Songorine suppresses cell growth and metastasis in epithelial ovarian cancer via the Bcl-2/Bax and GSK3β/β-catenin signaling pathways

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Abstract. Epithelial ovarian cancer (EOC) is the most frequent cause of cancer-associated mortality among all types of gynecological cancer. The high recurrence rate and the poor 5-year survival rate indicate that more effective therapeutic strategies are required. The aim of the present study was to investigate the role and potential mechanisms of songorine in treating EOC. EOC cells were cultured with different concentrations of songorine, following which MTT and flow cytometric analyses were conducted to measure cell viability and apoptosis. Wound healing and Transwell assays were used to detect cell migration and invasion abilities. Furthermore, associated molecules in the glycogen synthase kinase (GSK)-3β/β-catenin and B-cell lymphoma 2 (Bcl-2)/Bcl-2-associated X (Bax) signaling pathways were semi-quantified by western blotting. Finally, tumor size measurements, pathological observations, western blot analysis and toxicological evaluations were performed in SKOV-3 tumor-bearing BALB/c nude mice to investigate the efficacy and safety of songorine. As expected, songorine inhibited EOC cell survival, invasion and migration, promoted EOC cell apoptosis and suppressed mammalian EOC tumorigenic behavior. In particular, GSK3β inhibitor treatment restored the songorine-induced regulation of the GSK3β/β-catenin signaling pathway. Furthermore, in the *in vitro* and *in vivo* experiments, songorine consistently downregulated the expression of N-cadherin, vimentin, matrix metalloproteinase (MMP)-2, MMP-9, phosphorylated-GSK3β, β-catenin and Bcl-2, and upregulated the expression of E-cadherin, cleaved caspase-3, cleaved caspase-9 and Bax. In conclusion, songorine exerted its anticancer effect through the GSK3β/β-catenin and Bcl-2/Bax signaling pathways. These results highlight the potential use of songorine as a novel therapeutic agent for EOC.

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Introduction

Ovarian cancer is the most life-threatening form of gynecological cancer and epithelial ovarian cancer (EOC), the most frequent type of ovarian cancer, is a serious threat to female health (1). Due to a lack of pathognomonic clinical symptoms and effective early detection markers, up to 80% of patients with EOC with widespread metastases are diagnosed in the advanced stages (2). Clinically, conventional treatments mainly include surgery, chemotherapy and/or radiotherapy. These therapeutic approaches can elevate progression-free survival rates to 50%, however, the associated prognosis remains poor (3). Cell invasion and migration are the main factors contributing to the poor prognosis in EOC, whereas the underlying mechanisms driving the biological processes are complex and remain to be fully elucidated (4). It has been suggested that epithelial-mesenchymal transition (EMT) serves a critical role in the tumor invasion-metastasis cascade (5). EMT, a reversible cellular process that converts cells from the epithelial polarized phenotype to the mesenchymal fibroblastoid phenotype, is primarily associated with epithelial cell depolarization, loss of cell-to-cell contacts, and an increase in migratory motility and capacity (6). Unfortunately, tumor cells usually undergo EMT, which induces the invasive growth and metastasis of ovarian cancer (7). Therefore, the inhibition of EMT may be an effective strategy with the greatest potential for improving outcomes in patients with EOC.

Natural products have contributed to the development of a number of anticancer agents in recent decades. Due to their structural diversity, several agents used clinically are natural products or derivatives (8), including paclitaxel and vincristine (9,10). Associated studies have also shown that alkaloids are widely distributed in nature (11,12), with notable pharmacological activities in eliminating cancer, and inflammatory and oxidant activities (13-15). Furthermore, >450 alkaloids

are present in *Aconitum* species, which have been widely used in China, Japan and other regions for treating a number of disorders (16). The main alkaloid in *Aconitum soongaricum* Stapf is songorine (Fig. 1A), a C_{20}-diterpenoidaconitum alkaloid (17,18). Songorine possesses several properties, including anti-inflammatory, anti-arrhythmic and anti-central nervous system disorder properties (19). A recent study (20) revealed that songorine can significantly inhibit the survival of human liver cancer cells, which is suggestive of its potential activities in treating cancer. Based on this pharmacological study, it was hypothesized that songorine may maintain its therapeutic action in EOC. The aim of the present study was to evaluate the efficacy of songorine in regulating EOC cell proliferation, migration, invasion and apoptosis. The mechanisms underlying songorine in treating EOC were also discussed. Furthermore, the present study aimed to verify the pharmacological activity of songorine in murine xenograft models. Overall, the results may provide valuable information for drug development or potential strategies against EOC.

Materials and methods

Cell lines and experimental animals. The EOC cells (SKOV3 and A2780) and normal ovarian epithelial cells (IOSE-80) were obtained from the Shanghai Cell Bank of China Academy of Sciences (Shanghai, China). The SKOV3, A2780 and IOSE-80 cells were cultured in Dulbecco's modified Eagle's medium (DMEM), RPMI-1640 and DMEM (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), respectively, and then maintained in a humidified incubator with 5% CO_2 at 37˚C. In addition, 4-week old female BALB/C nude mice (n=24, 20±2 g) were kindly provided by Shanghai SLAC Experimental Animal Company (Shanghai, China) and housed in a pathogen-free environment at a constant temperature (23±2˚C) and humidity under a 12-h light/dark cycle on a full-balanced diet with free access to water and food.

Main reagents and instruments. The primary reagents used in the present study included the following: Songorine (Baoji Herbest Biotech Co., Ltd., Baoji, China); cisplatin (Haosen Pharmaceutical Co., Ltd., Lianyungang, China); SB216763, MTT and dimethyl sulfoxide (DMSO) (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany); an Enhanced Chemiluminescence (ECL) Western Blotting Kit (Beyotime Institute of Biotechnology, Beijing, China); Matrigel, crystal violet and a bicinchoninic acid kit (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China); a small Transwell chamber (Corning, Inc., Somerset, NJ, USA); alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), creatinine (CREA) and creatine kinase (CK) kits (Jiancheng Bioengineering Institute, Nanjing, China); phosphorylated (p)-glycogen synthase kina se (GSK)-3β (cat. no. 5558S), GSK-3β (cat. no. 12456S), β-catenin (cat. no. 8480S), Ki67 (cat. no. 9449S), N-cadherin (cat. no. 13116S), vimentin (cat. no. 5741S), matrix metalloproteinase (MMP)-2 (cat. no. 40994S), MMP-9 (cat. no. 13667S), B-cell lymphoma 2 (Bcl-2; cat. no. 15071S), E-cadherin (cat. no. 14472S), Bcl-2-associated X (Bax; cat. no. 5023S), cleaved caspase-3 (c-caspase-3; cat. no. 9664S), caspase-3 (9662S), c-caspase-9 (cat. no. 9505S), caspase-9 (cat. no. 9504S) and β-tubulin (cat. no. 2128S) primary antibodies (Cell Signaling Technology, Inc., Danvers, MA, USA); goat anti-rabbit immunoglobulin G (IgG) secondary antibody (cat. no. A0208; Bioworld Technology, Inc., St. Louis Park, MN, USA); a protein gel electrophoresis system (Tanon Science & Technology Co., Ltd., Shanghai, China); a light microscope (Olympus Optical Co., Ltd., Tokyo, Japan); an inverted fluorescence microscope (Nikon Instech Co., Ltd., Tokyo, Japan); and image Quant LAS 4000 (Ge Healthcare, Chicago, IL, USA).

