Sinomenine hydrochloride inhibits human hepatocellular carcinoma cell growth *in vitro* and *in vivo*: Involvement of cell cycle arrest and apoptosis induction

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Abstract. Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. However, therapies against HCC to date have not been completely effective. Sinomenine hydrochloride (SH), an anti-arthritis drug applied in clinical practice, has been reported to have *in vitro* anti-neoplastic activity in various cancer cells. Whether SH inhibits HCC remains unknown. For this purpose, in this study, MTT assay was used to determine cell growth. Flow cytometry, Hoechst staining, DNA fragmentation, western blot analysis, immunohistochemisty and TUNEL staining were performed to investigate the mechanisms involved. The *in vivo* activity of SH was determined using a mouse xenograft model. SH inhibited the growth of various types of human HCC cells *in vitro*. We found that SH promoted cell cycle arrest in the G1 phase and sub-G1 formation, associated with the increased p21/WAF1/Cip1 expression. Additionally, SH induced caspase-dependent apoptosis, which involved the disruption of mitochondrial membrane potential, the increased release of cytochrome *c* and Omi/HtrA2 from the mitochondria into the cytoplasm, the downregulation of Bcl-2 and the upregulation of Bax, the activation of a caspase cascade (caspase-8, -10, -9 and -3) and PARP, as well as the decreased expression of survivin. The SH-suppressed growth of human HCC xenografts *in vivo* occurred due to the decrease in proliferation and the induction of apoptosis, implicating the activation of caspase-3, the upregulation of p21 and the downregulation of survivin. These findings suggest that SH exhibits anticancer efficacy *in vitro* and *in vivo* involving cell cycle and caspase-dependent apoptosis and may serve as a potential drug candidate against HCC.

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Introduction

Hepatocellular carcinoma (HCC) is the sixth most commonly diagnosed malignancy and the third leading cause of cancer-related mortality, with an estimated 748,000 new cases and 696,000 deaths worldwide in 2008 (1). At present, patients with intermediate-advanced HCC are eligible for palliative treatments including transcatheter arterial chemoembolization (TACE) and the oral multikinase inhibitor, sorafenib (2,3). However, treatment benefits are still limited and the development of more effective pharmacological agents is expected (4).

Sinomenium acutum Rehd. *et* Wils. (Fam. *Menispermaceae*), a Chinese medicinal herb, has been traditionally used for the treatment of various diseases, such as rheumatism, fevers and neuralgia for centuries (5). Sinomenine (7,8-didehydro-4-hydroxy-3,7-dimethoxy-17-methylmorphinane-6-one) is the most abundant active component isolated from this herb and has previously been demonstrated to exert anti-inflammatory, anti-rheumatic, immunosuppressive and analgesic effects (5-10). Sinomenine hydrochloride (SH), a hydrochloride chemical form of sinomenine (Fig. 1A), has been successfully used in clinical practice in China for treating rheumatism and neuralgia with minimal side-effects (11). Recently, a number of studies have reported the anti-neoplastic potential of SH *in vitro* against a variety of human tumor cells, including leukemia (12), synovial sarcoma (13) and cancers of lung (14) and prostate (15).

As is currently known, cancer initiation and development are attributed to the disruption of the normal balance between cell proliferation and cell death (16). Cell proliferation is regulated by the cell cycle, which is governed by a number of cyclin-dependent kinases (CDKs) and their pivotal inhibitor, p21/WAF1/Cip1 (p21) (17). Apoptosis is a key mode of programmed cell death and its distinctive morphological changes include membrane blebbing, cell shrinkage, chromatin condensation, DNA cleavage and apoptotic bodies (18). It has been well-established that, in most circumstances, apoptosis is regulated and executed by the activated cysteine-aspartic proteases (caspases). Two major signaling pathways initiate

