

# Minecoside promotes apoptotic progression through STAT3 inactivation in breast cancer cells

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**Abstract.** Breast cancer is one of the most common malignant tumors in women worldwide, and is a major cause of mortality and morbidity in cancer patients. Constitutive activation of STAT3 has been found in a variety of malignant tumors, including breast cancer. Since STAT3 activation is capable of regulating various important features of tumor cells, identification of a novel STAT3 inhibitor is considered a potential strategy for treating breast cancer. The aim of the present study was to examine whether minecoside (MIN), an active compound extracted from *Veronica peregrina* L., exerts an antitumor effect by inhibiting STAT3 signaling pathway in MDA-MB-231 cells. The results revealed that MIN inhibited the constitutive STAT3 activation in a dose- and time-dependent manner. MIN also blocked the nuclear translocation of STAT3 and suppressed STAT3-DNA binding. In addition, MIN downregulated the STAT3-mediated expression of proteins such as Bcl-xL, Bcl-2, CXCR4, VEGF, and cyclin D1. Subsequently, MIN promoted the caspase-dependent apoptosis in MDA-MB-231 cells. Overall, results of the present study provide evidence that MIN exerted anticancer activity via inhibition of the STAT3 signaling pathway. Further studies using animal models are required to determine the potential of this molecule as an anticancer drug.

## Introduction

Breast cancer is one of the most common malignant tumors in women worldwide, and triple-negative breast cancer accounts for 15-20% of all breast cancer cases (1,2). At present, various

clinical trials have been conducted to treat and delay the progression of breast cancer, but mortality and morbidity among patients remain high (3). Several carcinogens and signaling pathways are known to be involved in the progression of breast cancer. Among them, signal transducer and activator of transcription 3 (STAT3) has been reported to play a pivotal role in breast cancer development (4). Unlike normal cells, in which the activity of STAT3 is strictly regulated, STAT3 activity in breast cancer contributes to tumorigenesis in a multifaceted manner (5). Interleukin-6 (IL-6), G-protein-coupled receptors (GPCRs), and Toll-like receptors (TLRs) are known to be involved in STAT3 activation in various types of cancer, including colon cancer (6,7). Once STAT3 signaling is activated, it induces carcinogenesis through the expression of various genes associated with apoptosis (survivin, Bcl-xL, and Bcl-2), proliferation (cyclin D1), and angiogenesis (VEGF) (8,9). Additionally, several protein tyrosine phosphatases (PTPs) promote the death of cancer cells by STAT3 inactivation (10,11). SH2 domain containing phosphatase 1/2 (SHP-1/2) and protein tyrosine phosphatase 1B (PTP-1B) are known to be associated with STAT3 inactivation. SHP-1 is highly expressed in normal lymphocytes, whereas its expression is decreased in most cancer cells (12). In addition, SHP-1 expression in cancer cells effectively suppresses target genes such as VEGF-1, cyclin D1, and survivin (13). Thus, dephosphorylation of STAT3 by increasing the expression of SHP-1 may be an efficient strategy for the treatment of various cancer types.

In previous decades, many researchers have suggested that natural compounds may act as potent anti-cancer drugs with high efficacy and low side effects. Various candidates have been studied and examined for their anti-cancer properties and underlying mechanisms. Minecoside (MIN), an active compound extracted from *Veronica peregrina* L., belongs to the family Scrophulariaceae (14). The entire plant has been used as a traditional drug for the treatment of menstrual irregularities, fractures, and traumatic injuries (14). Moreover, several compounds from *Veronica peregrina* L. have been reported to exhibit antioxidant activity (14). A recent study revealed that MIN suppressed the invasive capability of cancer cells by inhibiting CXCR4 expression via blocking NF- $\kappa$ B (15). However, the mechanism underlying the regulation of STAT3 activation by MIN has not been completely

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understood. Therefore, we investigated whether MIN could modulate the apoptosis of breast cancer cells by regulating the STAT3 signaling pathway.

## Materials and methods

**Purity analysis of minecoside.** Minecoside was isolated from the *Catalpa ovata* according to a previously protocol (16). Purity analysis of isolated minecoside was carried on Shiseido CapCell PAK C18 column (Sigma-Aldrich) particle size 5  $\mu$ m (150x4.6 mm) using a Waters 2695 system (Waters Corporation). The mobile phase consisted of water with 0.1% formic acid (solvent A), acetonitrile with 0.1% formic acid (solvent B), which were applied in the following gradient elution: 5% B (0-5 min), 5-95% B (5-30 min). The injection volume was 10  $\mu$ l, and the flow rate was 0.6 ml/min. The UV chromatogram of minecoside was acquired at 330 nm and integrated. Purity of minecoside was 90.4 $\pm$ 0.4%. The chemical structure of MIN is shown in Fig. 1A. As preliminary experiments with minecoside (MIN) had been performed by the authors, identical conditions were adhered to throughout the study (15).

