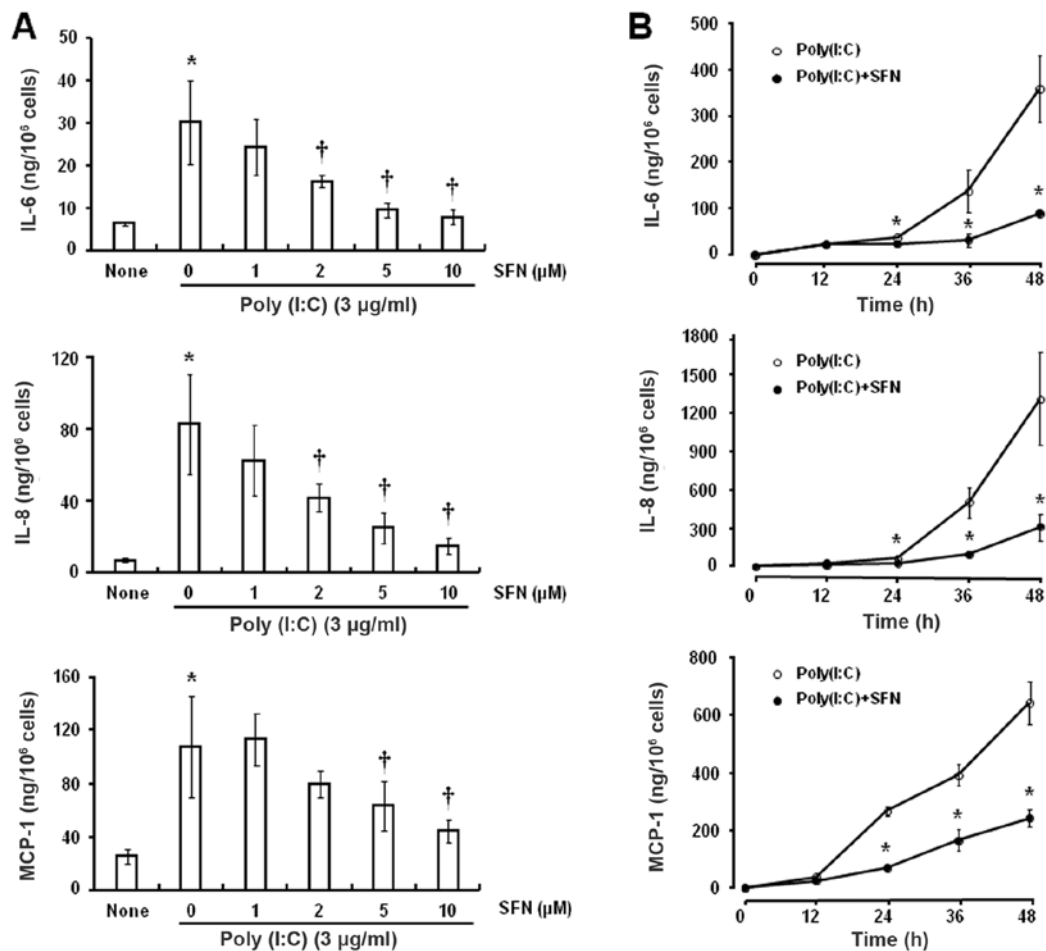


Figure 2. Inhibitory effects of SFN on the poly(I:C)-induced release of IL-6, IL-8 and MCP-1 by the HCFs. (A) Serum-deprived HCFs were treated with the corresponding concentrations of SFN for 24 h, then poly(I:C) (3  $\mu$ g/ml) was added for another 24 h. The results were assessed using ELISA. \* $P$ <0.05 compared with the control group (None). † $P$ <0.05 compared with that in the cells exposed to poly(I:C) alone. (B) Serum-deprived HCFs were incubated for 24 h with (solid circles) or without (open circles) SFN (10  $\mu$ M), then incubated for the specified time durations with poly(I:C) (3  $\mu$ g/ml). The release of these inflammatory factors was measured using ELISA. \* $P$ <0.05 compared with the cells incubated with poly(I:C) alone. SFN, sulforaphane; MCP-1, chemoattractant protein-1; poly(I:C), polyinosinic-polycytidylic acid; HCF, human corneal fibroblasts; IL, interleukin.

significant following exposure to poly(I:C) for  $\geq 24$  h compared with the respective times in the group without SFN treatment.