and propagate caspase activation (19,20): the extrinsic (death receptor) pathway originates from the recognition between death receptors (Fas and tumor necrosis factor-receptor 1) and their ligands and results in the activation of caspase-8 and -10 (20). The intrinsic (mitochondrial) pathway is triggered by the disruption of the mitochondrial transmembrane potential ($\Delta\psi_m$), permeabilization of the outer membrane, release of apoptogenic proteins such as cytochrome *c* (Cyt *c*) from the mitochondrial intermembrane space into the cytoplasm and subsequent activation of caspase-9 (21). Mitochondrial apoptosis is regulated by anti-apoptotic Bcl-2 and pro-apoptotic Bax which localize in the outer mitochondrial membrane (22). The extrinsic and intrinsic pathways converge at the activation of caspase-3, a key executioner of apoptosis (23).

The anticancer effects of various therapeutic drugs involve cell cycle arrest and apoptosis induction (24,25). SH has been demonstrated to inhibit cell proliferation and induce apoptosis in a variety of human tumor cells (12-15). However, the potential anticancer effect of SH in HCC and the underlying molecular mechanisms remain to be investigated. In this study, we report that SH inhibits HCC growth *in vitro* and *in vivo* by promoting p21-associated cell cycle arrest and inducing caspase-dependent apoptosis.

Materials and methods

Chemicals and reagents. Primary antibodies against poly(ADP-ribose) polymerase (PARP), caspase-3, -8, -9, -10 and Omi/HtrA2 were purchased from Cell Signaling Technology, Inc. Primary antibodies against proliferating cell nuclear antigen (PCNA), survivin, Cyt *c*, Bcl-2, Bax and p21 were obtained from Santa Cruz Biotechnology, Inc. Matrigel was obtained from BD Biosciences. The general caspase inhibitor, Z-VAD-FMK, was purchased from R&D Systems. SH (98% purity), provided by the Hunan Zhengqing Pharmaceutical Group Co. Ltd., was directly dissolved in DMEM containing 10% fetal bovine serum for *in vitro* studies and formulated in physiological saline for *in vivo* studies.

Cell culture. The human HCC cell lines, Hep3B, SMMC7721, HepG2, HHCC, Bel7402, MHCC97-H and MHCC97-L, were obtained from the Shanghai Institutes for Biological Sciences (SIBS). Cells were grown in DMEM supplemented with 10% fetal bovine serum and maintained in an incubator with a humidified atmosphere containing 5% CO_2 at 37˚C.

MTT assay for cell viability. We conducted 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays as recommended by the manufacturer. Briefly, exponentially growing cells were trypsinized, seeded at an initial density of $8x10^3$ per well in 96-well plates and incubated in standard growth medium for 24 h prior to being subjected to different treatments for the indicated periods of time. Cells were subsequently incubated in medium containing MTT (0.5 mg/ml) for an additional 4 h. The supernatant was removed and 150 μl DMSO were added to each well to dissolve the intracellular formazan compound. The absorbance was measured with an ELISA reader (Bio-Rad, Hercules, CA, USA) at 490 nm. The relative cell viability (%) was calculated as the percentage between SH-treated cells and the untreated controls.

Cell cycle distribution analysis. Cells were grown in 6-cm dishes at a density of $0.5x10^6$ cells/ml and treated with the desired concentrations of SH in complete medium for 48 h. Cells were then collected and fixed in 70% ethanol at -20˚C overnight. After being suspended in 0.5 ml of propidium iodide (PI) staining solution (50 μg/ml PI, 100 μg/ml RNase and 0.2% Triton X-100 in PBS) for 30 min at room temperature in the dark, cell cycle distribution was determined by a FACSCalibur flow cytometer (Becton-Dickinson).

Western blot analysis. Proteins were prepared as described previously (26). After protein quantification, equal amounts of protein (~30-60 μg) were electrophoretically separated by SDS-PAGE with 12% Tris-Glycine gels and transferred onto nitrocellulose membranes. After being blocked with 5% non-fat dry milk in TBST buffer (weight/volume) for 1 h, the membranes were incubated with the primary antibodies at 4˚C overnight, followed by incubation with a suitable horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h. Immunoreactive bands on the membranes were developed using an electrogenerated chemiluminescence (ECL) detection system followed by exposure to X-ray film.