Cell survival assay. Cell survival was measured using the MTT colorimetric method. Briefly, logarithmic phase cells were seeded into 96-well plates ($5x10^4$/ml) and incubated with different concentrations of songorine (0, 20, 40, 60, 80 or 100 μM) for 24 h at 37˚C. Subsequently, 20 μl MTT (5 mg/ml) was added into each well. Following 4 h of incubation at 37˚C, 150 μl DMSO was added to dissolve the formazan prior to measurements using a plate reader. Finally, the optical density value was measured at 490 nm. Six independent experiments were performed.

Wound scratch and Transwell chamber assays. The logarithmic phase cells were seeded in 6-well plates ($1x10^5$/ml) and incubated overnight to allow cells to attach. Scratches were created using a 200-μl pipette tip, creating a scratch along a marked line, and floating cells were washed away using PBS. Subsequently, fresh medium with different concentrations of songorine (0, 20, 40 or 80 μM) was added. Following culture (5% CO_2, 37˚C) for 24 h, the migrated cells were observed and the cell migration area was calculated.

The invasion assay was performed using Transwell invasion chambers (Costar; Corning, Inc.) pre-coated with Matrigel (BD Biosciences, San Jose, CA, USA). Firstly, 100 μl serum-free medium with logarithmic phase cells ($1x10^5$/ml) and different concentrations of songorine (0, 20, 40 or 80 μM) were added into the upper chamber. Following this, 600 μl medium containing 10% (v/v) fetal bovine serum (Gemini Bio-Products, West Sacramento, CA, USA) was loaded into the lower chamber. After 24 h, the non-invading cells were gently removed, and the invading cells on the bottom inserts were fixed with 4% paraformaldehyde (20 min) and stained with 0.1% crystal violet (12 min), successively. The number of cells was then counted per five fields of view under a light microscope.

Annexin V/propidium iodide (PI) staining assay. The logarithmic phase cells were seeded in 6-well plates ($1x10^5$/ml) and incubated overnight. Following treatment with different concentrations of songorine (0, 20, 40 or 80 μM) for 24 h, the cells were harvested and re-suspended ($1x10^6$ cells/ml) in binding buffer. The Annexin V-fluorescein isothiocyanate and PI were added into the cell suspension according to the instructions provided by the manufacturer of the apoptosis detection kit. Finally, cell apoptosis was detected by flow cytometry (BD Biosciences).

In vivo experiments. The *in vivo* investigation of songorine was performed using a xenograft model of ovarian cancer cells. Briefly, 200 μl resuspended serum-free PBS with $1x10^7$ viable

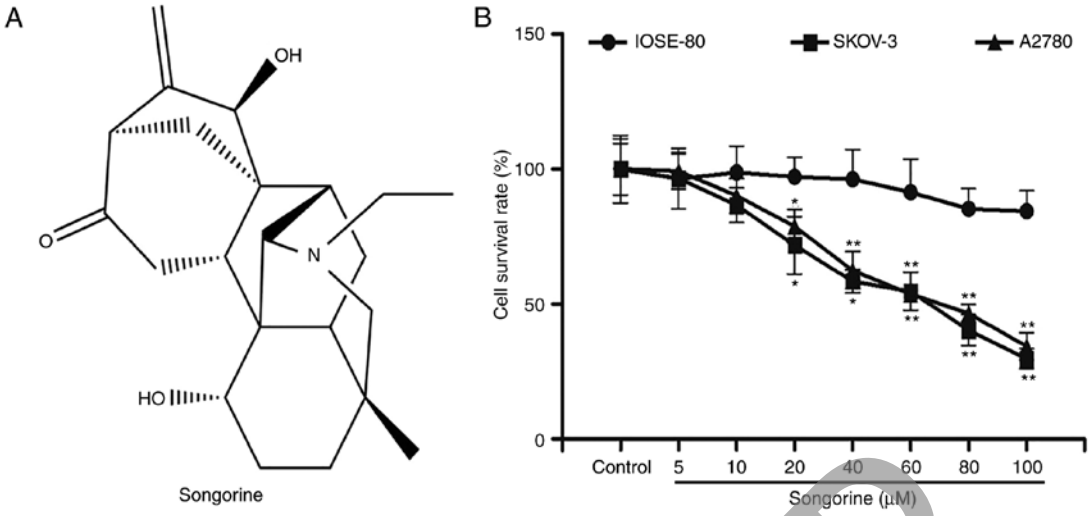

Figure 1. Songorine inhibits the cell viability of SKOV-3 and A2780 cells, but not IOSE-80 cells. (A) Chemical structure of songorine. Molecular formula: $C_{22}H_{31}NO_3$. Molecular weight: 357.494 g/mol. (B) SKOV3 and A2780 ovarian cancer cells, and normal ovarian IOSE80 cells were incubated with various concentrations of songorine (0, 5, 10, 20, 40, 60, 80 and 100 μM) for 24 h. An MTT assay was performed to measure cell viability. Values are presented as the mean ± standard deviation of three independent experiments. *P<0.05 and **P<0.01, vs. control group.

SKOV3 cells was injected subcutaneously into the right axillary region of the nude mice. When the tumor volume [volume = (length x width^2)/2] reached 100 mm^3, this was indicative of successful establishment of the ovarian cancer model. According to a previous study (21), the same administration dosages of songorine (0.25 and 2.5 mg/kg) were used in the present study. Subsequently, the mice were randomized into four groups (n=6): Mice were intraperitoneally injected with saline, songorine (0.25 or 2.5 mg/kg/day) and cisplatin (2 mg/kg/day) for 3 weeks. All animals were sacrificed under anesthesia following blood collection from the eye socket vein, and the subcutaneous tumors were then harvested. The blood supernatants were separated to detect the indicators of liver, renal and cardiac functions, whereas tumors were obtained for histomorphological observations and immunoblotting assays. All animal experimental procedures were performed in accordance with the International Standards of Animal Welfare and were approved by the Institute of Animal Care and Use Committee of Qingdao University (Qingdao, China).