**Materials.** Antibodies against phospho-STAT3 (1:1,000; rabbit, monoclonal; cat. no. 9145), STAT3 (1:1,000; rabbit, monoclonal; cat. no. 12640), p-JAK1 (1:1,000; rabbit, polyclonal; cat. no. 3331), p-JAK2 (1:1,000; rabbit, monoclonal; cat. no. 8082), p-Src (1:1,000; rabbit, polyclonal; cat. no. 2101), Src (1:1,000; rabbit, polyclonal; cat. no. 2108), SHP-1 (1:1,000; rabbit, monoclonal; cat. no. 3759), cleaved PARP (1:1,000; rabbit, monoclonal; cat. no. 5625), cleaved caspase-9 (1:1,000; rabbit, monoclonal; cat. no. 7237), cleaved caspase-3 (1:1,000; rabbit, polyclonal; cat. no. 9661), Bcl-2 (1:1,000; rabbit, monoclonal; cat. no. 3498),  $\beta$ -actin (1:1,000; rabbit, monoclonal; cat. no. 4970) and anti-rabbit IgG (1:5,000; rabbit, polyclonal; cat. no. 14708) were obtained from Cell Signaling Technology, Inc. CXCR4 antibody (1:10,000; rabbit, polyclonal; cat. no. ab227767) was obtained from Abcam. Antibodies to VEGF (1:1,000; rabbit, polyclonal; cat. no. sc-152), JAK1 (1:1,000; rabbit, polyclonal; cat. no. sc-277), JAK2 (1:1,000; rabbit, polyclonal; cat. no. sc-278), Bcl-xL (1:1,000; mouse, monoclonal; cat. no. sc-8392), Cyclin D1 (1:1,000; rabbit, polyclonal; cat. no. sc-718) and goat anti-mouse IgG (1:5,000; mouse, monoclonal; cat. no. 2355) were purchased from Santa Cruz Biotechnology, Inc. RIPA buffers were purchased from Cell Signaling Technology, Inc. DMEM, fetal bovine serum (FBS), and antibiotic-antimycotic mixture were purchased from Gibco BRL; Thermo Fisher Scientific, Inc. The DIG gel shift kit for EMSA was purchased from Roche Diagnostics.

**Cell culture.** MDA-MB-231 cells were obtained from the American Type Culture Collection and cultured in DMEM supplemented with 10% FBS and 1% antibiotics at 37°C in a humidified incubator with 5% CO<sub>2</sub>. For the western blot assay, MDA-MB-231 cells were treated with the indicated concentrations of MIN (0, 12.5, 25, and 50  $\mu$ M) for 24 h or at the indicated times (0, 6, 12, and 24 h) at 50  $\mu$ M concentration.

**Cell viability assay by Cell Counting Kit-8 (CCK-8).** To determine the optimal concentration of MIN capable of inhibiting STAT3 activity, MDA-MB-231 cells (5x10<sup>4</sup> cells/well) were

seeded into 96-well plates. MIN was added at various concentrations (0-100  $\mu$ M) at 37°C for 24 h. Subsequently, 10  $\mu$ l CCK-8 solution was added to each well, and the cells were incubated at 37°C for 2 h. The cell viability was determined by measuring the absorbance at 490 nm using a GloMax® Explorer Multimode Microplate Reader (Promega Corporation).

**Western blot assay.** As described in a previous study (17), whole-cell extracts were lysed with RIPA buffer and then the extracted proteins were separated by 10% SDS-PAGE and electrotransferred to PVDF membranes. The membranes were immunoblotted with the aforementioned primary and secondary antibodies.

**Electrophoretic mobility shift assay (EMSA).** As previously described (17), binding activity of STAT3 to consensus oligonucleotides was measured with extracting nuclear proteins from MIN-treated MDA-MB-231 cells using non-radioactive EMSA assay (DIG Gel Shift Kit; Roche Diagnostics). Oligonucleotide probes labeled with DIG containing consensus binding sites for STAT3 (5-CTTCATTTCCCGTAAATC CCTAAAGCT-3 and 5-AGCTTTAGGGATTACGGGAA ATGA-3) were used.

**Immunofluorescence assay.** As described in a previous study (15), immunofluorescence assay was performed to check STAT3 nuclear translocation. The cells were blocked with 5% BSA for 1 h and then incubated with the anti-STAT3 antibody at room temperature for 1 h. After being washed with PBS, the slides were incubated with the secondary antibody Alexa Flour 488 for 30 min and counterstained for nuclei with Hoechst-33342 at 37°C for 10 min. The fluorescence image was measured under x200 magnification using a fluorescence microscope (Nikon ECLIPSE Ti-U; Nikon Corporation).

