

## Cooperative inhibitory effect of sinomenine combined with 5-fluorouracil on esophageal carcinoma

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### Abstract

**AIM:** To investigate the inhibitory effects of sinomenine (SIN) combined with 5-fluorouracil (5-FU) on esophageal carcinoma *in vitro* and *in vivo*.

**METHODS:** Esophageal carcinoma (Eca-109) cells were cultured in DMEM. The single or combined growth inhibition effects of SIN and 5-FU on the Eca-109 cells were examined by measuring the absorbance of CCK-8 dye in living cells. Hoechst 33258 staining and an Annexin V/PI apoptosis kit were used to detect the percentage of cells undergoing apoptosis. Western blotting was used to investigate the essential mechanism underlying SIN and 5-FU-induced apoptosis. SIN at 25 mg/kg and 5-FU at 12 mg/kg every 3 d, either combined or alone, was injected into nude mice and tumor growth inhibition and side effects of the drug treatment were observed.

**RESULTS:** SIN and 5-FU, both in combination and individually, significantly inhibited the proliferation of

Eca-109 cells and induced obvious apoptosis. Furthermore, the combined effects were greater than those of the individual agents ( $P < 0.05$ ). Annexin V/PI staining and Hoechst 33258 staining both indicated that the percentage of apoptotic cells induced by SIN and 5-FU combined or alone were significantly different from the control ( $P < 0.05$ ). The up-regulation of Bax and down-regulation of Bcl-2 showed that the essential mechanism of apoptosis induced by SIN and 5-FU occurs *via* the mitochondrial pathway. SIN and 5-FU alone significantly inhibited the growth of tumor xenografts *in vivo*, and the combined inhibition rate was even higher ( $P < 0.05$ ). During the course of chemotherapy, no obvious side effects were observed in the liver or kidneys.

**CONCLUSION:** The combined effects of SIN and 5-FU on esophageal carcinoma were superior to those of the individual compounds, and the drug combination did not increase the side effects of chemotherapy.

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**Key words:** Esophageal carcinoma; Chemotherapy; Sinomenine; 5-Fluorouracil

**Core tip:** The cooperative inhibitory effects of sinomenine (SIN) and 5-fluorouracil (5-FU) on esophageal carcinoma *in vitro* and *in vivo* were investigated. SIN and 5-FU alone or in combination significantly inhibited the proliferation and induced apoptosis of esophageal carcinoma cells in a dose-dependent manner. The essential mechanism underlying SIN and 5-FU-induced apoptosis, as investigated by Western blotting, involved the mitochondrial pathway. No obvious side effects were observed in the liver and kidneys of nude mice. These results indicated that the combined effects of SIN and 5-FU on esophageal carcinoma were superior to the effects of the individual compounds, without an increase in side effects.

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## INTRODUCTION

Esophageal carcinoma is one of the most refractory and common malignant diseases worldwide and is associated with poor disease outcomes<sup>[1,2]</sup>. An estimated 482300 new esophageal cancer cases and 406800 deaths occurred globally in 2008<sup>[2]</sup>. Generally, the primary tumor of most patients can be cured with surgical resection, however, due to early distant metastases, the remainder of patients will eventually succumb to the disease. Despite the advances in surgical methods combined with perioperative treatment that have led to improved prognoses, the overall mortality rate remains low due to early distant metastases<sup>[3-5]</sup>. Systemic chemotherapy is regarded as one of the most effective treatments to improve survival. Although chemotherapy forms an important part of the multidisciplinary treatment approach for metastatic disease, the toxicity of the agents to normal tissues has been the main obstacle to successful treatment. Therefore, to enhance efficacy and reduce toxicity, combined treatments of several chemotherapy regimens are often used.

5-Fluorouracil (5-FU) is universally used as an anti-cancer agent in esophageal carcinoma. Cisplatin and fluorouracil combination therapy has been a standard choice for treating esophageal cancer, for which the median survival time is 9.2 mo for responders and 5.3 mo for non-responders, with a resulting 1-year survival rate of approximately 27.8%-37.6%<sup>[6-8]</sup>. To improve the prognosis of patients with esophageal cancer, more effective novel regimens with high therapeutic effect are urgently needed.

Sinomenine (SIN) is an immunosuppressive compound extracted from the Chinese medicinal plant *Sinomenium acutum* which has been successfully used in Chinese folk medicine to treat various autoimmune diseases for centuries<sup>[9]</sup>. Previous studies have indicated that SIN exhibits a wide range of significant pharmacological activities, including anti-inflammatory, anti-rheumatic, anti-arrhythmic, anti-angiogenesis, analgesic and immunosuppressive effects<sup>[10-12]</sup>. Lu *et al.*<sup>[13]</sup> observed that SIN can promote cell cycle arrest in the G1 phase, which was associated with increased p21/WAP/Cip expression. Additionally, SIN induces caspase-dependent apoptosis, which is involved in the disruption of mitochondrial membrane potential. SIN can also significantly inhibit basic fibroblast growth factor (bFGF)-induced angiogenesis, an effect that may render the compound a prime candidate for possible anti-cancer agents<sup>[12]</sup>.

