

Sinomenine inhibits proliferation of SGC-7901 gastric adenocarcinoma cells via suppression of cyclooxygenase-2 expression

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Abstract. Sinomenine (SIN) is a bioactive alkaloid extracted from the Chinese medicinal plant *Sinomenium acutum*. Results of studies have shown that the anti-inflammatory, immunosuppressive and anti-arthritic effects of SIN are partially attributed to the inhibition of cyclooxygenase-2 (COX-2) expression. COX-2 overexpression is associated with enhanced proliferation and angiogenesis of gastric cancer (GC). SGC-7901 cells were treated with different concentrations of SIN in order to observe its effect on the proliferation of human gastric adenocarcinoma cells and to explore the potential underlying molecular mechanism via the detection of COX-2 expression. Celecoxib was used as the positive control. Morphological alterations of the cells were observed microscopically. Cell proliferation was evaluated using MTT assay. COX-2 expression was detected using semi-quantitative RT-PCR and Western blotting. The results showed that SIN inhibited the proliferation of SGC-7901 cells in a time- and dose-dependent manner. In the presence of SIN or celecoxib, SGC-7901 cells became round and detached morphologically, indicating cell apoptosis. The expression of COX-2 was inhibited by SIN in a dose-dependent manner at both the mRNA and protein levels. Our findings indicate that the protective effects of SIN are mediated through the inhibition of COX-2 expression. These findings suggest a novel therapy to treat inflammation-mediated gastric adenocarcinoma.

Introduction

Sinomenine (SIN; 7,8-didehydro-4-hydroxy-3,7-dimethoxy-17-methylmorphinan-6-one) is a biomonomer alkali derived from the Chinese medicinal plant *Sinomenium acutum*. Traditionally, SIN has been used in the treatment of rheumatoid arthritis due to its anti-inflammatory effect (1). Previous studies demonstrated that SIN has cardioprotective (2) and

immunosuppressive effects (3,4). *In vitro* studies indicated that the suppression of cyclooxygenase-2 (COX-2) expression is one of the possible mechanisms for the anti-inflammatory characteristic of SIN (5). Furthermore, in the pioneer experiment conducted by Zhang *et al* SIN was found to inhibit the proliferation of HeLa cells, possibly by inhibiting the expression of COX-2 (6).

COX is a key enzyme mediating the conversion of arachidonic acid to prostaglandins. Two distinct COX enzymes have been identified: COX-1, a constitutive enzyme, and COX-2, an inducible form (7). COX-1 is a housekeeping molecule that can be detected in most cells and tissues under normal conditions and is involved in maintaining homeostasis by regulating normal physiological functions, such as immune response, acid secretion and blood supply. The expression of COX-2 is rapidly induced by growth factors, oncogenes, carcinogens, mitogens and lipopolysaccharides (8). The majority of the data from animal and human studies indicate that COX-2 is crucial to inflammation and oncogenesis. COX-2 is up-regulated in transformed cells and in a variety of solid tumors such as lung, colorectal, pancreatic and breast cancers (9-12). COX-2 inhibitors induce apoptosis in various cancer cells both *in vitro* and *in vivo* (13). COX-2 is considered to be a potential preventive and therapeutic target for malignancies (14).

Gastric cancer is one of the most common causes of cancer-related mortality in China and other Asian countries (15). At present, surgery and chemotherapy are the standard treatment modalities utilized in gastric cancer (16). However, the 5-year survival of gastric cancer patients is estimated to be only 30%. To improve the prognosis of GC, the development of novel strategies based on its molecular alterations is required. The majority of gastric adenocarcinomas have a high-level expression of COX-2 (17-19). Both angiogenesis and *Helicobacter pylori* infection have been reported to be associated with the COX-2 expression in gastric cancer patients (20). The knockdown of COX-2 in a SGC-7901 gastric adenocarcinoma cell line by RNA interference inhibits proliferation and induces apoptosis (21), indicating that suppression of COX-2 may be developed into an effective approach for the treatment of gastric cancer. The majority of selective COX-2 inhibitors have pronounced side effects that limit the administration of these drugs. In the present study, the inhibitory effect of SIN on the proliferation of SGC-7901 gastric adenocarcinoma cells was observed. Additionally,

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the question of whether the suppression of COX-2 expression is a potential mechanism for SIN on the proliferation of SGC-7901 cells was investigated.