Spectrophotometer detection. The levels of ALT, AST, BUN, CREA and CK in the blood supernatants of each group were measured according to the operating steps described in the instructions of the commercial assay kits.

Hematoxylin and eosin (H&E) staining. The tumor samples were collected and dehydrated in a graded ethanol series prior to paraffin embedding. For histopathological analysis, serial paraffin sections (5-μm) were stained with H&E. The morphological changes were observed under a light microscope (Eclipse TE200; Nikon Instech Co., Ltd.) by investigators blinded to the experimental groups.

Immunohistochemistry. The sections were deparaffinized, rehydrated and then immersed in 100 μl 3% hydrogen peroxide for 15 min at room temperature. Following antigen retrieval in boiled sodium citrate buffer, the sections were successively incubated with the rabbit anti-Ki67 antibody (dilution 1:500) at 4˚C overnight and goat anti-rabbit IgG (dilution 1:500) for 30 min at room temperature. Subsequently, the sections were visualized by incubating with DAB solution. The expression level of Ki67 was assessed under a light microscope (magnification, x400) and presented as the average staining intensity of five fields.

Immunoblotting assay. Each group of cells or tumor tissues were washed with PBS three times, and proteins were then extracted using a total protein assay kit. A total of 30 mg of denatured protein was separated by 10% SDS-PAGE and electrotransferred onto PVDF membranes (Merck KGaA Co., Ltd., Darmstadt, Hessen, Germany), and then incubated with specific antibodies of p-GSK-3β, GSK-3β, β-catenin, N-cadherin, vimentin, MMP-2, MMP-9, Bcl-2, E-cadherin, Bax, c-caspase-3, caspase-3, c-caspase-9, caspase-9 and β-tubulin (dilution 1:1,000) overnight at 4˚C. The PVDF membranes were washed three times in PBST, and then incubated with horseradish peroxidase-linked secondary antibody (dilution 1:5,000; cat. no. BA1054; Wuhan Boster Biological Technology, Ltd., Wuhan, China) for 1 h at room temperature. The specific proteins on the blots were visualized using ECL and quantified using the Image Quant LAS 4000 system. β-tubulin was used as the loading control.

Statistical analysis. Quantitative data are expressed as the mean ± standard deviation. Data were analyzed using one-way analysis of variance (ANOVA), followed by Dunnett's post hoc test using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Songorine suppresses the survival of EOC cells. To preliminarily examine the effect of songorine on EOC, human EOC cells were treated with a range of concentrations of songorine.

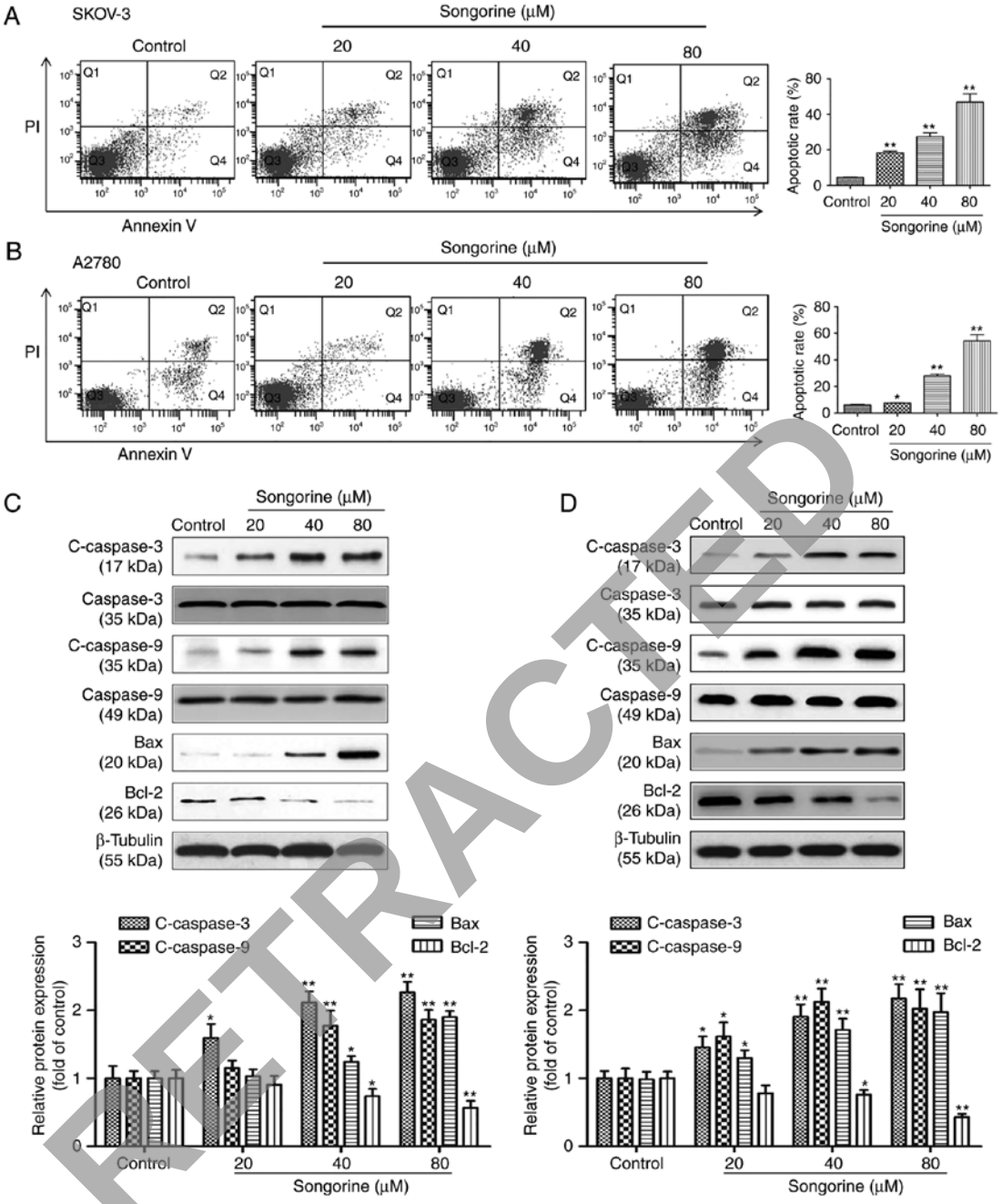

Figure 2. Songorine induces apoptosis of SKOV-3 and A2780 cells via the Bcl-2/Bax signaling pathway. The apoptotic rates of (A) SKOV-3 and (B) A2780 cells were measured by flow cytometry and double staining with Annexin V and PI. The expression levels of apoptosis-associated proteins were detected in (C) SKOV-3 and (D) A2780 cells treated with songorine for 24 h. Values are presented as the mean ± standard deviation of three independent experiments. *P<0.05 and **P<0.01, vs. control group. Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X; PI, propidium iodide; c-caspase, cleaved caspase.