The aim of this study was to evaluate the effects of SIN combined with 5-FU in terms of their activity

against esophageal carcinoma *in vitro* and *in vivo*.

## MATERIALS AND METHODS

### Chemicals and cell culture

SIN (C<sub>19</sub>H<sub>23</sub>NO<sub>4</sub> · 0.3 CHCl<sub>3</sub>) and 5-FU (C<sub>4</sub>H<sub>3</sub>FN<sub>2</sub>O<sub>2</sub>) were purchased from Sigma (Sigma-Aldrich, St. Louis, MO, United States) and dissolved at 100 mmol/L in dimethylsulfoxide (DMSO) for storage at -20 °C. The esophageal carcinoma cell line Eca-109 was obtained from China Center for Type Culture Collection. The growth medium consisted of DMEM (Gibco BRL Gaithersburg, MD, United States) containing 10% fetal bovine serum. Cell cultures were maintained at 37 °C in a 95% humidified atmosphere of 5% CO<sub>2</sub> in air.

### Cell proliferation assay

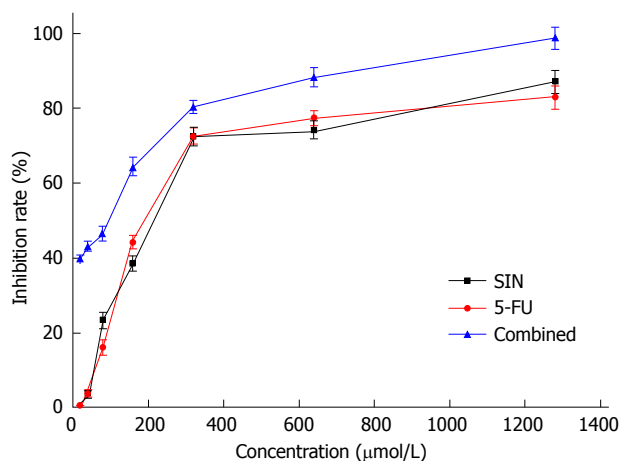
Cell proliferation *in vitro* was determined using the WST-8 Cell Counting Kit-8 (Beyotime Biotechnology, Jiangsu, China) as previously described<sup>[14]</sup>. Generally, cells were seeded in 96-well micro plates at a density of  $5 \times 10^3$ /well in 0.1 mL growth medium. After exposure to different concentrations of SIN (40, 80, 160, 320, 640 μmol/L), 5-FU (40, 80, 160, 320, 640 μmol/L) or SIN + 5-FU for 48 h, 10 μL CCK-8 solution was added to each well. The plates were then incubated for an additional 2 h at 37 °C. The optical density at 450 nm was measured by a microplate reader (BIO-RAD iMark). The procedure was performed in triplicate.

### Annexin V/PI staining for apoptosis

The amount of phosphatidylserine exposed on the extracellular membrane of the apoptotic cells was quantified by the Annexin V-FITC kit. Following incubation with SIN (160 μmol/L) and 5-FU (160 μmol/L) alone or combined for 48 h, adherent cells were harvested by mild trypsinization, washed twice with cold PBS and resuspended in 500 μL binding buffer. After adding 5 μL Annexin V-FITC conjugate and 10 μL Propidium Iodide (PI), the cells were incubated for 15 min at room temperature in the dark. Flow cytometric analysis was performed immediately with FACSCalibur using the CellQuest software.

### Hoechst 33258 assay for apoptosis

The morphological features of apoptotic cells were detected by Hoechst 33258 staining following the manufacturer's protocol (Beyotime Biotechnology, Jiangsu, China). Cells were seeded on sterile cover glasses placed in the 6-well plates. After exposure to SIN (160 μmol/L), 5-FU (160 μmol/L) alone or combined for 48 h, the cells were fixed, washed and stained with 0.5 mL Hoechst 33258 staining solution for 5 min at room temperature in the dark. Then washed twice with PBS and stained nuclei were scored and categorized according to the condensation and staining characteristics of chromatin under a fluorescence microscope (Olympus, Shinjuku-ku, Tokyo, Japan). Ten random fields per dish were observed and



**Figure 1** The individual and combined effects of sinomenine and 5-Fluorouracil on the proliferation of esophageal carcinoma cells. The proliferation of Esophageal carcinoma (Eca-109) cells was inhibited by SIN and 5-FU in a dose dependent manner ( $P < 0.05$ ). SIN and 5-FU had significant synergistic effects on inhibiting the proliferation of Eca-109 cells especially at lower concentrations. SIN: Sinomenine; 5-FU: 5-Fluorouracil.

counted.