**TUNEL assay.** Detection of DNA fragments *in situ* using terminal deoxyribonucleotidyl transferase-mediated dUTP-digoxigenin nick end labeling (TUNEL) assay kits (Roche Diagnostics) was applied to investigate active cell death. Briefly, cells were treated with 50  $\mu$ M MIN for 24 h, then washed with PBS. The cells were fixed with freshly prepared 4% paraformaldehyde for 1 h at room temperature and treated with 0.2% Triton X-100 solution for 2 min on ice. Intracellular DNA fragments were then labeled by exposing the cells to TUNEL reaction mixture for 1 h at 37°C in a humidified atmosphere, with protection from light. The cells were washed with PBS twice, then transferred to slides and analyzed under a fluorescence microscope (Nikon ECLIPSE Ti-U; Nikon Corporation).

**Statistical analysis.** Experimental data are presented as the mean  $\pm$  SEM obtained from three independent experiments. Statistical significance was assessed by one-way analysis of variance (ANOVA) followed by Tukey's honest significant difference test or Students' t-test using graph pad prism 6 software package (GraphPad Software Inc.) and ImageJ (imagej.nih.gov/ij). The statistics of the cell viability assay and western blot were determined by triplicate experiments using one-way ANOVA with Tukey's post hoc test. The statistical analysis of STAT3 translocation was determined by Students' t-test. P<0.05 was considered to indicate a statistically significant difference.