An MTT assay was used to measure the cell viabilities of SKOV-3 and A2780 cells. As expected, songorine inhibited SKOV-3 and A2780 cell viability in a concentration-dependent manner (Fig. 1B). In addition, the half-maximal inhibitory concentration values of songorine were 55.63 μM for SKOV-3 cells and 64.42 μM for A2780 cells. To verify whether songorine was hypotoxic to normal ovarian cells, IOSE-80 cells subsequently underwent songorine treatment. The results demonstrated that songorine exerted no significant inhibitory effects on the normal ovarian cell survival (Fig. 1B). Based on the above results, it was hypothesized that songorine is effective for the treatment of EOC and provides a favorable safety profile. To confirm this hypothesis, the underlying pharmaco-mechanisms of songorine were determined *in vivo* and *in vitro*.

Songorine promotes the apoptosis of EOC cells via the Bcl-2/Bax signaling pathway. To further verify whether the cytotoxic properties of songorine were generated by apoptosis, the degree of cell apoptosis and associated mechanisms were visualized using flow cytometric analysis and immunoblotting assays, respectively. As shown in Fig. 2A and B, the flow cytometry results revealed that treatment of the SKOV-3 and A2780 cells with songorine dose-dependently increased the

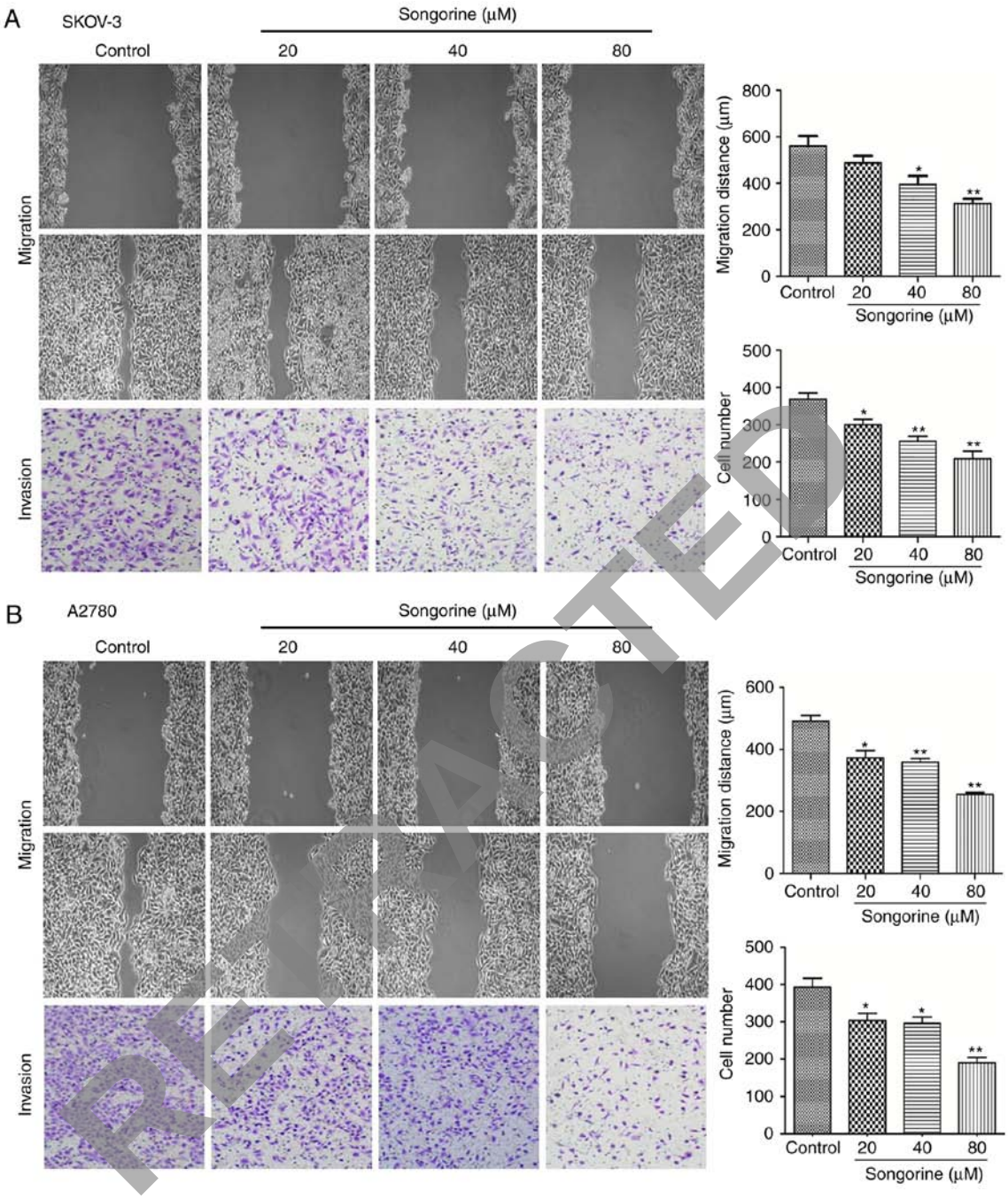

Figure 3. Songorine reduces the migration and invasion of SKOV-3 and A2780 cells. A wound healing assay was performed to observe the effects of songorine treatment on (A) SKOV-3 and (B) A2780 cell migratory abilities. The migration distance of SKOV-3 and A2780 cells was quantified at 0 and 24 h. A Transwell assay was used to detect the effects of songorine treatment on SKOV-3 and A2780 cell invasive abilities. The number of migrated SKOV-3 and A2780 cells in each group was counted. Values are presented as the mean ± standard deviation of three independent experiments. *P<0.05 and **P<0.01, vs. control group.

numbers of early and late apoptotic cells. The percentages of apoptotic cells were 18.4, 27.5 and 46.9% in total SKOV-3 cells treated with 20, 40 and 80 μM songorine, respectively. The apoptotic rates of the A2780 cells were 7.5, 27.8 and 54.4%, respectively. Apoptosis is a type of programmed cell death which is caspase-dependent and mainly regulated by the Bcl-2/Bax signaling pathway. The results of the present study demonstrated that songorine treatment decreased the expression of Bcl-2, but increased the expression levels of c-caspase-3, c-caspase-9 and Bax in a dose-dependent manner (Fig. 2C and D). Therefore, regulation of the Bcl-2/Bax signaling pathway by songorine represents a strategy to influence the apoptotic mechanism in SKOV-3 and A2780 cells.