### Western blot analysis

Whole protein extracts were prepared and analyzed by western blotting using a wet transfer system (Bio-Rad, Hercules, CA, United States). Protein samples (20 μg) were resolved by electrophoresis of SDS-polyacrylamide gels and electro-transferred to nitrocellulose membranes. Transfer efficiency and homogeneous loading was assessed by Ponceau stain. Membranes were blocked for 1 h in 10% non-fat milk, incubated with titrated primary antibody overnight at 4 °C and then labeled with the horseradish peroxidase conjugated secondary antibody for 2 h at ambient temperature. The following antibodies were used: Bax, Bcl-2, GAPDH (Santa Cruz Biotechnology, CA, United States) 1:1000. Antibody reaction was revealed using an enhanced chemiluminescence system (Millipore, Bedford, MA, United States) and then exposed with Kodak X-ray film. Protein band intensity was determined using the CMIASWIN video imaging system (Bio-Rad, Hercules, CA, United States).

### Xenograft tumor model

Animal experiments, which complied with the NIH Guide for the Care and Use of Laboratory Animals, were approved by the committee on Animal Experiment of Wuhan University. Esophageal carcinoma tumors were established in male BALB/c nude mice (4-6 wk, Laboratory Animal Central of Wuhan University) by serial subcutaneous transplantation of Eca-109 cells ( $1 \times 10^7$  cells/mouse). Eighteen mice bearing xenografted tumors were obtained and a further six mice inoculated with saline acted as controls. When the tumors reached approximately 100 mm<sup>3</sup> in size, the mice were divided into four groups ( $P < 0.05$ ): SIN group (25 mg/kg), 5-FU group (12 mg/kg), combination group, and saline control group. Treatment was ad-

ministered *via* intratumoral injection every 3 d. Tumor size was measured 2-3 times per week in two dimensions by calipers, and the volumes were calculated using the following formula: volume (mm<sup>3</sup>) = [length × (width)<sup>2</sup> × π/6].

### Hematoxylin and eosin staining and TUNEL assay

For histologic analysis, tumor tissues were prepared according to a standard protocol, and the paraffin-embedded tumor tissue sections were subsequently stained with hematoxylin and eosin (HE). For TUNEL assay, an in situ apoptosis detection kit (Roche, Branchburg, NJ, United States) was used to detect apoptotic cells in tumor tissue sections. The apoptotic cells were identified, counted (nine random fields per slide), and analyzed under light microscopy (Olympus, Japan).

### Evaluation of side effects

Blood samples were collected by cardiac puncture. The biomarkers of liver and renal injury, such as the activity of alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN) and serum creatinine (Cr), were detected using an OLYMPUS AU5400 analyzer (OLYMPUS, Japan).

### Statistical analysis

All data were expressed as the mean ± SE of the mean, and then subjected to the unpaired Student's *t* test. Statistical significance was defined as a value of  $P < 0.05$ .

## RESULTS

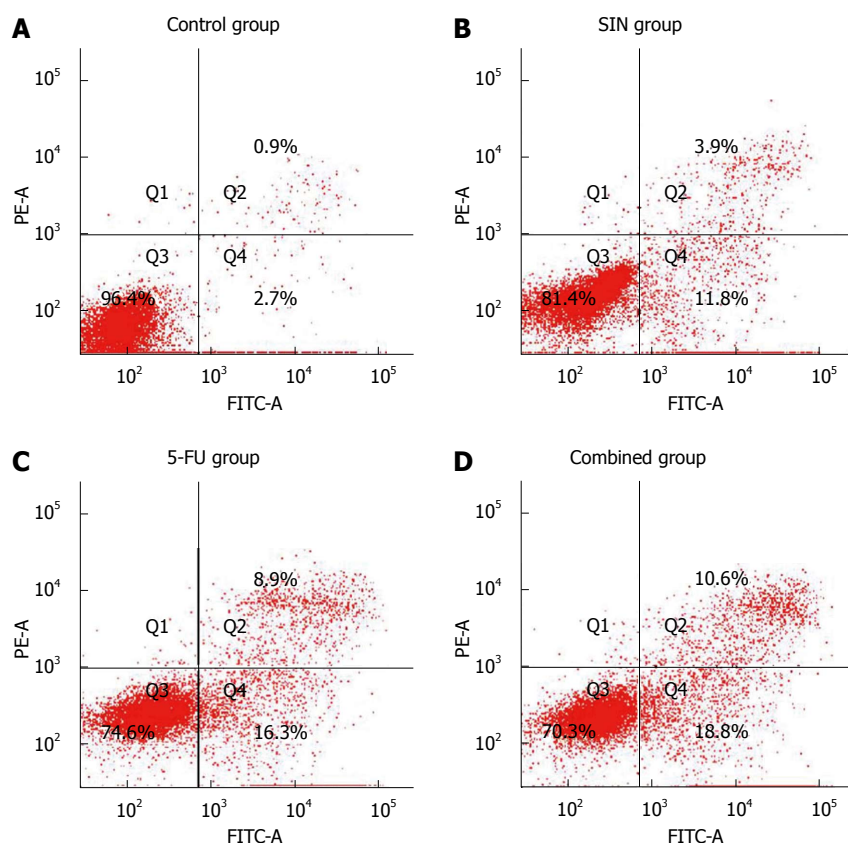
### Inhibition of Eca-109 cell proliferation by SIN and 5-FU

The inhibition of Eca-109 cell proliferation by SIN and 5-FU was assessed by the WSK-8 cell viability assay after 48 h of drug exposure, following 24 h culture in a drug-free medium. As shown in Figure 1, the growth of Eca-109 cells was significantly inhibited in a dose-dependent manner ( $P < 0.05$ ). Significant differences were observed for each concentration of the combined treatment (SIN:5-FU ratio was 1:1) compared to SIN or 5-FU given individually ( $P < 0.05$ ), especially at lower concentrations.