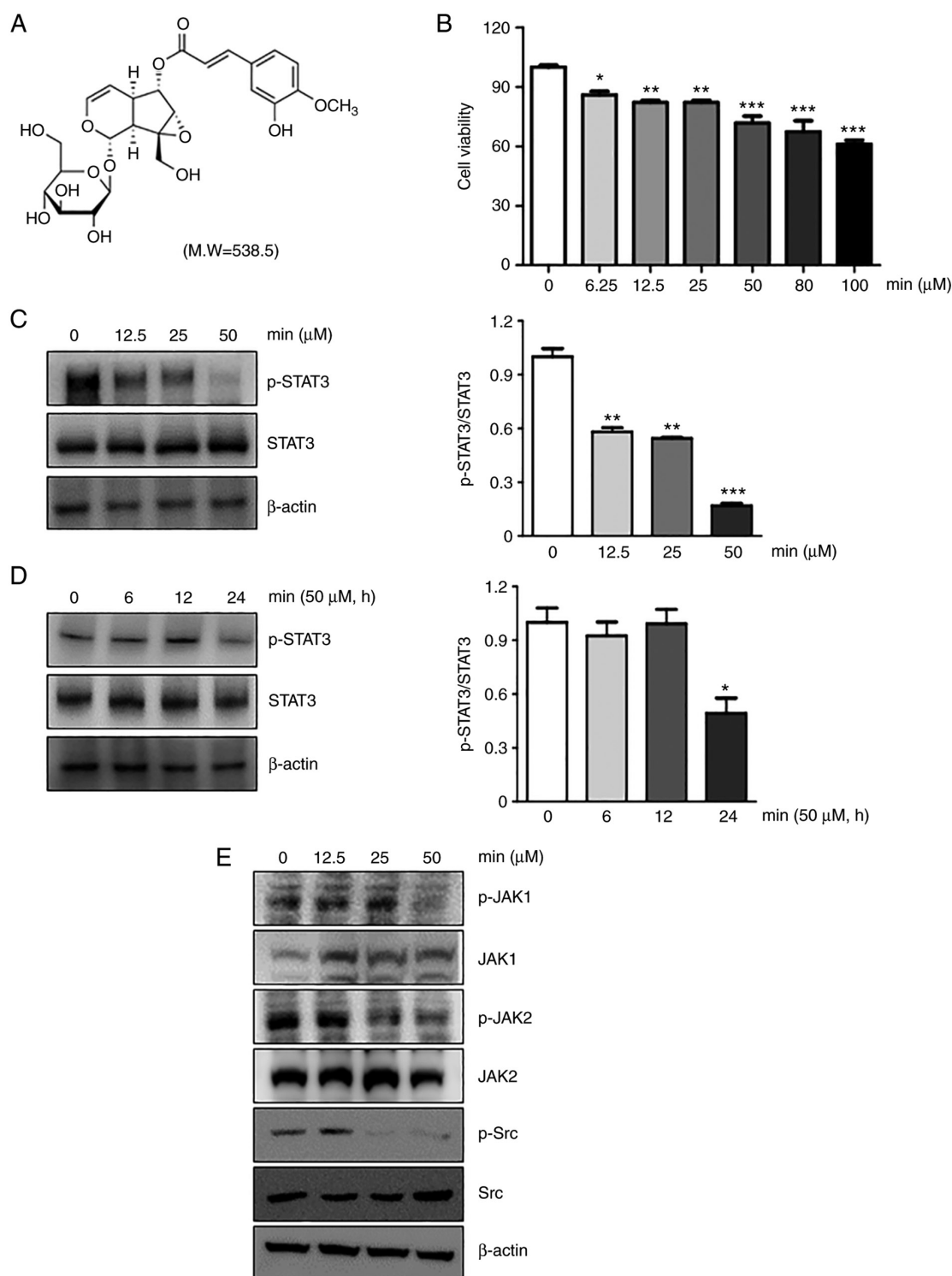


Figure 1. MIN downregulates constitutive activation of STAT3 in MDA-MB-231 cells. (A) Chemical structure of minecoside (MIN) (B) MDA-MB-231 cells ( $1 \times 10^4$  cells/ml) were treated with the indicated concentrations of MIN. The cell viability was assessed by CCK-8 assays and the results are expressed as percentage of surviving cells over control cells. (C) MIN inhibits STAT3 phosphorylation in a dose-dependent manner. MDA-MB-231 cells were treated with the indicated concentrations of MIN for 24 h. Subsequently, western blotting was performed as described in Materials and methods. (D) MIN inhibits STAT3 phosphorylation in a time-dependent manner. Western blotting was performed after MDA-MB-231 cells were treated with 50  $\mu$ M MIN for the indicated times (0, 6, 12 and 24 h). (E) MIN suppresses p-JAK1, p-JAK2 and p-Src expression in a dose-dependent manner. After MDA-MB-231 cells were treated with the indicated concentrations of MIN for 24 h, the protein expression of p-JAK1, p-JAK2 and p-Src was observed by western blot analysis. Data indicate mean  $\pm$  SEM of three independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs. control.

## Results

*MIN downregulates constitutive activation of STAT3 in MDA-MB-231 cells.* Since constitutive activation of STAT3 is

found in numerous cancer types, including >40% of primary breast tumors (4), and a recent study has reported anti-cancer effects of MIN in breast cancer (15), whether MIN affects STAT3 activation was examined in the present study. To