**Effects of SFN on poly(I:C)-induced changes in MMP production by HCFs.** Poly(I:C) increased the release of MMP-1 and MMP-3 by HCFs, which was sensitive to inhibition by SFN, in a dose-dependent manner (Fig. 3A). The inhibitory effects of SFN were statistically significant at concentrations  $\geq 1$   $\mu$ M compared with 0  $\mu$ M SFN. In addition, SFN (10  $\mu$ M) significantly attenuated the poly(I:C)-induced release of MMP-1 and MMP-3 after exposure to poly(I:C) for 36 and 48 h compared with the respective times in the group without SFN treatment (Fig. 3B).

**Effect of SFN on poly(I:C)-induced expression of TLR3 in HCFs.** RT-qPCR analysis revealed that incubation of serum-deprived HCFs with poly(I:C) (3  $\mu$ g/ml) for 4 h induced significant upregulation of TLR3 mRNA expression level (Fig. 4). Furthermore, prior exposure of the cells to SFN (10  $\mu$ M) for 24 h prevented this effect of poly(I:C). SFN also reduced the basal abundance of TLR3 mRNA in the cells (Fig. 4).

**Effects of SFN on poly(I:C)-associated activation of MAPK and AP-1 protein expression level by HCFs.** Western blot analysis showed that exposure of HCFs to poly(I:C) for 90 min increased the expression level of the phosphorylated forms of ERK, p38, c-Jun NH<sub>2</sub>-terminal kinase (JNK) and c-Jun; however, there was no marked effect on the total amounts of these proteins (Fig. 5), which indicated that poly(I:C) activated the MAPK and AP-1 signal pathways. SFN (10  $\mu$ M) inhibited the poly(I:C)-induced phosphorylation of ERK (Fig. 5A), p38 (Fig. 5B) and c-Jun (Fig. 5D), however, there was no marked effect on that of JNK (Fig. 5C).

**Effects of SFN on poly(I:C)-mediated activation of I $\kappa$ B- $\alpha$  and NF- $\kappa$ B by HCFs.** Western blot analysis revealed that SFN (10  $\mu$ M) suppressed the poly(I:C)-induced phosphorylation of the NF- $\kappa$ B inhibitor I $\kappa$ B- $\alpha$  in HCFs (Fig. 6A). Furthermore, the immunofluorescence analysis showed that, whereas the p65 subunit of NF- $\kappa$ B was localized predominantly in the cytoplasm of HCFs under control conditions, it was localized to the nucleus following exposure of the cells to poly(I:C) for 90 min, and the effect of poly(I:C) was partially prevented by SFN (Fig. 6B). Thus, these results indicated that SFN