Materials and methods

Cell cultures and reagents. SGC-7901 gastric adenocarcinoma cells were cultured with Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco), 100 U/ml penicillin and 100 μ g/ml streptomycin. Cultures were maintained at 37°C in a humidified incubator in an atmosphere of 5% CO₂. Cells were passaged at 1:3 every 3 days. SIN and celecoxib (Sino-American Biotech, Henan, China) were dissolved in dimethylsulfoxide (DMSO; Sigma, St. Louis, MO, USA), stored at -20°C and diluted in DMEM in different proportions (DMSO density of <0.1%). The morphological and growth patterns of the cells were dynamically observed under an inverted microscope (Olympus IX-50; Olympus Optical, Tokyo, Japan).

MTT assay. Following the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, 5×10^3 cells were seeded in 96-well plates and cultured for 24 h at 37°C and 5% CO₂. Media containing various concentrations of SIN were added to the wells 24 h later to reach final concentrations of 125, 250, 500 and 1,000 μ mol/l. Celecoxib at a final concentration of 50 μ mol/l was used as a positive control. For the DMSO control, DMSO was added to a final concentration of 1‰ to exclude the possible effect of DMSO on cell proliferation. For the blank control, no reagent was added. Drug treatment was continued for another 24, 48, 72 or 96 h, and 5 mg/ml MTT (Sigma) was added to the wells. All of the groups were incubated for 4 h at 37°C. The supernatant was removed and crystals were dissolved in 200 μ l DMSO. The absorbance was examined with an automated microplate reader (Bio-Tek, Winooski, VT, USA) at an absorption wavelength of 490 nm. Only the medium was added to the negative control well, which was used to zero the absorbance. Three wells were set up for each group and three independent experiments were conducted.

Reverse transcription-polymerase chain reaction (RT-PCR). The relative expression of COX-2 mRNA was evaluated using a semi-quantitative reverse transcriptase PCR kit (Takara, Otsu, Shiga, Japan). Total RNA was isolated from SGC-7901 cells using a TRIzol reagent (Promega, Madison, WI, USA). Reverse transcription of total RNA (2 μ g) was performed in 20 μ l volume according to the manufacturer's instructions. The primers used for COX-2 were: 5'-CGAGGTGTATGTATGAGTGTG-3' (forward) and 5'-TCTAGCCAGAGTTTCACCGTA-3' (reverse). β -actin was amplified as an internal control using the primers: 5'-GTAAAGACCTCTATGCCATCA-3' (forward) and 5'-GGACTCATCGTACTCCTGCT-3' (reverse), resulting in products of 550 and 227 bp, respectively. Each PCR product was visualized by staining with ethidium bromide after electrophoresis on 2% agarose gels under ultraviolet light. The gel images were photographed (Olympus) and relative densities were analyzed using the Bandscan software.

Western blotting. All groups of SGC-7901 cells were collected in 1.5-ml Eppendorf tubes when the cells were treated with drugs for 48 h. The total protein was extracted with RIPA lysis buffer containing proteinase inhibitors. Protein concentration was determined using the Bradford assay. The protein (100 μ g) of each sample was separated on 10% sodium dodecyl sulfate (SDS) polyacrylamide gel and transferred to nitrocellulose membranes. Non-specific binding was blocked by 5% skimmed milk for 2 h at room temperature. The membranes were incubated with primary antibody against COX-2 and β -actin (1:1,000 dilution; Sigma) for 4 h at room temperature or overnight at 4°C. After washing with PBST followed by incubation with peroxidase-conjugated goat anti-mouse IgG as secondary antibody (1:2,000 dilution; Sigma) for 1 h at room temperature, protein was detected using enhanced chemiluminescence solution, and by exposing membranes to Kodak X-ray film. The expression of β -actin was detected as an internal control.

Statistical analysis. Statistical analysis was performed using SPSS software (SPSS13.0). Statistical analyses of the data were performed using one-way analysis of variance (ANOVA) followed by a *post hoc* test. Data were shown as the mean \pm standard deviation. $P < 0.05$ was considered to be statistically significant.

Results

Cell morphology. After SGC-7901 cells were treated with different concentrations of drugs, proliferation of SGC-7901 cells was inhibited, the number of cells decreased significantly and cell growth was retarded. Morphologically, the cells detached from the bottle and became round. Achromatolysis, deflation and pyknosis of the nucleus was observed. This phenomenon was most obvious in the 1,000 μ mol/l SIN and celecoxib-positive groups. SGC-7901 cells grew more rapidly in the DMSO control group (Fig. 1).