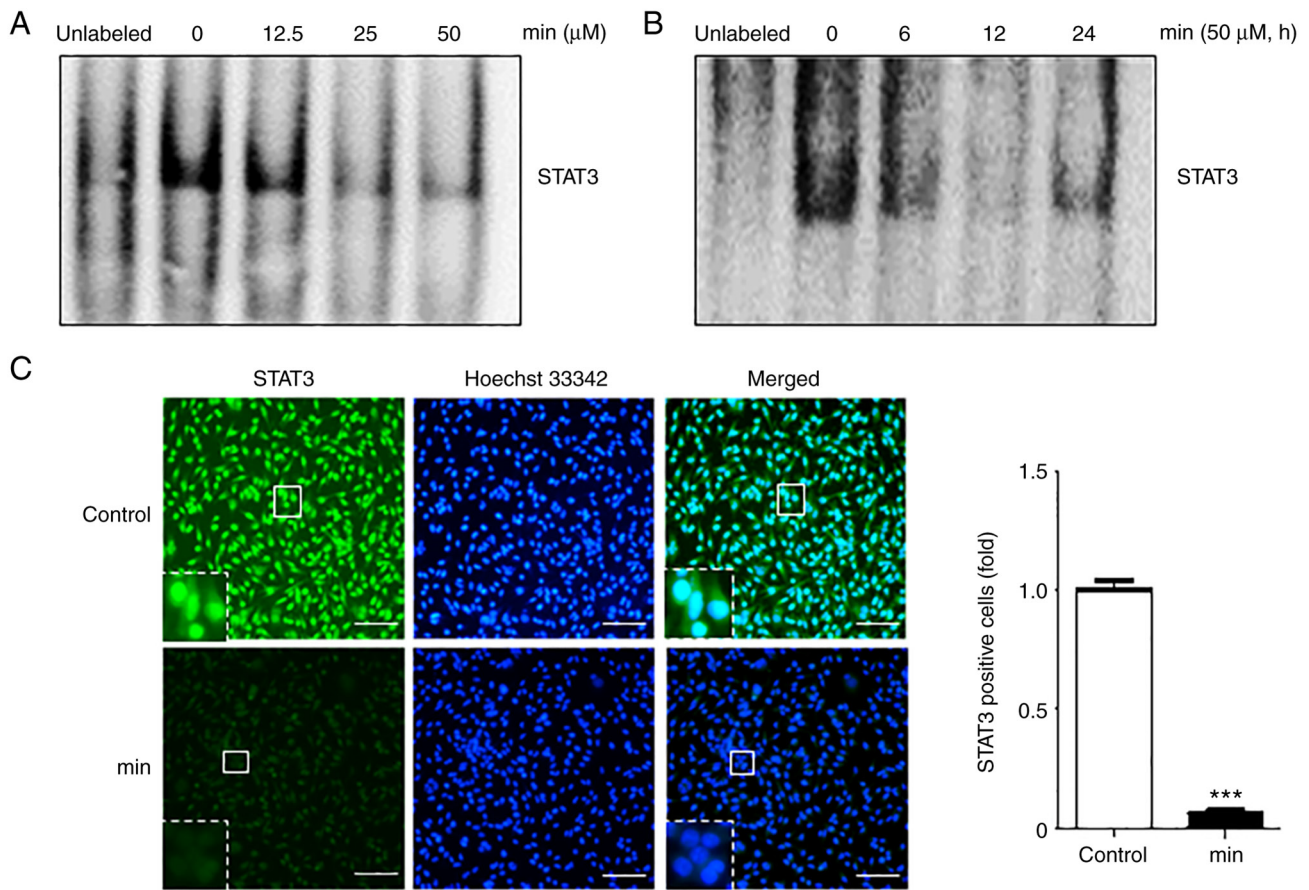


Figure 2. MIN suppresses DNA binding of STAT3 in MDA-MB-231 cells. (A) MIN suppresses STAT3-DNA binding activity in a dose-dependent manner. MDA-MB-231 cells were treated with the indicated concentrations of MIN for 24 h and STAT3-DNA binding activity was measured by EMSA. (B) MDA-MB-231 cells were treated with 50  $\mu$ M MIN for the indicated times and analyzed for nuclear STAT3 levels by EMSA. (C) MIN inhibits nuclear translocation of STAT3 in MDA-MB-231 cells. After treatment with MDA-MB-231 cells with 50  $\mu$ M MIN for 24 h, nuclear translocation of STAT3 was measured by immunofluorescence. Scale bars: 100  $\mu$ m (magnification, x200). The third panels show the merged images of the first and second panels. The fold of STAT3 positive cells with fluorescence was quantified using ImageJ. \*\*\*P<0.001 vs. control.

determine the optimal concentration of MIN, the cell viability at the indicated concentrations of MIN (0, 6.25, 12.5, 25, 50, 80 and 100  $\mu$ M) were evaluated. Based on the results, 50  $\mu$ M of MIN was selected as an optimal concentration and used throughout the study (Fig. 1B). As shown in Fig. 1C and D, MIN reduced the phosphorylation of STAT3 in a dose- and time-dependent manner in MDA-MB-231 cells. The reduction ratio of phosphorylated STAT3 to STAT3 was from 15 to 55%.

*MIN downregulates upstream signaling pathway responsible for STAT3 activation.* Janus kinases 1/2 (JAK1/2) and Src kinase were reported to contribute to STAT3 activation and are involved in cancer cell growth (18). Thus, whether MIN could inhibit the upstream signaling molecules that activate STAT3 was examined. The results showed that MIN suppressed the constitutive activation of JAK1, JAK2, and Src kinase at 50  $\mu$ M in MDA-MB-231 cells (Fig. 1E).

*MIN suppresses DNA binding of STAT3 in MDA-MB-231 cells.* Based on the fact that the phosphorylation of STAT3 regulates gene transcription through dimerization, nuclear translocation, and DNA binding (19), whether MIN can regulate the DNA binding activity of STAT3 was investigated.

After cells were treated with the indicated concentrations of MIN for 24 h or with 50  $\mu$ M of MIN for different time periods, EMSA was performed to examine the DNA binding activity of STAT3. The results showed that MIN suppressed STAT3-DNA binding in a dose- and time-dependent manner (Fig. 2A and B).

To check whether MIN inhibits STAT3-DNA binding by blocking STAT3 nuclear translocation, an immunofluorescence assay was performed. As shown in Fig. 2C, 50  $\mu$ M MIN suppressed the translocation of STAT3 into the nucleus as compared to the control groups.

*MIN induces the expression of SHP-1.* Previous findings showed that, several PTPs can modulate the STAT3 signaling pathway in various cancer cells (20). Therefore, whether the regulation of STAT3 activity by MIN was due to PTP induction was examined. Several studies reported that loss of SHP-1 in cancer cells improves STAT3 signaling (12,21). In addition, SHP-1 was proposed as a promising drug target in the development of STAT3 inhibitors because it can dephosphorylate JAKs (22) as well as STAT3 (11). Thus, whether MIN modulated SHP-1 expression in MDA-MB-231 cells was examined. As shown in Fig. 3A, MIN treatment increased SHP-1 expression in a concentration-dependent manner.