Songorine attenuates the migration and invasion of EOC cells via the GSK3β/β-catenin signaling pathway. To investigate the underlying antimetastatic effect of songorine on EOC cells, wound healing and Transwell chamber assays were conducted. The results demonstrated that the migration distances of the SKOV-3 and A2780 cells treated with songorine were dose-dependently decreased (Fig. 3A and B). In addition, the number of SKOV3 and A2780 cells that invaded the Transwell

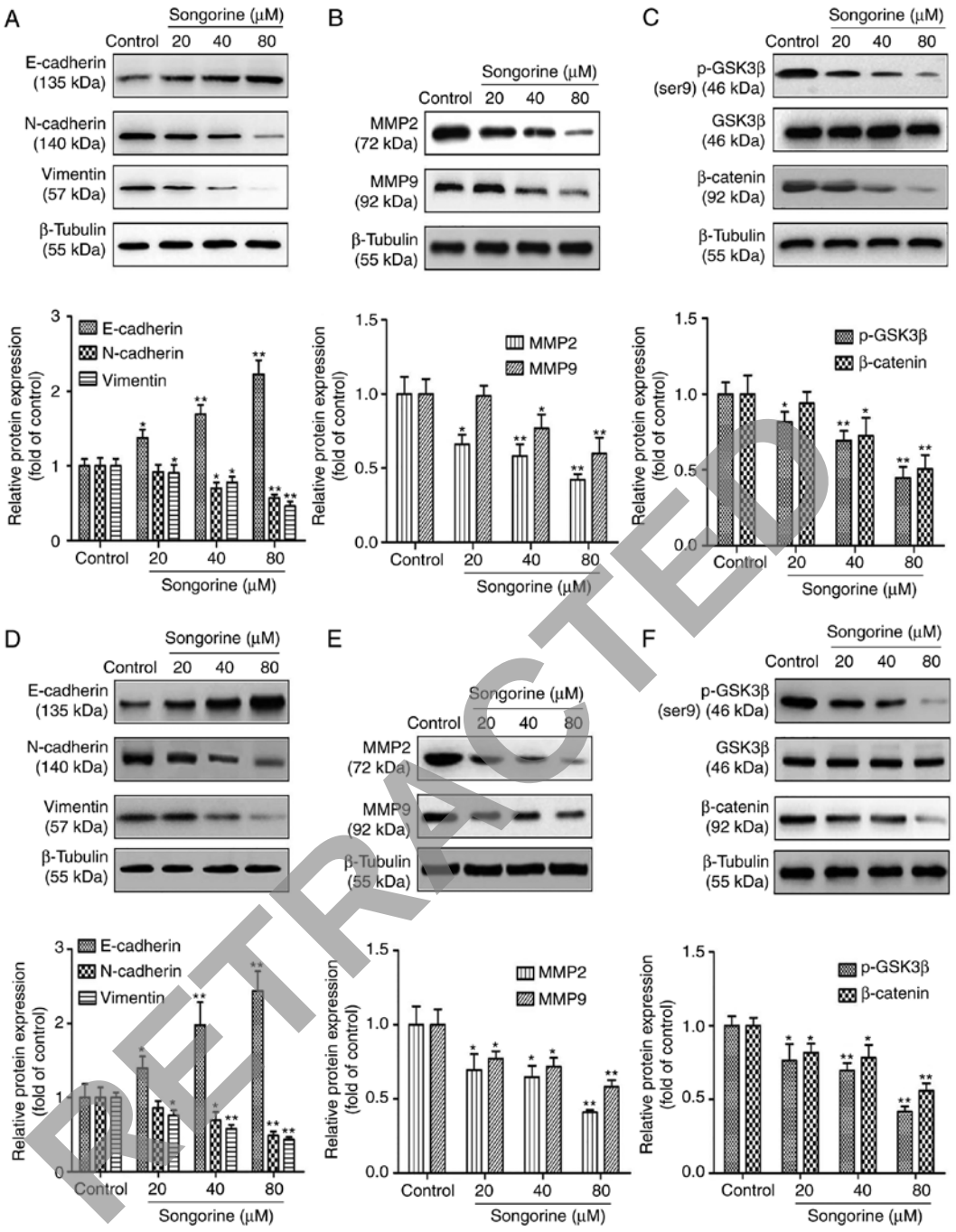

Figure 4. Songorine downregulates theGSK3β/β-catenin signaling pathway in SKOV-3 and A2780 cells. (A) Western blot analysis of the expression of E-cadherin, N-cadherin and vimentin in SKOV-3 cells following treatment with songorine. (B) Western blot analysis of the expression of MMP-2 and MMP-9 in SKOV-3 cells following treatment with songorine. (C) Effects of songorine treatment on the GSK3β/β-catenin signaling pathway in SKOV-3 cells were analyzed via western blotting. (D) Western blot analysis of the expression of E-cadherin, N-cadherin and vimentin in A2780 cells following treatment with songorine. (E) Western blot analysis of the expression of MMP-2 and MMP-9 in A2780 cells following treatment with songorine. (F) Effects of songorine treatment on the GSK3β/β-catenin signaling pathway in A2780 cells were also analyzed via western blotting. Representative blots are presented with the densitometry results; β-tubulin served as an invariant control for equal loading. Values are presented as the mean ± standard deviation of three independent experiments. *P<0.05 and **P<0.01, vs. control group. GSK3β, glycogen synthase kinase 3β; p-GSK3β, phosphorylated GSK3β; MMP, matrix metalloproteinase.

chamber were markedly reduced by songorine (Fig. 3A and B). Therefore, as songorine effectively inhibited the migration and invasion abilities of the EOC cells, the present study subsequently measured metastasis-associated protein expression by western blot analysis. As shown in Fig. 4A and D, the mesenchymal markers of SKOV3 and A2780 cells, including vimentin and N-cadherin, were significantly downregulated, whereas the epithelial marker E-cadherin was markedly upregulated. The results also indicated that songorine markedly reduced the levels of MMP-2 and MMP-9 in the SKOV3 and A2780 cells (Fig. 4B and E). Taken together, the results suggested that songorine exerted an antimetastatic effect by interfering with the EMT process and secretion of MMPs in the two cancer cell lines. To further elucidate the possible regulatory mechanisms, the present study investigated the GSK3β/β-catenin signaling pathway. The results of the