Cell proliferation. We found that the proliferation of SGC-7901 cells was inhibited to various extents in all of the experimental groups and the celecoxib-positive control group (Fig. 2). The DMSO control group was not depressed. SIN inhibited the growth of SGC-7901 cells in a dose-dependent manner and the number of cells decreased following the increased concentration of SIN. Compared to that of the blank control group, the growth of cells treated with SIN decreased significantly ($P < 0.05$ by ANOVA and Tukey's *post hoc* test to detect significantly different means). A significant difference was also observed between the celecoxib-positive control group and the blank control and SIN groups ($P < 0.05$). The DMSO control group showed no effects on SGC-7901 cells; in a group comparison between the various densities of SIN, the high-dose group resulted in a markedly reduced growth of SGC-7901 cells as compared to that of the low-dose treated group ($P < 0.05$). Concomitantly, SGC-7901 cells treated with SIN for 24-96 h resulted in an obviously increased inhibitory rate of cell growth. We observed that the highest inhibitory rate among the SIN groups was 93.89% in the 1,000 μ mol/l SIN group at 96 h. Moreover, the inhibitory action of SIN on SGC-7901 cells occurred in a time-dependent manner ($P < 0.05$).

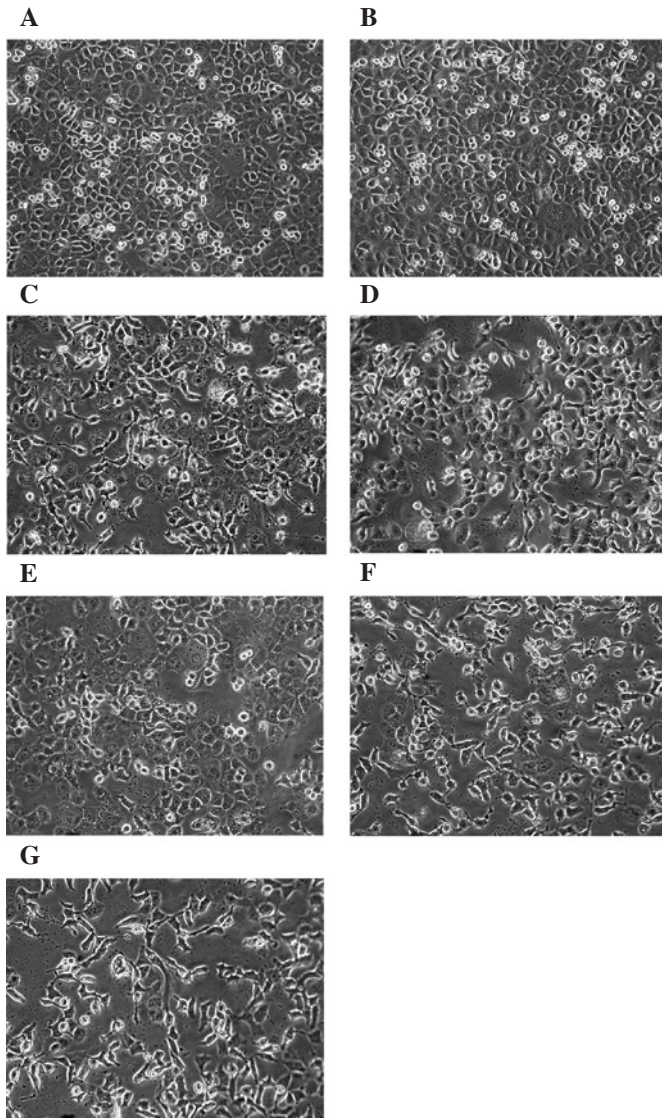


Figure 1. SIN suppressed SGC-7901 cell growth in a dose-dependent manner. Cell growth was inhibited, the number of cells markedly decreased and the shape of SGC-7901 cells became round and detached from the bottle. (A) Blank control group. (B) DMSO control group. SIN groups, (C) 125 $\mu\text{mol/l}$; (D) 250 $\mu\text{mol/l}$; (E) 500 $\mu\text{mol/l}$ and (F) 1,000 $\mu\text{mol/l}$. (G) Positive control group (50 $\mu\text{mol/l}$ celecoxib).