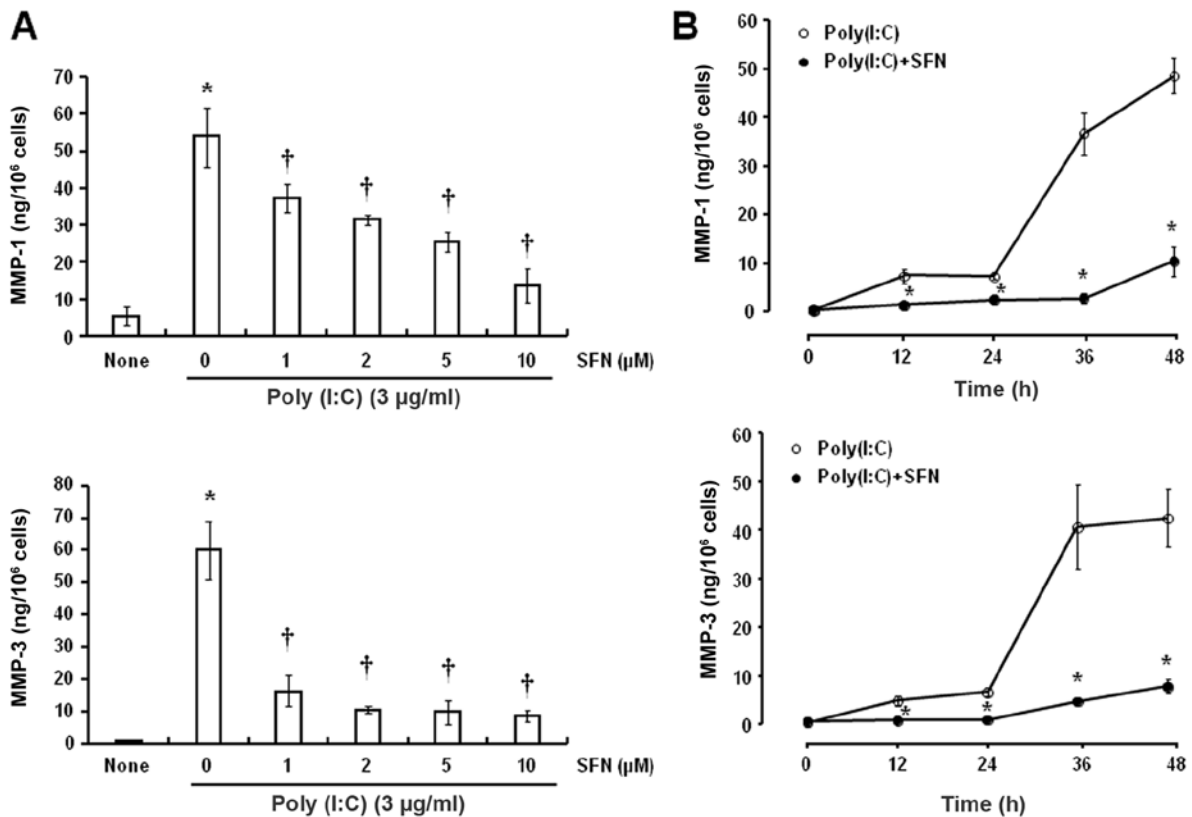


Figure 3. Inhibitory effects of SFN on the poly(I:C)-enhanced secretion of MMP-1 and MMP-3 by the HCFs. (A) Serum-deprived HCFs were incubated with the corresponding doses of SFN for 24 h, then incubated with or without poly(I:C) (3  $\mu$ g/ml) for a further 48 h. The measurement of MMP-1 and MMP-3 was determined using ELISA. \* $P$ <0.05 compared with the control group (None); † $P$ <0.05 compared with that in cells exposed to poly(I:C) alone. (B) Serum-deprived HCFs were treated with SFN [10  $\mu$ M (solid circles)] or without [0  $\mu$ M (open circles)] for 24 h, then incubated for the specified time points with poly(I:C) (3  $\mu$ g/ml), following which the culture supernatants were analyzed using ELISA. \* $P$ <0.05 compared with the respective times in the group with poly(I:C) alone. SFN, sulforaphane; poly(I:C), polyinosinic-polycytidylic acid; HCF, human corneal fibroblasts; MMP, matrix metalloproteinases.