### Apoptosis induced by SIN and 5-FU

Apoptosis induced by SIN and 5-FU was confirmed by Annexin V/PI staining to quantify the amount of phosphatidylserine exposed on the apoptotic cellular membrane. As shown in Figure 2, the percentage of Annexin V-positive/PI-negative cells increased progressively in Eca-109 cells incubated at low concentrations of SIN (160 μmol/L) and 5-FU (160 μmol/L) for 48 h. SIN and 5-FU alone significantly induced apoptosis, as compared with the control group ( $P < 0.05$ ); furthermore, the combined treatment effect was stronger than that of SIN and 5-FU given individually ( $P < 0.05$ , Figure 2).

The morphological features of apoptotic cells were detected by Hoechst 33258 staining. The apoptotic nuclei were assessed by changes revealed by Hoechst staining and were identified by condensed chromatin as well as



**Figure 2** Quantitative flow cytometric measurements of G<sub>2</sub>/M arrest and apoptosis in esophageal carcinoma cells treated with sinomenine and 5-Fluorouracil alone or in combination. The density plots illustrate four cell populations (live, apoptotic, necrotic, and late apoptotic/dead) defined by their fluorescence profiles. SIN and 5-FU alone significantly induced apoptosis compared with the group control ( $P < 0.05$ ), and the combined effects were stronger than those of SIN or 5-FU alone ( $P < 0.05$ ). SIN: Sinomenine; 5-FU: 5-Fluorouracil.

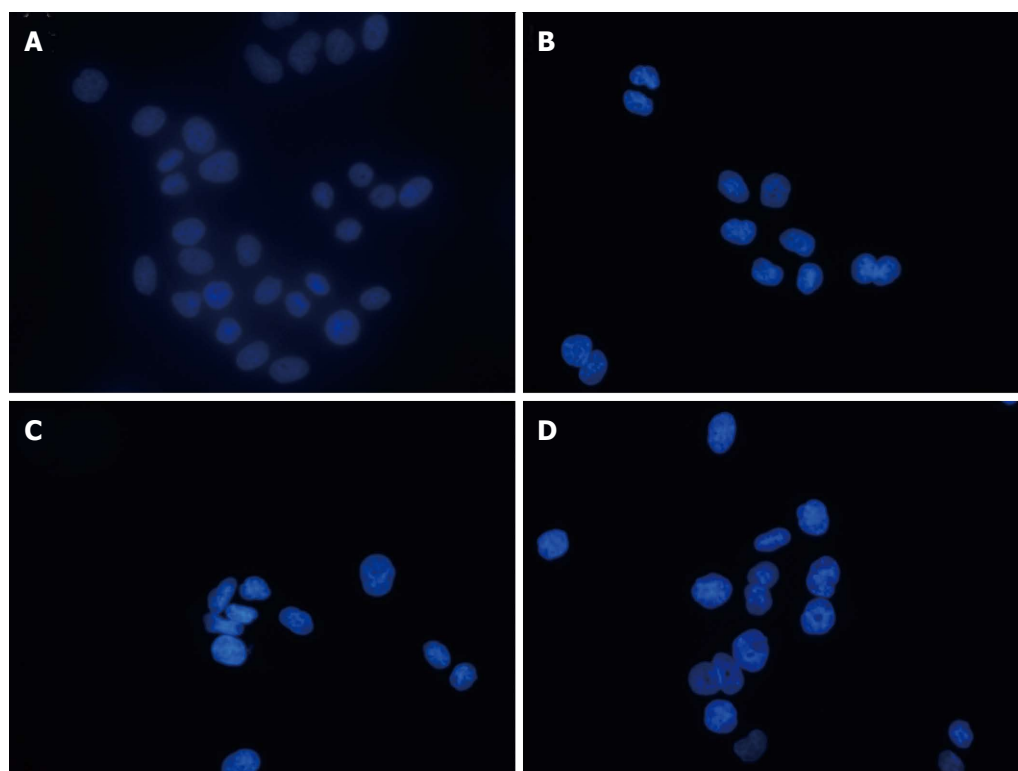
nuclear fragmentation with formation of apoptotic bodies. Ten random fields per dish were observed and counted under a fluorescence microscope. The mean values are expressed as the percentage of apoptotic nuclei per field. Significant changes were detected in the number of apoptotic cells, and the percentage of apoptotic cells induced by SIN and 5-FU combined or alone were significant when compared with the group control ( $P < 0.05$ , Figure 3); furthermore, the apoptotic rate of the combined treatment was greater than those of the individual treatments ( $P < 0.05$ , Figure 3).