SIN inhibits COX-2 expression in human gastric adenocarcinoma cells. To determine COX-2 expression in response to SIN treatment, RT-PCR was performed and SGC-7901 cells were examined (Fig. 3). SIN at a concentration of 125 $\mu\text{mol/l}$ caused a decrease in the expression of COX-2 mRNA, which began 48 h after the initial treatment was administered and occurred in a dose-dependent manner in SGC-7901 cells compared to the blank control group ($P<0.05$). The DMSO control group exhibited no effects on the expression of COX-2 mRNA in SGC-7901 cells. The celecoxib-positive control group was significantly different from the blank control group ($P<0.05$).

Western blotting verified the expression of COX-2. Western blot analysis revealed that COX-2 protein was expressed in gastric cancer cells (Fig. 4). No significant difference was observed between the blank and DMSO control groups. Compared to the blank control group, the expression of

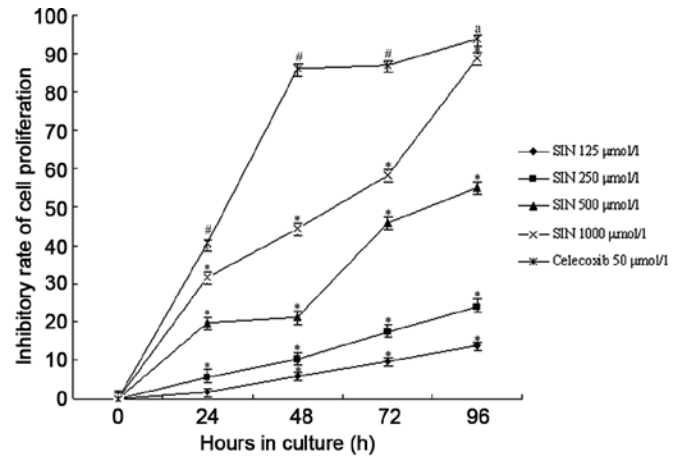


Figure 2. Effects of proliferation on SGC-7901 cells treated with SIN. A MTT assay showed that the inhibitory rate of SGC-7901 cells decreased with SIN. A higher inhibition rate corresponded to higher drug doses. The cells treated with 1,000 $\mu\text{mol/l}$ SIN were clearly inhibited. A higher inhibition rate corresponded to longer drug treatment times. Proliferation of SGC-7901 cells was inhibited in a dose- and time- dependent manner. * $P<0.05$ indicates a significant difference between the various concentrations of the SIN groups. * $P<0.05$ compared to the SIN groups. * $P<0.05$ vs. SIN groups (125, 250 and 500 $\mu\text{mol/l}$).

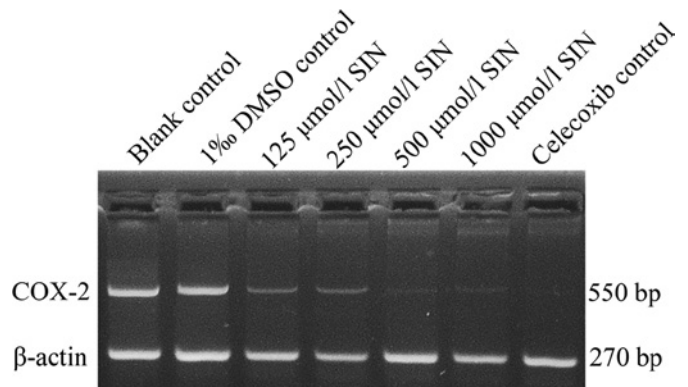


Figure 3. RT-PCR analysis of COX-2 mRNA. β -actin was used as an internal control. RT-PCR analysis revealed that SIN rapidly inhibited the expression of COX-2 mRNA in SGC-7901 cells. The levels of COX-2 mRNA decreased in a dose-dependent manner.

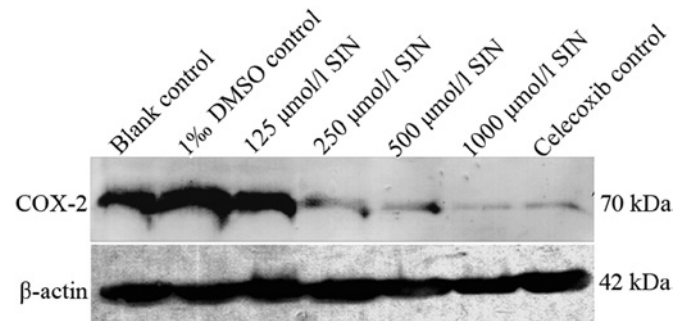


Figure 4. The expression of COX-2 in SGC-7901 cells analyzed by Western blotting. COX-2 and β -actin antibodies showing bands with the 70 and 42 kDa expected size, respectively. The expression of COX-2 was found to decrease with increase of the drug dose.