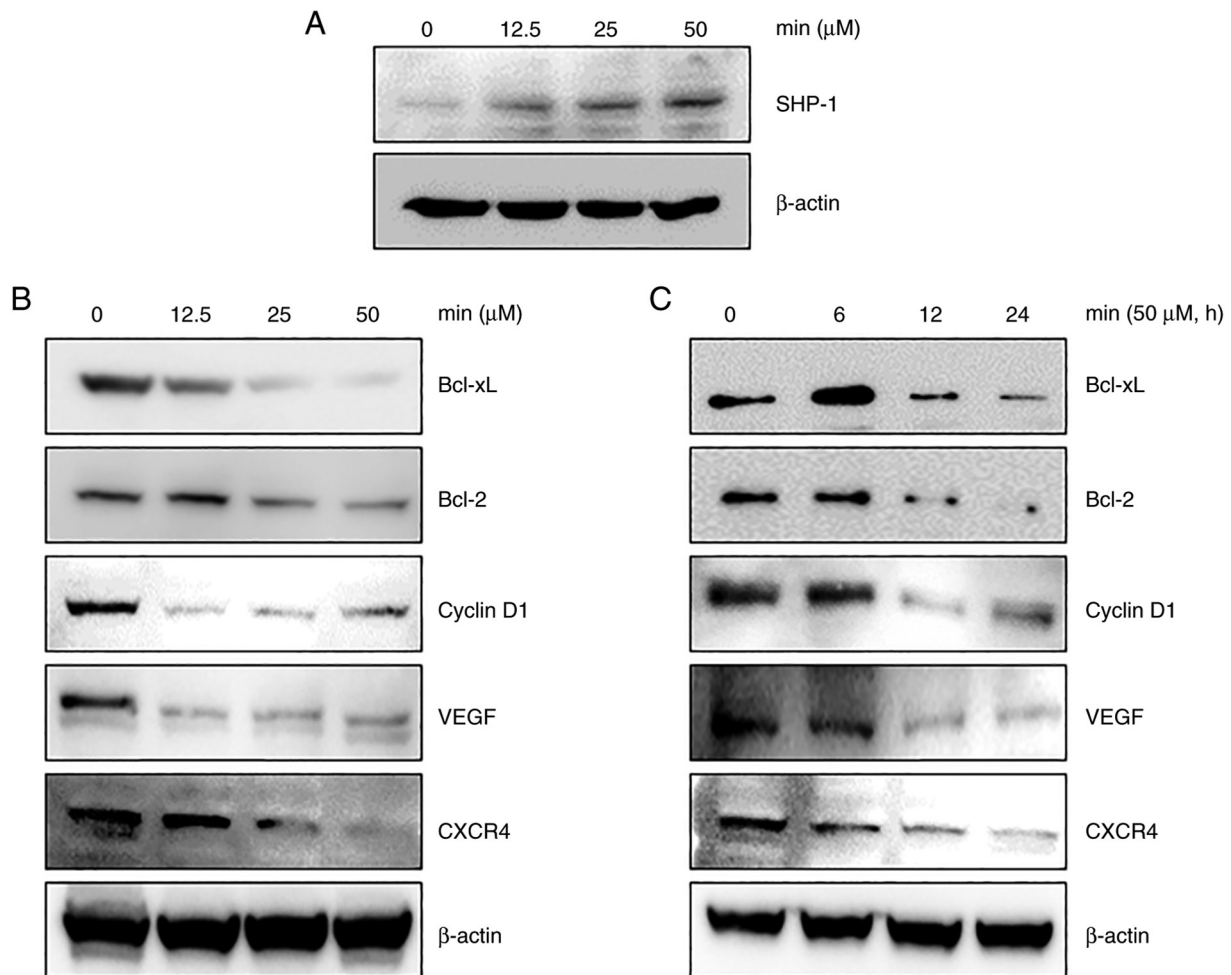


Figure 3. MIN inhibits the expression of various genes involved in growth, angiogenesis, invasion, and metastasis. (A) MIN induces the expression of SHP-1 protein in MDA-MB-231 cells. After MDA-MB-231 cells were treated with indicated concentrations of MIN for 24 h, whole cell extracts were prepared and the protein expression of SHP-1 was observed by western blot analysis. (B and C) MIN suppresses various gene products involved in survival, apoptosis, and invasion.

*MIN inhibits the expression of various genes involved in growth, angiogenesis, invasion, and metastasis.* STAT3 activation induces the expression of various genes involved in cancer cell survival and proliferation (9). The anti-apoptotic proteins Bcl-2 (23), Bcl-xL (24), invasive protein CXCR4 (25), angiogenic protein VEGF (26), and cell cycle control protein cyclin D1 (27) are known to be induced upon STAT3 activation. Thus, whether MIN affects the expression of these genes by regulating STAT3 activity was investigated. As shown in Fig. 3B and C, MIN downregulated the expression of Bcl-xL, Bcl-2, cyclin D1, VEGF, and CXCR4 in a dose- and time-dependent manner.