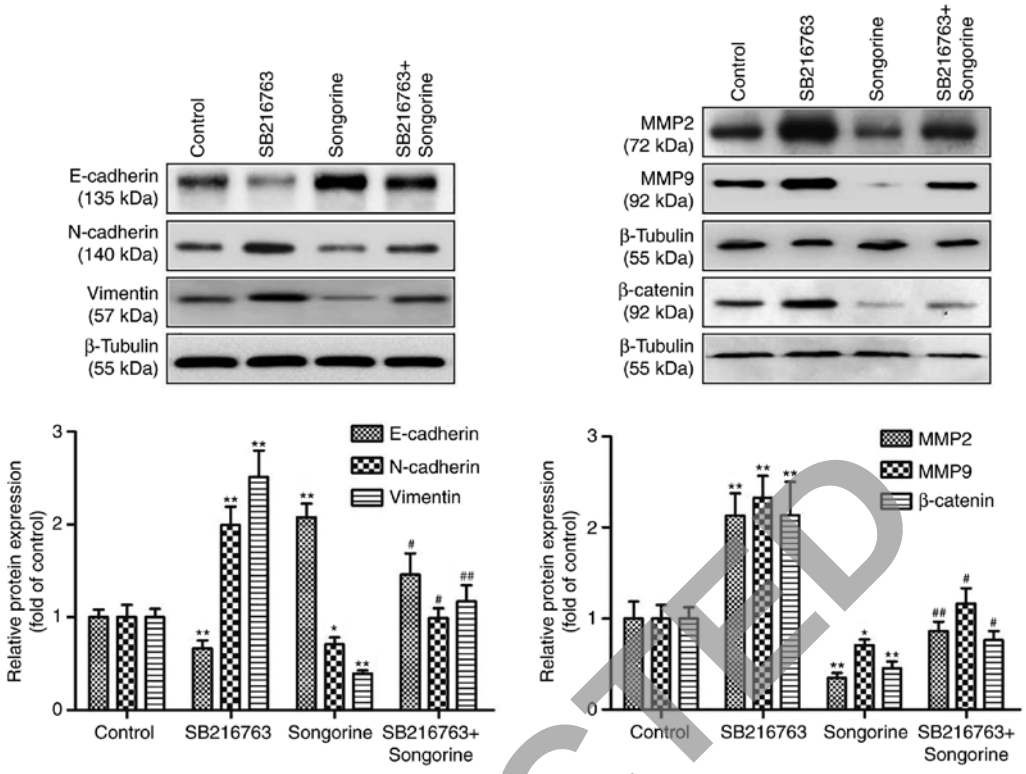

Figure 5. Songorine represses the EMT process and secretion of MMPs potentially by targeting GSK3β. The SKOV-3 cells were treated with 10 μM SB216763, with or without 40 μM songorine, following which cell lysates were subjected to western blotting and probed for the expression levels of E-cadherin, N-cadherin, vimentin, MMP-2, MMP-9 and β-catenin; β-tubulin served as the loading control. Representative blots are presented with the densitometry results. Values are presented as the mean ± standard deviation of three independent experiments. *P<0.05 and **P<0.01, vs. control group; $^{\#}$P<0.05 and $^{\#\#}$P<0.01, vs. songorine group. EMT, epithelial-mesenchymal transition; MMP, matrix metalloproteinase; GSK3β, glycogen synthase kinase 3β.

western blot analysis demonstrated that songorine suppressed the phosphorylation of GSK3β, and thus induced the degradation of β-catenin in a time-dependent manner (Fig. 4C and F), indicating the involvement of the GSK3β/β-catenin pathway in the songorine-induced reduction in cell metastatic abilities. The results revealed significant differences in cell migration and invasion, which suggest that the songorine-mediated inhibition of the EMT processes and secretion of MMPs in EOC cells may be closely associated with downregulation of the GSK3β/β-catenin signaling pathway.

Songorine inhibits EMT processes and the secretion of MMP potentially by targeting GSK3β. To verify whether songorine suppressed the EMT processes and the secretion of MMPs in EOC cells by targeting GSK3β, the present study subsequently used SB216763 (a GSK3β inhibitor) for SKOV-3 cell pretreatment. The results demonstrated that SB216763 significantly reversed the effects of songorine by upregulating the expression of β-catenin (Fig. 5). In particular, in the songorine-treated SKOV-3 cells, SB216763 markedly reduced the levels of the epithelial marker E-cadherin but increased the levels of the mesenchymal markers N-cadherin and vimentin, and MMP-2 and MMP-9 (Fig. 5). These results suggest that songorine inhibited the migration and invasion abilities of EOC cells by alleviating EMT processes and levels of MMPs, potentially via targeting GSK3β.

Songorine attenuates tumorigenic activity in a xenograft model of ovarian cancer. To further investigate the associated pharmacodynamic activities *in vivo*, SKOV-3 tumor-bearing BALB/c nude mice were injected with songorine. Reduced tumor size and tumor weight were observed following treatment (Fig. 6A). Pathological analysis of the H&E-stained samples indicated the typical pathological characteristics of tumors, including large and irregularly shaped nuclei, closely packed together. However, songorine effectively improved pathological conditions by inducing cell shrinkage, fragmentation, sparse arrangement and chromatin disappearance (Fig. 6B). In addition, the immunohistochemical assay revealed that songorine at different concentrations effectively decreased the expression of the proliferation marker Ki67 in tumor tissues (Fig. 6C). These morphological changes were indicative of the positive role of songorine in suppressing tumor growth, and promoting tumor necrosis to varying degrees. As detected by western blotting, the protein expression levels of N-cadherin, vimentin, MMP-2, MMP-9, p-GSK3β, β-catenin and Bcl-2 were significantly decreased, whereas the protein expression levels of E-cadherin, c-caspase-3, c-caspase-9 and Bax were markedly increased in the songorine-treated nude mice (Fig. 6D-F). Therefore, the results suggested that songorine inhibited EOC tumorigenic activities *in vivo* by reducing cell migration and invasion via the GSK3β/β-catenin signaling pathway, and inducing cell apoptosis through the Bcl-2/Bax signaling pathway. Finally, blood biochemical analysis revealed that all biochemical parameters of the mice treated with songorine were within ranges similar to those of the mice in the control group (Fig. 6G). These results suggested that songorine induced no significant systemic toxicity *in vivo* at the dose used.