COX-2 was decreased in various densities of the SIN group in a dose-dependent manner ($P < 0.05$). In contrast to the blank control group, the expression of COX-2 was decreased in the celecoxib-positive control group ($p < 0.05$).

Discussion

Gastric cancer is the most common cause of cancer-related mortality worldwide. Numerous molecular studies have been performed to investigate the developmental mechanism of gastric cancer and COX-2 expression in the pathogenesis of gastric cancer. COX-2 was found to play a significant role in gastric cancer by various pathways. Additionally, the correlation between COX-2 and clinicopathological characteristics, such as tumor size, stage, invasion and lymph node metastasis, of gastric cancer have been identified. COX-2 overexpression protects cancer cells against various apoptotic stimuli (22). The up-regulation of COX-2 is closely related to gastric cancer metastasis through the promotion of lymphangiogenesis and the angiogenesis of gastric cancer (23). Findings of studies have demonstrated that COX-2 is constitutively overexpressed in gastric cancer (24). The relationship between *Helicobacter pylori* infection and gastric cancer has also been demonstrated. Thus, *Helicobacter pylori* infection is thought to contribute to the development of gastric cancer via COX-2, which may be due to the stimulation of tumor growth and angiogenesis (25). Several molecular pathways have been hypothesized in the development of gastric cancer. Previous studies indicated that the COX-2-PGI₂-PPAR δ pathway was also involved in tumorigenesis (26). VEGF is one of the most significant mediators of the COX-2 pathway (27). COX-2 produced by cancer cells is correlated with the elevation of Bcl-2 protein and inhibition of apoptosis in gastric cancer tissue.

In the present study, we observed that COX-2 was highly expressed in gastric cancer cells, a result that is consistent with findings of other studies. COX-2 selective inhibitors have been shown to induce apoptosis in gastric cancer (28). Our study found that SIN was suppressed COX-2 expression in SGC-7901 cells, which grew slowly and became round. In their study, Zhang *et al* found that SIN inhibited the proliferation of HeLa cells as a COX-2 selective inhibitor (6). This inhibition may relate to SIN blockage of the cell cycle and induction of apoptosis, the mechanism of which may constitute the inhibition of COX-2 expression in a dose-dependent manner. Studies have also shown that SIN mediated the down-regulation of COX-2 expression and the production of induced PGE₂ in PC-12 cells by suppressing the activity of NF- κ B (5). To assess whether the inhibition of COX-2 expression is involved in gastric cancer cells, MTT assay, RT-PCR analysis and Western blotting were performed to test cell viability, COX-2 mRNA and protein expression, respectively.

The results of this study suggest that SIN has an inhibitory effect on the growth of gastric cancer. Based on our observation of cell morphology, we found that SIN effectively inhibited the growth of SGC-7901 cells. Compared to the control group, the number of cells decreased significantly in the SIN groups and the proliferation of SGC-7901 cells was inhibited. The highest inhibitory rate was 93.89% in the 1,000 μ mol/l SIN group at 96 h. The preliminary inhibitory effect of SIN on gastric cancer cells was demonstrated by this

result. We showed that SIN was capable of reducing up-regulated mRNA and the protein levels of COX-2. COX-2 mRNA was significantly decreased compared to the blank control group. SIN down-regulated the COX-2 protein expression in a dose-dependent manner in gastric cancer cells. The present results indicate that the inhibitory effect of SIN on gastric cancer cells may be activated by the COX-2 pathway. COX-2 is a key enzyme in prostaglandin synthesis. PGE₂ may promote the growth of gastric cancer cells and induce Foxp3 expression independently of TGF- β and IL-10 in the gastric cancer microenvironment (29). SIN may also inhibit PGE₂ synthesis by suppressing the expression of COX-2. Further investigation is required to identify the signal transduction pathway of COX-2. Blocking this pathway using SIN may facilitate tumor therapeutics.

In conclusion, the present study suggests that SIN is involved in inhibiting the proliferation of gastric cancer cells *in vitro* and that its therapeutic mechanism is related to the inhibition of COX-2 expression. The findings of this study suggest that SIN has a preliminarily therapeutic effect on gastric cancer, indicating that SIN is an effective candidate drug for treating gastric cancer.

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