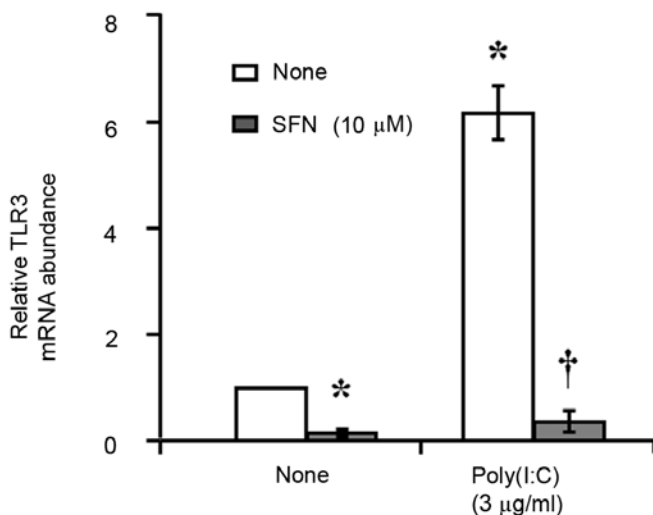


Figure 4. Inhibitory effect of SFN on the poly(I:C)-induced upregulation of TLR3 mRNA expression level in the HCFs. Serum-deprived HCFs were incubated first for 24 h in the absence or presence of SFN (10  $\mu$ M), then for an additional 4 h in the absence or presence of poly(I:C) (3  $\mu$ g/ml). The TLR3 mRNA expression level in the cells was determined using reverse transcription-quantitative PCR analysis. The data are presented as the mean  $\pm$  SD from 3 replicates, from 3 independent experiments. \* $P$ <0.05 compared with that in cells incubated without treatment; † $P$ <0.05 compared with that in cells exposed to poly(I:C) alone. SFN, sulforaphane; poly(I:C), polyinosinic-polycytidylic acid; TLR, Toll-like receptor; HCF, human corneal fibroblasts.

attenuated the activation of NF- $\kappa$ B signaling induced by poly(I:C) in HCFs.

*Effect of SFN on the activation of AKT associated with poly(I:C) in HCFs.* Finally, the effect of SFN on the activation of the phosphoinositide 3-kinase (PI3K)-Akt signaling pathway was investigated with poly(I:C) in HCFs. Western blot analysis showed that the phosphorylation of Akt induced by exposure of HCFs to poly(I:C) for 30 min was inhibited by SFN (10  $\mu$ M; Fig. 7).

## Discussion

Viral stromal keratitis is not only an infectious disease but also a chronic immunopathological condition (1). Viral stromal keratitis was found to upregulate the mRNA expression of TLR3 and subsequent production of various cytokines and chemokines, such as IL-6, IL-8 and MCP-1, in corneal fibroblasts (32). Poly(I:C), an analogue of the viral dsRNA, was found to activate TLR3 and has been adopted as an experimental tool to model the effects of HSV-1 infection (10,12,13). The cytokines, IL-8 and IL-6 and the chemokine, MCP-1 have key roles in the inflammatory response, by attracting neutrophils, monocytes and macrophages (17-19). SFN reportedly inhibited the release of IL-6 induced by lipopolysaccharide in microglial cells (26) in a rat model of endometriosis (24),