Hoechst staining. Hoechst staining was performed using an Apoptosis-Hoechst Staining kit (Beyotime), according to the manufacturer's instruction. Exponentially growing cells were seeded in 6-multiwell plates at a density of $5x10^5$ cells per well, maintained in culture overnight and exposed to SH at 2 μmol/ml for 48 h. Cells were fixed with 4% paraformaldehyde, stained with Hoechst 33528 and then visualized under a fluorescence microscope (Olympus).

DNA fragmentation assay and quantitative analysis for apoptosis. Cells were plated on 6-cm dishes at a density of $0.5x10^6$ cells/ml, grown for 24 h and treated with increasing concentrations of SH for 48 h. Cells were then collected and centrifuged. For the detection of DNA ladders, intracellular DNA was extracted using an Apoptosis DNA Ladder Detection kit (Nanjing KeyGen Biotech. Co. Ltd.) according to the manufacturer's instructions. DNA samples were dissolved in TE buffer (pH 8.0), separated by electrophoresis on a 1.5% agarose gel with ethidium bromide (0.5 μg/ml) and then photographed using a molecular imager (Bio-Rad). Quantitative analysis of apoptosis by flow cytometry was assessed using an Annexin V-FITC Apoptosis Assay kit (Joincare Biosciences, Zhuhai, China), as described in the manufacturer's recommendations. At least 10,000 cells were analyzed for each sample.

Mitochondrial membrane potential measurement. We examined $\Delta\psi_m$ using a Mitochondrial Membrane Potential Assay kit with JC-1 (Beyotime) as described in the manufacturer's instructions. Cells at a density of $0.5x10^6$ cells/ml were seeded on 6-cm dishes, grown for 24 h, treated with different concentrations of SH for 48 h and stained with JC-1, followed by flow cytometry for the quantitative analysis of $\Delta\psi_m$. At least 10,000 events per sample were recorded.

Preparation of cytosolic fractionation. Briefly, cells were lysed with permeabilization buffer (250 mmol/l sucrose, 20 mmol/l HEPES/KOH (pH 7.4), 1 mmol/l EGTA, 1 mmol/l EDTA,

1 mmol/l DTT, 0.1 mmol/l PMSF, 1 μg/ml chymostatin, 1 μg/ml leupeptin, 1 μg/ml antiparin and 1 μg/ml pepstatin A). The lysates were centrifuged at 500 x g for 10 min (4˚C). The supernatants were further centrifuged at 13,000 x g for 30 min (4˚C) and the supernatants containing cytosolic fraction were collected.

Caspase-3 activity assay. The activity of caspase-3 was assessed using a Caspase Colorimetric Assay kit (R&D Systems), following the instructions provided by the manufacturer. Briefly, cells were washed with ice-cold PBS and lysed in a lysis buffer. After being centrifuged at 10,000 x g for 10 min (4˚C), cell lysates were incubated with 50 μl of reaction buffer and 5 μl of caspase-3 colorimetric substrate (DEVD-p-nitroaniline) for 2 h at 37˚C. The optical density of the color reaction was spectrophotometrically measured at 405 nm.