#### **SIN and 5-FU induce apoptosis through activation of the mitochondrial pathway**

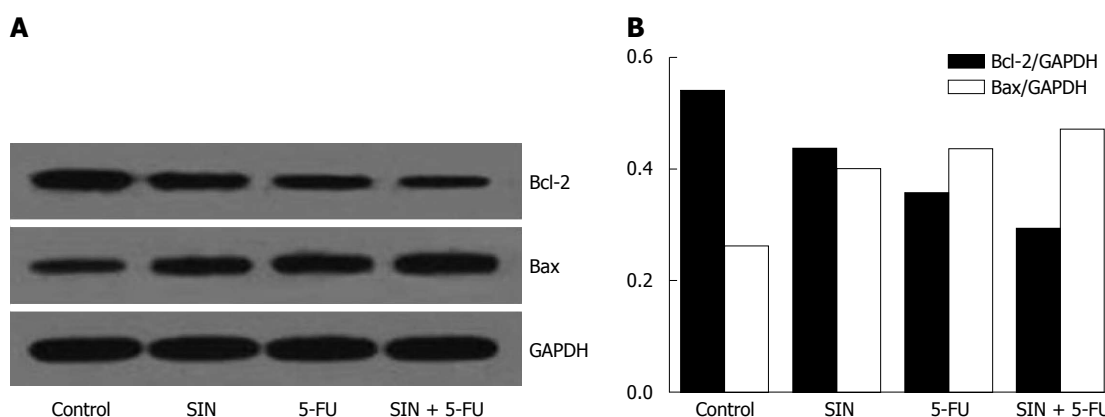
To further investigate the essential mechanism underlying SIN- and 5-FU-induced apoptosis, their effects on the mitochondrial pathway were examined. As shown in Figure 4, SIN (160  $\mu\text{mol/L}$ ) and 5-FU (160  $\mu\text{mol/L}$ ) treatment combined or alone caused an increase in Bax/GAPDH protein levels and a decrease in Bcl-2/GAPDH levels in Eca-109 cells, which led to a decrease in the antiapoptotic/proapoptotic (Bcl-2/Bax) protein ratio. The apoptotic effect in the combined treatment group was significantly greater than that of the individual treatment groups.

#### **Effect of SIN and 5-FU on tumor development in nude mice**

The effects of SIN and 5-FU on the growth of primary tumor xenografts in nude mice were examined. None of the mice died during the course of the experiment, and all 24 mice successfully grew tumor xenografts. After 14 d growth, the tumor xenografts reached a mean size of 100  $\text{mm}^3$ . The mice were then randomly divided into four treatment groups, and no significant differences were observed in tumor size in the different groups at the start of the study. The results demonstrated that the tumor volumes and weights in the treatment groups were both significantly reduced compared with the saline control group ( $P < 0.05$ , Tables 1 and 2), while the extent of tumor reduction differed for each group. The combination treatment induced greater tumor growth suppression than did SIN or 5-FU alone ( $P < 0.05$ , Table 1), and a difference in tumor weight was also observed between groups ( $P < 0.05$ , Table 2). When compared with the control group, the tumor volume inhibition rate for the combination group was 91.22%, whereas the inhibition rates for the SIN and 5-FU groups were 64.68% and 71.68%, respectively (Table 1). When compared with the control group, the tumor weight inhibition rate for the combination group was 83.38%, whereas the inhibition



**Figure 3** Apoptosis of esophageal carcinoma induced by sinomenine and 5-Fluorouracil alone or in combination. A: Control group; B: SIN group; C: 5-FU group; D: Combined group; apoptotic features were assessed by observing chromatin condensation and fragment staining. SIN: Sinomenine; 5-FU: 5-Fluorouracil.



**Figure 4** Sinomenine and 5-Fluorouracil alone or in combination induced apoptosis in esophageal carcinoma cells through the mitochondrial pathway. Western blotting analysis results of Bcl-2 and Bax protein after SIN (160  $\mu\text{mol/L}$ ) and 5-FU (160  $\mu\text{mol/L}$ ) treatment. GAPDH was used as a loading control. SIN: Sinomenine; 5-FU: 5-Fluorouracil; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

rates for the SIN and 5-FU groups were 63.10% and 61.97%, respectively (Table 2). The results suggest that SIN combined with 5-FU exhibits significant anti-tumor potential *in vivo*.