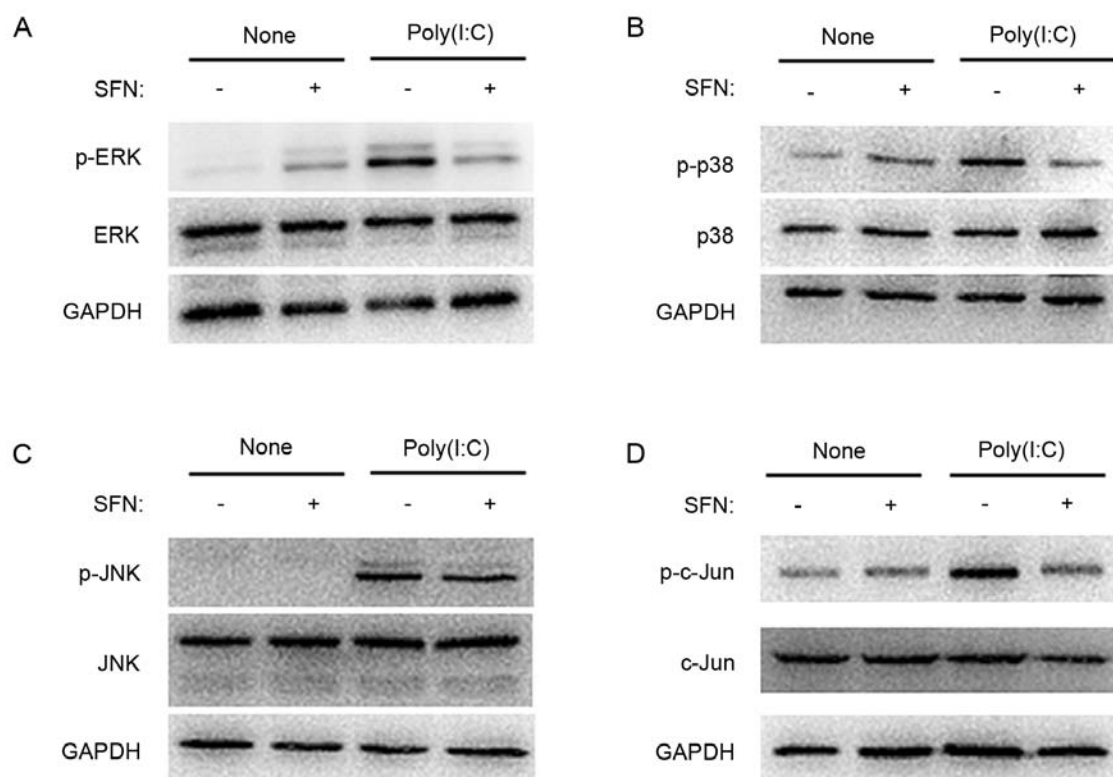


Figure 5. Effects of SFN on the poly(I:C)-associated activation of the MAPK and AP-1 signaling pathway in the HCFs. Serum-deprived HCFs were incubated first for 24 h with or without SFN (10 and 0  $\mu$ M), then for 90 min with or without poly(I:C) (3  $\mu$ g/ml). The measurements of total or activated (A) ERK, (B) p38, (C) JNK and (D) c-Jun and GAPDH (loading control) were determined using western blot analysis. AP-1, activator protein-1; SFN, sulforaphane; p-, phosphorylated; poly(I:C), polyinosinic-polycytidylic acid; HCF, human corneal fibroblasts.