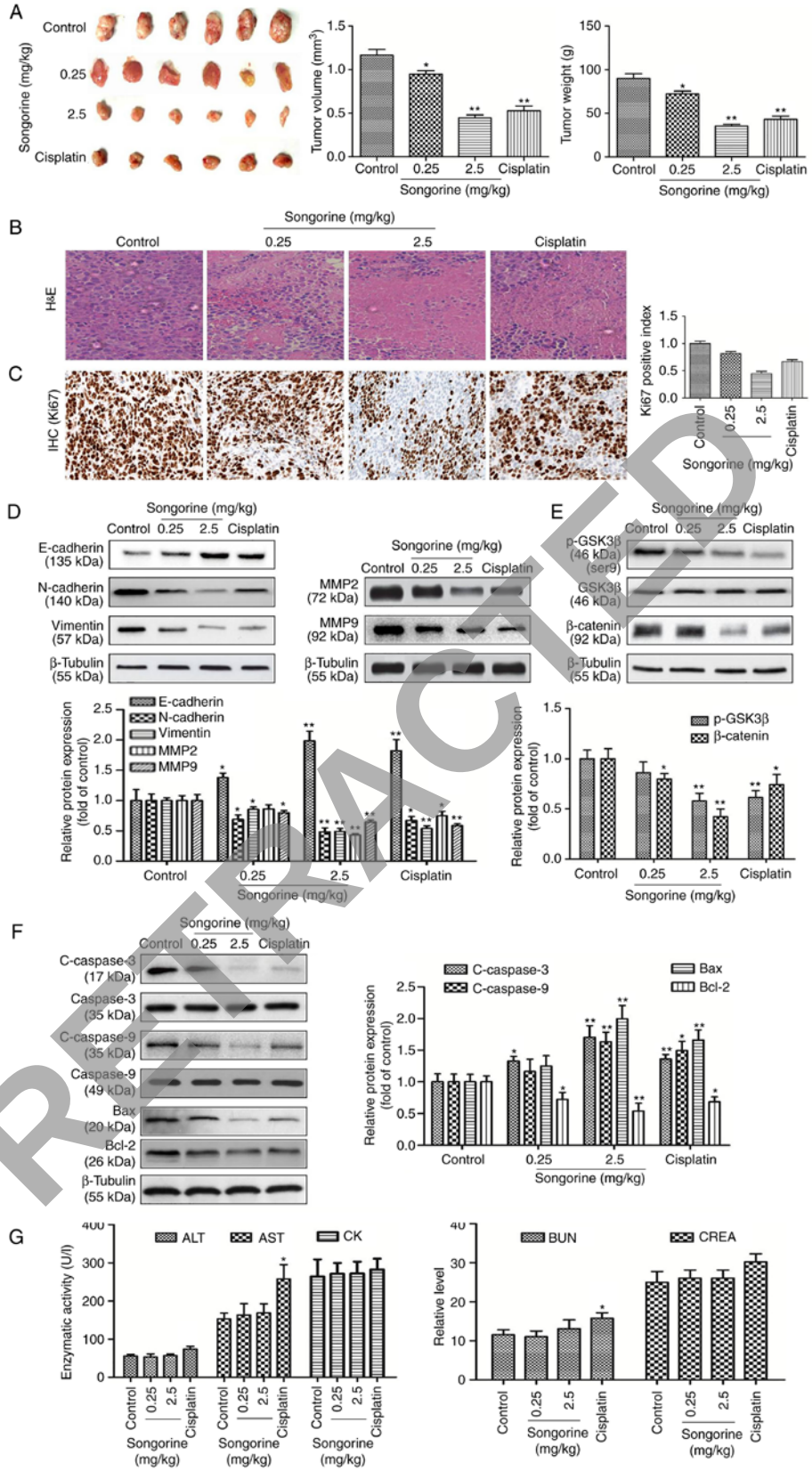

Figure 6. Songorine attenuates tumorigenic activity *in vivo*. (A) Tumors extracted following subcutaneous growth. The tumor volume and weight were measured in each group. (B) H&E staining of the tumor tissues of SKOV-3 tumor-bearing BALB/c nude mice following songorine treatment. (C) An IHC assay was performed to detect the expression of the proliferation marker Ki67 in tissue sections. (D) Expression levels of E-cadherin, N-cadherin, vimentin, MMP-2 and MMP-9 in tumor tissues were analyzed via western blotting with β-tubulin serving as the internal control. (E) Expression levels of GSK3β, p-GSK3β and β-catenin in tumor tissues were analyzed via western blotting with β-tubulin serving as the internal control. (F) Expression levels of caspase-3, c-caspase-3, caspase-9, c-caspase-9, Bax and Bcl-2 in tumor tissues were analyzed via western blotting with β-tubulin serving as the internal control. Representative blots are presented with the densitometry results. (G) Cardiac, liver and renal toxicities of songorine were evaluated by measuring the CK, ALT, AST, BUN and CREA levels. Values are presented as the mean ± standard deviation of three independent experiments. *P<0.05 and **P<0.01, vs. control group. MMP, matrix metalloproteinase; GSK3β, glycogen synthase kinase 3β; p-, phosphorylated; c-, cleaved; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X; CK, creatine kinase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; CREA, creatinine; H&E, hematoxylin and eosin; IHC, immunohistochemistry.

Discussion

EOC accounts for ~90% of all ovarian tumors, making it one of the major gynecological malignancies worldwide (22). In the last 50 years, the lack of improvement in the rates of mortality highlight the requirement for novel chemotherapy strategies. The *Aconitum* genus is a member of the Ranunculaceae family and is important in traditional medicine in general and in traditional Chinese medicine in particular (16). Notably, songorine is one of the main aconitum alkaloids extracted from *Aconitum soongaricum* L, with notable pharmacological effects and promising preclinical potential (19). In the present study, different concentrations of songorine significantly suppressed the proliferation of EOC cells, but exerted hypotoxicity towards normal ovarian epithelial cells. The results demonstrated the potential therapeutic values of songorine in treating EOC, and the associated pharmacological mechanisms require further investigation.