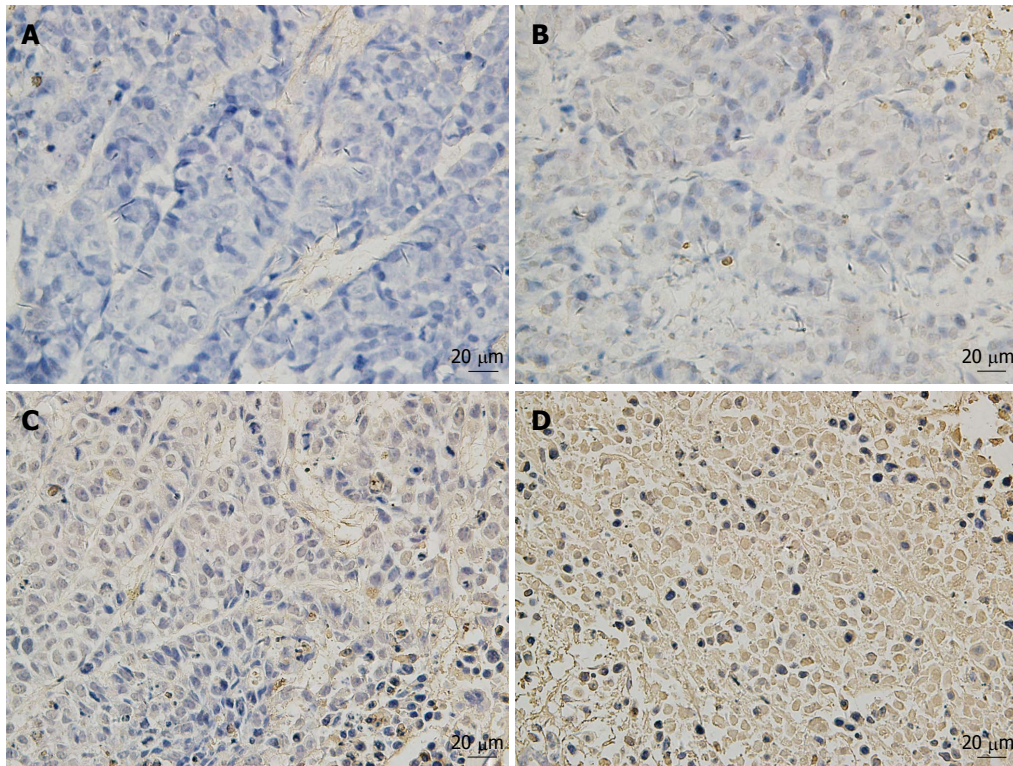
HE staining and TUNEL analysis of the subcutaneous primary tumor sections indicated that SIN and 5-FU individually or in combination induced significant apoptosis of Eca-109 cells *in vivo*, as compared with the saline control group ( $P < 0.05$ , Figure 5), although the degree of apoptosis differed for each group; the apoptotic rate in the combination treatment group was greater than that in the SIN alone and 5-FU alone groups.

The above data indicated that the effects of SIN combined with 5-FU against esophageal carcinoma *in vivo* were superior to that of SIN and 5-FU used individually.

#### Evaluation of side effects

At the end of the experiment, the nude mice were sacrificed and necropsied. No obvious metastasis, hemorrhage, or injury of the liver and kidneys was observed by visual examination.

Blood samples collected by cardiac puncture were used to monitor hepatic and renal toxicity. As biomarkers of liver and renal injury, the activity of ALT and AST,



**Figure 5** Detection of apoptotic cells in xenograft tumor tissue by the terminal deoxynucleotidyl transferase dUTP nick end labeling assay. A: Control group; B: SIN group; C: 5-FU group; D: Combined group; SIN and 5-FU induced significant apoptosis of esophageal carcinoma cells *in vivo* compared with the saline control group ( $P < 0.05$ ), and the apoptotic rate of the combination treatment group was more significant than that of the SIN alone and 5-FU alone groups. SIN: Sinomenine; 5-FU: 5-Fluorouracil.

**Table 1** Inhibitory effect of sinomenine and 5-Fluorouracil on esophageal carcinoma tumor volume in nude mice

Group	<i>n</i>	Volume (mm <sup>3</sup> )	Inhibition rate (%)
SIN	6	161.94 (40.96) <sup>bd</sup>	64.68
5-FU	6	129.82 (16.39) <sup>bd</sup>	71.68
SIN + 5-FU	6	40.27 (5.55) <sup>d</sup>	91.22
Control	6	540.20 (66.04)	

Data presented as mean (SD) and are expressed as inhibition rate (%) = (1 - mean of tumor volume of tests/mean of tumor volume of control) × 100%. There were 6 mice per group. <sup>b</sup> $P < 0.05$ , significantly different from SIN + 5-FU group; <sup>d</sup> $P < 0.05$ , significantly different from control group. SIN: Sinomenine; 5-FU: 5-Fluorouracil.