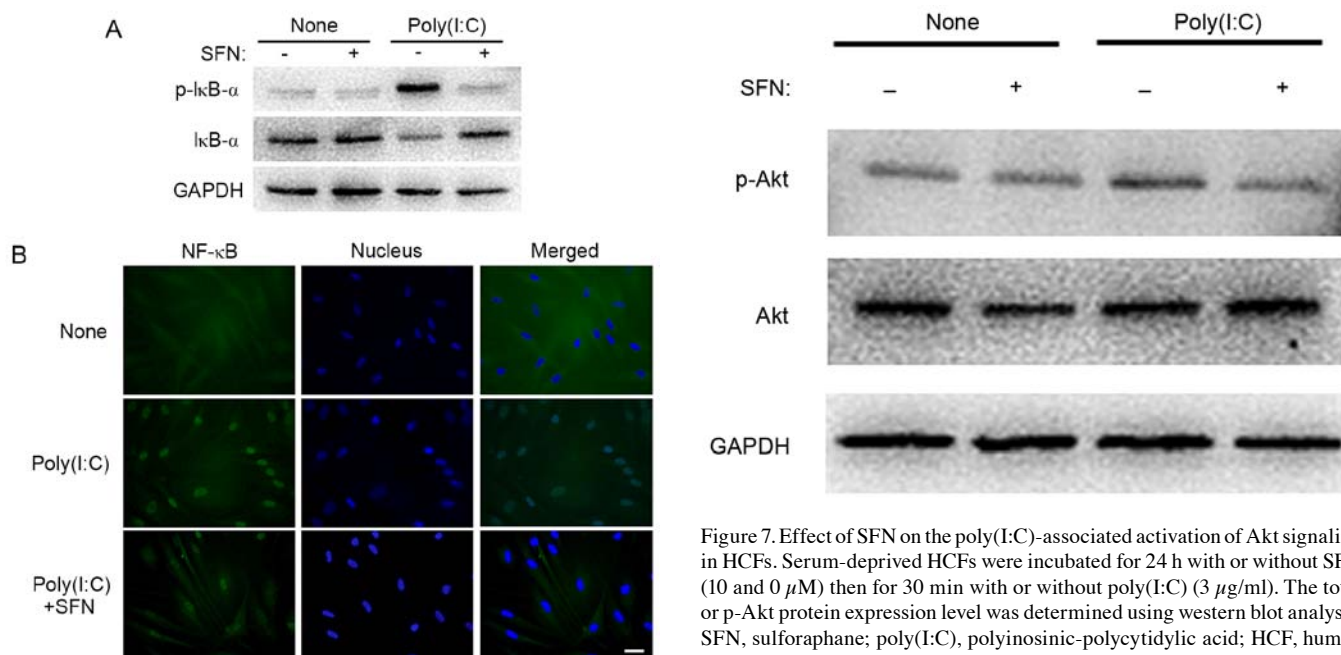


Figure 6. Effect of SFN on the poly(I:C)-associated activation of NF- $\kappa$ B signaling in the HCFs. Serum-deprived HCFs were incubated first for 24 h with or without SFN (10 and 0  $\mu$ M) and incubated for an additional 90 min with or without poly(I:C) (3  $\mu$ g/ml). (A) Measurement of total IκB or p-IκB- $\alpha$  and GAPDH (loading control) protein expression level determined using western blot analysis. (B) Cells were examined using immunofluorescence staining with NF- $\kappa$ B p65 antibodies (green fluorescence) and for nuclear staining with DAPI (blue fluorescence). Scale bar, 50  $\mu$ m. SFN, sulforaphane; poly(I:C), polyinosinic-polycytidylic acid; HCF, human corneal fibroblasts; p-, phosphorylated.

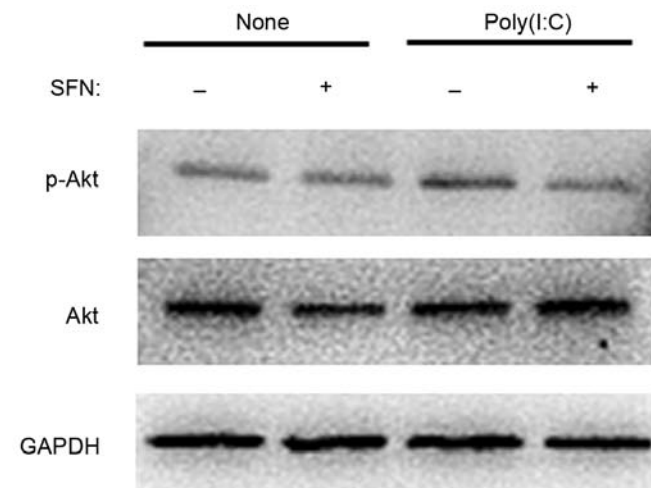


Figure 7. Effect of SFN on the poly(I:C)-associated activation of Akt signaling in HCFs. Serum-deprived HCFs were incubated for 24 h with or without SFN (10 and 0  $\mu$ M) then for 30 min with or without poly(I:C) (3  $\mu$ g/ml). The total or p-Akt protein expression level was determined using western blot analysis. SFN, sulforaphane; poly(I:C), polyinosinic-polycytidylic acid; HCF, human corneal fibroblasts; p-, phosphorylated.

and in a mouse model of acute lung cancer (25). The present study found that SFN inhibited the poly(I:C)-induced production of IL-6, IL-8 and MCP-1 by HCFs. This suggested that SFN could be a promising drug candidate for therapeutic modulation of the inflammatory response in viral stromal keratitis.

Viral stromal keratitis has been associated with the proteolytic degradation of stromal collagen, which can lead to corneal ulceration and ultimately, to the loss of corneal transparency (21). MMPs are zinc-dependent enzymes that degrade extracellular matrix proteins, of which MMP-1 and MMP-3 are two major types and significantly contribute to corneal ulcers (21). Upregulation of MMP-1 or MMP-3 expression has been associated with varicella zoster virus (33) and cytomegalovirus (34) infection. Expression of MMP-3 was also found elevated in the brain stem of HSV-1-infected mice (35). The protein expression of MMPs was induced in viral keratitis (36,37) and contributed to tissue infiltration by polymorphonuclear leukocytes (38). These various observations indicated the important role of MMPs in the pathogenesis of disease associated with viral keratitis. Consistent with a previous study (39), the present study found that poly(I:C) induced the concentration of MMP-1 and MMP-3 in corneal fibroblasts. This suggested that cells associated with lesions of viral keratitis may contribute to remodeling of the extracellular matrix and consequently corneal ulceration by producing MMPs. In previous studies, SFN was shown to prevent the upregulation of MMP-1 induced by ultraviolet irradiation in the skin of mice (40), and inhibited the production of MMP-1 and MMP-3 stimulated by IL-1 $\beta$  in synovial fibroblasts associated with rheumatoid arthritis (41). In the present study, SFN was found to suppress the poly(I:C)-associated release of these two MMPs by HCFs. Further studies are required to determine the effect of SFN on viral corneal ulceration; however, these results indicated that this agent may prove an effective treatment for this condition.