In clinical practice, the invasion and migration of ovarian cancer are the most difficult and important concerns, as they frequently cause EOC-associated mortality (23). EMT is a necessary physical function of the mammalian embryonic development process, however, studies have indicated that EMT induces cancer cell invasion and migration by accelerating cellular and micro-environmental changes (24-26). Following EMT, cancer cells lose normal epithelial polarity and gain mesenchymal traits, which is accompanied by the downregulation of epithelial markers, including E-cadherin, and upregulation of interstitial markers, including N-cadherin and vimentin (24,27,28). Subsequently, the cell-cell connections and cell-matrix contacts are destroyed, and the malignant cells migrate and invade the surrounding matrix to exacerbate tumor progression and invasion (29). In the present study, when EOC cells were exposed to songorine, their relative migration and invasion abilities were markedly reduced. In addition, songorine induced the upregulation of E-cadherin, which was accompanied by the downregulation of N-cadherin and vimentin. Accordingly, these results indicated that songorine suppressed EOC cell migration and invasion abilities by attenuating EMT. By contrast, MMP-2 and MMP-9 are expressed in cancer cells during malignant invasion and migration (30). They are important proteolytic enzymes for the degradation of extracellular matrix, and can induce cancer cell penetration into the basement membrane (31). A previous study demonstrated that reductions in the levels of MMP-2 and MMP-9 in ovarian cancer alleviated cell metastatic activity (32). This is consistent with the results of the present study, as MMP-2 and MMP-9 were significantly decreased when cell metastasis was suppressed in songorine-treated EOC cells. Collectively, these results suggest that songorine has the potential to repress EOC cell migration and invasion by restraining the EMT progress and secretion of MMPs.

Previous studies have demonstrated that the Wnt/β-catenin signaling pathway serves a crucial role in cellular differentiation, migration, innovation and proliferation. Additionally, stimulation of the Wnt/β-catenin signaling pathway is closely associated with cancer development (33). Following activation of the Wnt/β-catenin signaling pathway during neoplastic transformation, cell migration and invasion are subsequently exacerbated (34). Increased GSK3β protein stability may accelerate the degradation of β-catenin (35) and interfere with the occurrence of EMT, which indirectly causes the upregulation of E-cadherin, and the downregulation of N-cadherin and vimentin (36,37). Furthermore, the degradation of β-catenin results in the downregulation of MMP secretion, which in turn causes the inhibition of tumor invasion and migration (38). Subsequently, western blotting analysis confirmed that the expression levels of p-GSK3β and β-catenin were downregulated by songorine. Therefore, it was hypothesized that inhibition of the GSK3β/β-catenin signaling pathway induced by songorine may contribute to the disturbance in malignant cell migration and invasion. To investigate whether the songorine-mediated alteration of EOC cell migration and invasion is associated with the phosphorylation of GSK3β, the present study performed measurements following SB216763 (GSK3β inhibitor) treatment. As the xenograft models were established using SKOV-3 cells in follow-up research. SB216763 was provisionally used for incubation with SKOV-3 cells. As a result, SB216763 significantly increased the levels of p-GSK3β, β-catenin, N-cadherin, Vimentin, MMP-2 and MMP-9, and decreased the expression of E-cadherin in EOC cells. Specifically, GSK3β inhibitor treatment restored the songorine-induced regulative effects. Taken together, it was concluded that songorine inhibits EOC cell invasion and migration by targeting GSK3β. However, SB216763 acted as a competitive inhibitor of GSK3β, it partially verified the songorine as a potential inhibitor of GSK3β. To confirm GSK3β as the target of songorine, gene-silencing and biolayer interferometry technologies may be applied in further investigations.

An increasing body of evidence has revealed that apoptosis is an essential target of cancer chemotherapy (39,40). Apoptosis, a type of programmed cell death, serves a crucial role in the normal physiological activity of the body (41). During the apoptotic process, the caspase protein family is mainly involved in the cleavage and activation of each other. Active caspase-9 triggers the activation of downstream 'executioner' caspase-3, which facilitates cell death (42). Bcl-2 is an integral membrane protein that is mainly located on the outer membrane of mitochondria and induces the release of caspase-3/-9. Proteins in this family exhibit either pro-apoptotic or anti-apoptotic activities, with the Bcl-2/Bax ratio serving as a marker to determine cell susceptibility to apoptosis (43). Notably, the results of the present study showed that songorine increased the expression levels of Bax, caspase-3 and caspase-9, but downregulated the expression of Bcl-2. Furthermore, the results revealed that the number of EOC cells undergoing apoptosis was significantly increased by songorine. These novel findings indicated that songorine induced Bax/Bcl-2 hyperfunctioning in EOC cells, which suggested that songorine promoted cell apoptosis via Bcl-2/Bax signaling.

Upon confirming that songorine significantly inhibits EOC cell migration and invasion, and induces cell apoptosis *in vitro*, the present study further examined the therapeutic effects of songorine *in vivo*. For the xenografts in nude mice, SKOV3 cells were inoculated to form subcutaneous transplant tumors, as these cells germinate as moderately well-differentiated adenocarcinoma similar to primary ovarian cancer (44). Treatment with songorine in the xenografted nude mice led to notably reduced tumor size, and it also caused the inactivity or regression of tumor cells. These results indicate that songorine

serves a potential role in the treatment of EOC. Following further analysis, the promotion of Bax/Bcl-2 hyperfunctioning was accompanied by increased levels of c-caspase-3 and c-caspase-9 following songorine treatment. In addition, the expression of E-cadherin in the tumors was upregulated, whereas the expression levels of p-GSK3β, β-catenin, MMP-2, MMP-9, N-cadherin and vimentin were downregulated following songorine treatment. Taken together, the results of the present study highlight the positive effects of songorine in treating EOC by influencing cell survival, apoptosis, migration and invasion. Additionally, hematologic toxicity analysis revealed the hypotoxicity of songorine *in vivo*, which support the potential clinical value of songorine as a novel antitumor agent for EOC in the future.

In conclusion, to the best of our knowledge, the present study is the first to demonstrate the potential role of songorine in treating EOC. Treatment with songorine suppressed the migration and invasion properties of EOC cells, and the underlying mechanism may be associated with inhibition of the EMT process and secretion of MMP via the GSK3β/β-catenin signaling pathway. The results also indicated that the Bcl2/Bax signaling pathway may be a critical target of songorine-mediated apoptosis in anti-EOC activities. Taken together, these results are valuable for understanding the pharmacodynamic actions of songorine in treating EOC. However, this mechanism requires additional elucidation and confirmation in future studies.

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Availability of data and materials

The datasets used in the present study are available from the corresponding author upon reasonable request.

Authors' contributions

HZ and YW conceived and designed the study, and wrote the manuscript. HZ, RD and PZ performed the experiments. RD reviewed and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research.

Ethics approval and consent to participate

All experimental protocols in the present study were approved by the Institutional Animal Care and Use Committee of Qingdao University (Qingdao, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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