**Table 2** Inhibitory effect of sinomenine and 5-Fluorouracil on esophageal carcinoma tumor weight in nude mice

Group	<i>n</i>	Weight (g)	Inhibition rate (%)
SIN	6	0.218 (0.061) <sup>bd</sup>	63.10
5-FU	6	0.225 (0.088) <sup>bd</sup>	61.97
SIN + 5-FU	6	0.098 (0.066) <sup>d</sup>	83.38
Control	6	0.593 (0.070)	

Data presented as mean (SD) and are expressed as inhibition rate (%) = (1 - mean of tumor weight of tests/mean of tumor weight of control) × 100%. There were 6 mice per group. <sup>b</sup> $P < 0.05$ , significantly different from SIN + 5-FU group; <sup>d</sup> $P < 0.05$ , significantly different from control group. SIN: Sinomenine; 5-FU: 5-Fluorouracil.

as well as the urea and Cr values, were determined to evaluate potential toxicity. The hepatic and renal toxicity induced by SIN and 5-FU alone or in combination is shown in Table 3. Compared to the control group, there were no significant increases in the values of ALT, AST, urea, and Cr ( $P > 0.05$ , Table 3), and no significant differences were observed between the SIN alone, 5-FU alone and combination treatment groups ( $P > 0.05$ , Table 3). At necropsy, the livers and kidneys of the mice from the different treatment groups appeared smooth and normal in color. There was no significant difference in the liver and renal volume or weight between the treated groups and the control group, and the histological pathology examination showed no obvious lesions.

## DISCUSSION

Esophageal carcinoma is one of the most aggressive malignancies and remains a major public health threat globally. In 2008, the number of new esophageal cancer cases was estimated to be 482300 which accounts for 3.8% of all cancers, while the number of deaths was 406800 which accounts for approximately 5.4% of global cancer mortalities<sup>[2]</sup>. More effective treatments, as well as methods for earlier diagnosis, have led to improved survival over recent decades. However, patients with esophageal cancer still exhibit rapid progression and poor prognosis, with a natural disease history of 6-8 mo and a 5-year survival rate of 5%-7%<sup>[15]</sup>, owing to extensive local inva-

**Table 3** Effect of sinomenine combined with 5-Fluorouracil or alone on hepatic and renal function

Group	ALT (U/L)	AST (U/L)	Urea ( $\mu$ mol/L)	Cr ( $\mu$ mol/L)
SIN	45.50 (7.37)	143.67 (28.82)	8.18 (2.04)	16.63 (3.41)
5-FU	45.33 (6.02)	145.33 (18.53)	8.04 (1.20)	15.73 (4.30)
SIN + 5-FU	46.17 (7.17)	146.83 (19.55)	8.28 (1.44)	16.68 (2.68)
Tumor control	45.83 (3.76)	144.67 (17.32)	7.98 (0.70)	16.74 (4.46)
Normal control	46.00 (5.00)	145.17 (11.27)	8.09 (1.03)	16.29 (4.51)

Data presented as mean (SD), with  $n = 6$  mice/group. Groups were treated as follows: SIN (25 mg/kg); 5-FU (12 mg/kg); SIN (25 mg/kg) + 5-FU (12 mg/kg); Tumor control (saline of equal volume); Normal control. No differences were observed in ALT, AST, Urea, and Cr among the groups ( $P > 0.05$ ). SIN: Sinomenine; 5-FU: 5-Fluorouracil; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; Cr: Serum creatinine.

sion, lymph node involvement and distant metastases at the time of diagnosis<sup>[16]</sup>. Consequently, more effective chemotherapies have become an important means of extending the survival of esophageal cancer patients.

5-FU is widely used in chemotherapeutic regimens, including those for cancers of the gastrointestinal tract and breast. Although 5-FU-based chemotherapy improves the overall survival of patients with esophageal carcinoma, the response rate is extremely low, and even the combination of 5-FU with newer chemotherapies such as cisplatin and doxorubicin only generates response rates of 25%-35%, with 1-year survival rates of 27%-37%<sup>[6,7]</sup>. To improve the prognosis of patients with esophageal cancer, researchers have undertaken the investigation of novel drugs and combinations of chemotherapeutics.

Over the last few years, interest in exploring the use of traditional medicines for the prevention or treatment of tumors has increased. SIN is an alkaloid derived from the stem of the Chinese medical plant *Sinomenium acutum*, which has been used in traditional Chinese medicine for over 2000 years to treat various rheumatoid diseases with minimal side effects<sup>[17,18]</sup>. The chemical structure of SIN has been clarified, and its potential value in treating rheumatoid arthritis has been recognized by Western medicine. Many studies have indicated that SIN has a wide range of significant pharmacological actions, such as anti-inflammatory, anti-arrhythmic, anti-angiogenesis and immunosuppressive effects. Previous studies have demonstrated that inflammation can affect the angiogenesis and invasion of various types of tumors<sup>[19,20]</sup>. Kok *et al.*<sup>[12]</sup> found out that SIN possesses anti-angiogenic activity in several systems both *in vitro* and *in vivo*. Similarly, in a recent study, SIN was demonstrated to induce apoptosis by modulating NF- $\kappa$ B expression and activity<sup>[21]</sup>, and was able to significantly inhibit NF- $\kappa$ B activity even at  $> 10$  ng/mL<sup>[13]</sup>. Based on these results, SIN was hypothesized to enhance the sensitivity of various cancers to anti-cancer drugs.