*MIN induces caspase-dependent apoptosis in MDA-MB-231 cells.* To further examine the apoptotic progression induced by MIN, major proteins involved in the caspase pathway were examined using western blot analysis. The results showed that MIN upregulated the expression of cleaved forms of caspase-9, caspase-3, and PARP in a dose- and time-dependent manner (Fig. 4A and B). To confirm MIN-induced apoptosis, a TUNEL assay, which is a commonly used method for identifying apoptosis, was performed. The TUNEL analysis revealed that MIN increased the number of apoptotic cells (Fig. 4C). Taken together, these results

strongly suggested that MIN induced caspase-dependent apoptosis in MDA-MB-231 cells.

## Discussion

Although the specific role of STAT3 in the onset and progression of breast cancer is not fully defined, the activation of IL-6/JAK2/STAT3 pathways is well known to promote carcinogenesis (4,28). In particular, breast cancer is associated with inflammatory conditions with high levels of growth factors and cytokines (6). For example, inflammatory cytokines, such as IL-6, are known to promote cancer cell growth, invasion, migration, metastasis, angiogenesis, and drug resistance through the JAK/STAT3 pathway (23,26,29). Based on the aforementioned studies, we investigated whether MIN promoted apoptosis by inhibiting constitutive STAT3 activation in breast cancer cells.

The results showed that, MIN suppressed constitutive STAT3 activation in MDA-MB-231 cells. The phosphorylation of STAT3 at Tyr705, an important step in STAT3 activation, was inhibited by MIN in a dose- and time-dependent manner. STAT3 phosphorylation is mediated through the activation of non-receptor protein tyrosine kinases such as JAK1 and JAK2. Src kinase is also known to play