TLRs contribute to the initiation and modulation of inflammation in the eye (30). Upregulation of TLR3 mRNA expression in the human cornea has been association with HSV-1 infection (15). TLR3 is a specific receptor for dsRNA and would not be expected to detect DNA derived from a DNA virus, such as HSV-1 (15). However, dsRNA is produced by most viruses during their replication cycle and is considered a molecular marker of viral infection (42). As a synthetic analog of viral dsRNA, poly(I:C) is recognized by TLR3 (43). The present study found that poly(I:C) upregulated the TLR3 mRNA expression level in HCFs and that SFN attenuated this effect. Thus, the inhibitory effects of SFN on cytokine, chemokine and MMP expression in HCFs, exposed to poly(I:C) may be mediated by attenuation of the TLR3 signaling pathway.

Stimulation of TLR3 initiates a cascade of intracellular signaling, including that mediated by MAPKs and PI3K-Akt, and results in the activation of NF- $\kappa$ B or AP-1 (14). All of these signaling pathways have been associated with inflammation, including that of the cornea (13,16,44,45). NF- $\kappa$ B, a transcription factor, mediates the mRNA expression level of inflammation-related genes, including cytokines, chemokines and adhesion molecules (46). Under basal conditions, NF- $\kappa$ B is bound to the inhibitor, I $\kappa$ B in the cytoplasm (47). The phosphorylation of I $\kappa$ B, induced by inflammatory stimuli, triggers its degradation and consequently transfers the active NF- $\kappa$ B to the nucleus, where NF- $\kappa$ B, in turn, activates the mRNA expression level of cytokines, chemokines and MMP-related genes (13,16). The JNK, p38 MAPKs and ERK are important pathways in the regulation of various cell activities, such as cell proliferation, differentiation and migration, and are

significantly associated with inflammation, innate immunity and apoptosis (48,49). The activation of TLRs by components of pathogens induces the phosphorylation of MAPKs, which can then lead to I $\kappa$ B phosphorylation and activation of NF- $\kappa$ B (50). The transcription factor, AP-1, also mediates the mRNA expression level of inflammatory genes (51). Activation of the AP-1 component c-Jun triggers the release of inflammatory mediators, and MMPs in human synoviocytes (45). It has been shown that the activation of the PI3K-Akt signaling pathway was associated with the regulation of MCP-1 mRNA and protein expression in human retinal pigment epithelial cells (52). Poly(I:C) was confirmed to induce the activation of Akt in HCFs (44). In the present study, SFN was demonstrated to inhibit the poly(I:C)-induced activation of ERK, p38, c-Jun and AKT, and the degradation of I $\kappa$ B- $\alpha$  and the nuclear translocation of NF- $\kappa$ B in HCFs. This suggested that the anti-inflammatory effects of SFN in these cells may be mediated by the attenuation of signaling by the MAPK, AP-1, PI3K-Akt and NF- $\kappa$ B pathways.

In conclusion, the present study showed that SFN inhibited the poly(I:C)-associated release of proinflammatory cytokines, chemokines and MMPs in HCFs, potentially through suppression of the TLR3, MAPK (ERK and p38), NF- $\kappa$ B, AP-1 and Akt signaling pathways. SFN may be a potential treatment for corneal viral infection by limiting immune cell infiltration. Further research is warranted to investigate the potential efficacy of SFN for the treatment of viral stromal keratitis.

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#### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

#### Authors' contributions

YaL, YeL and XZha contributed to the study design. PL, HZ and LC performed the experiments. PL, XZhe and XY analyzed the data. XZhe and YaL wrote the first draft of the manuscript. All authors read and approved the final manuscript.

#### Ethics approval and consent to participate

Not applicable.

#### Patient consent for publication

Not applicable.



## Competing interests

The authors declare that they have no competing interests.

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