In the present study, we investigated the inhibitory effects of SIN combined with 5-FU treatment on esophageal carcinoma *in vitro* and *in vivo*. We found that SIN and 5-FU alone can significantly inhibit the proliferation of

Eca-109 cells in a dose-dependent manner ( $P < 0.05$ ). In addition, the combined effect of SIN and 5-FU on the proliferation of human esophageal carcinoma was superior to that of SIN or 5-FU alone *in vitro* and *in vivo*. We also examined the apoptotic effect induced by SIN combined with 5-FU or administered individually. The results indicated that SIN and 5-FU alone significantly induced apoptosis compared with the control, and that the combined treatment effects were stronger than the individual effects of SIN and 5-FU.

SIN, as a typical anti-arrhythmic drug, has also been reported to inhibit the proliferation and induce apoptosis in various tumors<sup>[22]</sup>. However, the exact mechanisms underlying this apoptotic effect are poorly understood. Apoptosis is a tightly controlled type of cell death, with characteristic effects such as cell shrinkage, membrane blebbing and DNA fragmentation. The signals for apoptosis can be either initiated extrinsically through the death receptor pathway or intrinsically through the mitochondrial pathway<sup>[23,24]</sup>. Mitochondria are considered to play a pivotal role in apoptosis. The mitochondrial pathway has been shown to be an important signaling pathway for apoptosis, and SIN has been proven to utilize this pathway to induce the apoptosis of cancer cells<sup>[25-27]</sup>. 5-FU is well known to inhibit the thymic pyrimidine nucleotidase of tumor cells and affect DNA stability<sup>[28]</sup>. Moreover, 5-FU has been demonstrated to induce the apoptosis of various cancer cells such as breast and colon cancer, with resulting changes in p53<sup>[29]</sup>, caspase-3<sup>[30]</sup> and caspase-8<sup>[31]</sup>. In this study, we confirm that SIN combined with 5-FU treatment can induce apoptosis through the mitochondrial pathway.

A mouse tumor xenograft model was established, and the animals were administered chemotherapy consisting of SIN (25 mg/kg) and 5-FU (12 mg/kg) every 3 d. Similar effects on the proliferation and apoptosis of esophageal cancer cells induced by SIN and 5-FU were also observed *in vivo*. No adverse effects such as gastroenterol disturbance, hemorrhage or kidney dysfunction were observed. The model was also used to evaluate the potential histological pathology and hepatic and renal toxicity resulting from treatment, with no significant differences found between the treatment and control groups. Thus, we conclude that SIN combined with 5-FU demonstrates an anti-cancer effect with no increase in side effects *in vivo*.

In conclusion, SIN combined with 5-FU exhibited a significantly superior anti-cancer effect in comparison to SIN or 5-FU alone. In addition, this study demonstrated that SIN and 5-FU did not increase toxicity *in vivo* when used in combination. Above all, the present study indicates the potential for the combined use of SIN and 5-FU in clinical treatment.

## ACKNOWLEDGMENTS

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for assistance in data collection.

## COMMENTS

### Background

Esophageal carcinoma is one of the most refractory and common malignant diseases and is associated with poor outcome. To enhance the effect of systemic chemotherapy and reduce the toxicity, combined treatments with several regimens are often used. 5-Fluorouracil (5-FU) is universally used as an anti-cancer agent in esophageal carcinoma. Sinomenine (SIN) is an immunosuppressive compound extracted from the Chinese medicinal plant *Sinomenium acutum*; this compound has a wide range of significant pharmacological actions and has been hypothesized to enhance the sensitivity of various cancers to anti-cancer drugs.

### Research frontiers

As an alkaloid derived from a medical plant, SIN has been used in traditional Chinese medicine for over 2000 years. In the area of SIN treatment for cancer, the crucial areas of research are to confirm its effects, investigate the agent's mechanism of action and identify combined systemic chemotherapy regimens to prevent the growth of cancer.

### Innovations and breakthroughs

Few studies have described the anti-inflammatory, anti-rheumatic, anti-cancer, and anti-angiogenesis effects of SIN. The results of this study suggest that the combined effects of SIN and 5-FU on the growth of esophageal carcinoma are superior, with no observed increase in side effects. The essential mechanism underlying SIN- and 5-FU-induced apoptosis, as investigated by Western blotting, involved the mitochondrial pathway.

### Applications

In this study, the combined effects of SIN and 5-FU, as well as their essential mechanism were investigated in esophageal carcinoma. The combined use of these two medicines generated a superior anti-cancer effect without increased side effects. This finding may help to provide novel combined systemic chemotherapy in the treatment of esophageal carcinoma.

### Terminology

SIN: SIN is an alkaloid derived from the stem of the Chinese medical plant *Sinomenium acutum*, which has been used in traditional Chinese medicine for over 2000 years to treat various rheumatoid diseases. The chemical structure of SIN has been clarified, and its potential value in the treatment of immunological and other diseases is recognized by Western medicine.

### Peer review

This is a valuable original study in which the authors examine the inhibitory effects of SIN combined with 5-FU on esophageal carcinoma Eca-109 cells *in vitro* and *in vivo*. The results are exciting and suggest that the combined use of SIN and 5-FU is superior to the effects of the individual agents. This study is important for enhancing the efficacy and reducing the toxicity of chemotherapy regimens for patients with esophageal cancer.

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