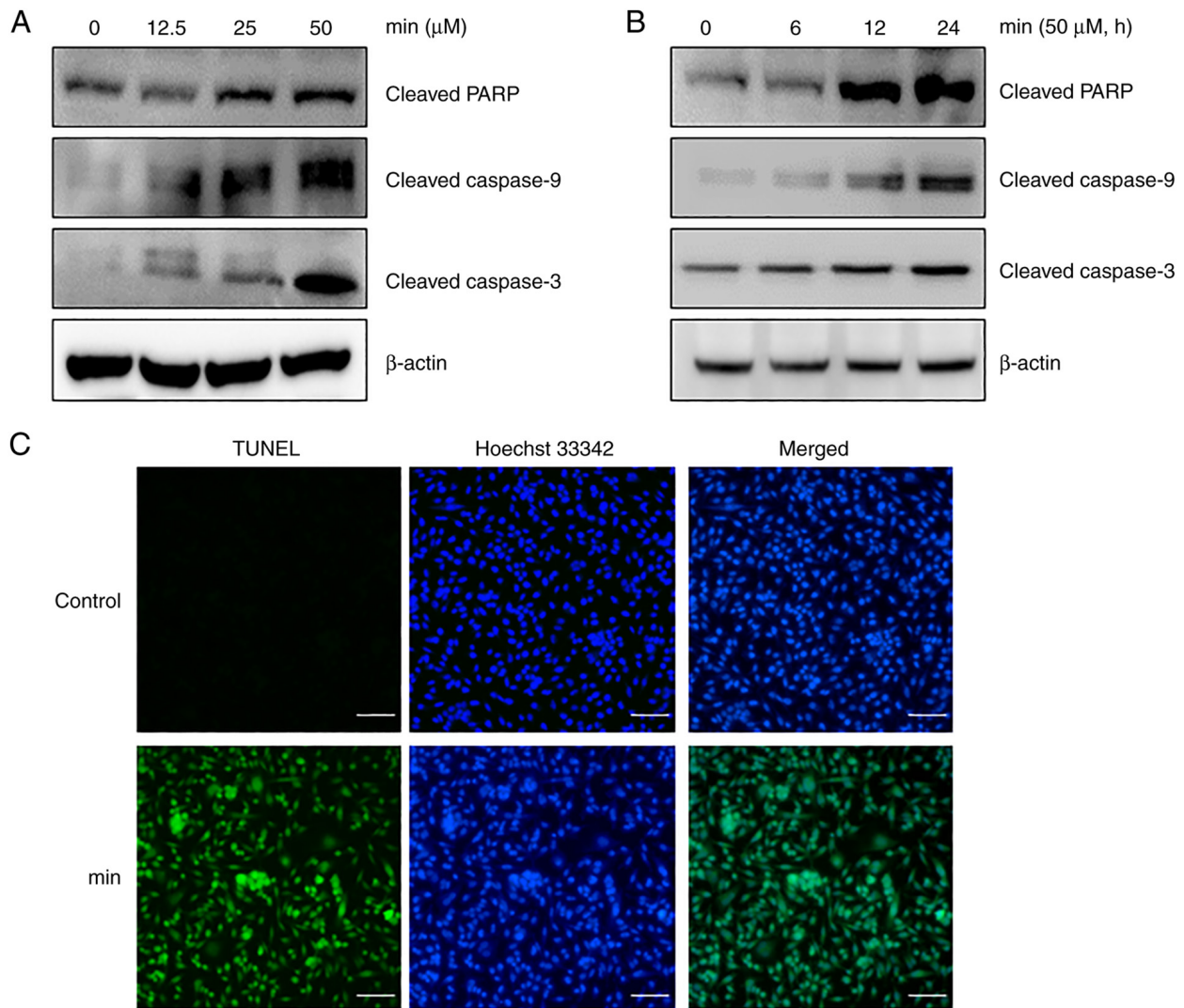


Figure 4. MIN induces caspase-dependent apoptosis in MDA-MB-231 cells. (A and B) MIN induces cleavage of caspase-9, -3, and PARP. Whole cell extracts were subjected to western blotting against a cleaved form of caspase-9, caspase-3 and PARP. (C) MDA-MB-231 cells were treated with 50  $\mu$ M MIN for 24 h, fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and stained with labeled TUNEL reagent. The third panels show the merged images of the first and second panels. Scale bars: 100  $\mu$ m (magnification, x200).

an important role in the phosphorylation of STAT3 (30,31). Indeed, although upstream kinases are important in tumorigenesis, STAT3, which is considered a key transcription factor for gene expression involved in malignancy, was the primary focus of the present study. The results demonstrated that MIN inhibited JAK1, JAK2, and Src kinase activation, suggesting that MIN suppresses STAT3 phosphorylation via the inactivation of upstream kinases. Since the phosphorylation of STAT3 regulates gene transcription through dimerization, nuclear translocation, and DNA binding (19), whether MIN can affect STAT3 translocation and DNA binding activity was investigated. The results revealed that MIN suppressed STAT3-DNA binding as well as the nuclear translocation of STAT3.

Several PTPs modulate the STAT3 signaling pathway in various cancer cells (20). In particular, the SH2 domain-containing phosphatase-1 (SHP-1) possesses a tumor-suppressive potential by virtue of negatively regulating STAT3 signaling (12); it is considered an antagonist of tyrosine kinases related to tumor growth and anti-apoptosis (32). SHP-1

expression is known to be reduced or abolished in estrogen receptor-negative breast cancer cell lines. In the present study, whether the inhibitory effect of MIN on STAT3 activation was related to SHP-1 expression was examined. The results showed that, MIN treatment increased SHP-1 expression in a concentration-dependent manner. The increase in SHP-1 expression by MIN was confirmed via the association with the inhibition of constitutive STAT3 activation in subsequent experiments. Besides SHP-1, the involvement of other PTPs in the regulation of STAT3 by MIN should be investigated in future studies.

STAT3 activation is observed in various malignant tumors, including lung, breast, liver, colon, prostate, stomach, pancreas, kidney, and brain cancers (33). This is because STAT3 activation upregulates a variety of cellular signaling required for cell survival (Bcl-xL, Bcl-2, c-myc, Mcl-1, and survivin), proliferation (cyclin D1), invasion (MMP-9, CXCR4, Rho, and Rac), and angiogenesis (VEGF) (23,25,28,33). Findings of the present study showed that MIN also downregulated STAT3-mediated protein

expression such as cyclin D1, Bcl-2, Bcl-xL, CXCR4, and VEGF. In a previous study, the inhibitory effect of MIN on CXCR4 expression was found to be mediated via the blockade of transcription factor NF- $\kappa$ B (15). A gene that contains only NF- $\kappa$ B binding sites may be responsive to NF- $\kappa$ B, but not STAT3. However, a gene that contains both NF- $\kappa$ B and STAT3 binding sites may be regulated by both in a cooperative manner (34). Thus, investigation of the key role of MIN on crosstalk between STAT3 and NF- $\kappa$ B is imperative.

Since MIN inhibited the expression of proteins related to proliferation (cyclin D1), and cell survival (Bcl-2, Bcl-xL), whether MIN affected apoptotic progression was examined. The results showed that MIN upregulated caspase-dependent apoptosis in MDA-MB-231 cells. A major limitation of the present study was that the inhibitory effect of MIN was not examined in other cancer cells or in an animal model.

In conclusion, the results of the present study provide evidence that MIN exerts anticancer activity via inhibition of the JAK/STAT3 signaling pathway, especially in breast cancer cells. Further studies using animal models are required to determine the potential of this molecule as an anticancer drug.

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#### Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

#### Authors' contributions

BP and BK conceived the study. BK developed the methodology, and obtained and validated the data. BK performed the experiments. BP and KL analyzed and interpreted the data. BK and BP prepared the original draft. BP and KL revised the draft. BK and BP confirm the authenticity of all the raw data. 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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