Tumor xenograft study. Four-week-old male BALB/c athymic nude mice, weighing 18-23.5 g, were obtained from Shanghai SLAC Laboratory Animal Co. Ltd. (Shanghai, China) and housed in sterile laminar flow rooms with 12-h light and dark cycles at a temperature range of 19-23˚C and a humidity of 40-60% in the Laboratory Animal Centre of Xi'an Jiaotong University. All experimental procedures were conducted in accordance with the institutional guidelines for conduct and animal welfare. Mice were subcutaneously injected with $5x10^6$ of human HCC Hep3B cells suspended in 100 μl of mixture containing serum-free DMEM and matrigel (5:1) in the right hind legs. Thirty mice were randomly divided into the following 4 groups: control group (n=6), SH 50 mg/kg group (n=8), SH 100 mg/kg group (n=8) and SH 150 mg/kg group (n=8). After 24 h, mice in the control group were intraperitoneally (i.p.) injected with 0.1 ml of physiologic saline per 10 g body weight daily and mice in other 3 groups were i.p. injected with 50, 100 and 150 mg/kg doses of SH in 0.1 ml of saline daily for 24 days. Mice and their average food consumption were weighed daily to determine the effects of SH on their general health. Tumor size was also measured daily with vernier caliper and tumor volume was calculated as 0.5236 x length x width x width and expressed as mm^3. At the end of the experiment, mice were sacrificed and tumors were immediately removed, weighed and then fixed in 4% paraformaldehyde.

Immunohistochemistry staining. Paraformaldehyde-fixed tissues were embedded in paraffin blocks and cut into 4-μm sections. Haematoxylin and eosin (H&E) staining was conducted according to conventional procedures. Immunohistochemistry staining was carried out using a Dako Autostainer (Dako, Carpinteria, CA, USA) as described previously (27). Briefly, the sections were dewaxed, rehydrated, subjected to antigen retrieval by pressure cooking for 5 min and incubated with serum for 15 min to block endogenous enzyme and non-specific antigens. The sections were incubated with appropriately diluted primary antibodies at 4˚C overnight and then with DakoCytomation EnVision-HRP reagent for 30 min, followed by incubation with diaminobenzidine (DAB) in a dark room. After counterstaining with hematoxylin, the sections were then dehydrated. The relative protein expression was evaluated by the average percentage of positive cells (number of positive cells x100/total number of cells) in 5 different random microscopic fields (x400) in each tumor sample.

In situ apoptosis detection by TUNEL staining. Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) assay was performed using an *In Situ* Cell Death Detection kit, POD (Roche Diagnostics) according to the protocol supplied by the manufacturer. The average apoptotic index of each tumor sample was calculated as described above.

Statistical analysis. SPSS 16.0 was used to analyze statistical data. Data are expressed as the means ± SE. Statistical comparisons were performed using one-way ANOVA or the Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

SH inhibits growth of human HCC cells. The effect of SH on the growth of HCC cells were evaluated using MTT assay. We employed 7 HCC cell lines representing different degrees of differentiation and from diverse genetic backgrounds to investigate the extent at which SH inhibits the growth of HCC cells. In all the HCC cell lines, treatment with SH at 0.5, 1 and 2 μmol/ml for 24, 48 and 72 h significantly inhibited cell growth in a concentration- and time-dependent manner (Fig. 1B-H).

SH induces cell cycle arrest and upregulates p21 protein expression in human Hep3B and SMMC7721 HCC cells. We hypothesized that the SH-induced cell growth inhibition may be associated with the modulation of cell cycle progression. To determine this possibility, following SH treatment, the cells were stained with PI and then subjected to flow cytometry to analyze cell cycle distribution. In the Hep3B and SMMC7721 cells, it was shown that an accumulation of cells in the G1 phase, accompanied by a decrease in the number of cells in the S phase and an increase in the number of cells in the sub-G1 phase, was caused by SH in a concentration-dependent manner following 48 h of treatment (Fig. 2A). To elucidate the mechanism of the cell cycle arrest induced by SH, we further investigated the effect of SH on the cell cycle regulatory protein, p21. Our results revealed that the protein level of p21 was significantly elevated following 48 h of SH treatment in a concentration-dependent manner (Fig. 2B).

SH induces apoptosis in Hep3B and SMMC7721 cells. We then assessed whether SH causes apoptotic cell death in Hep3B and SMMC7721 cells. As shown in Fig. 3A, significant morphological changes associated with apoptosis, e.g., formation of condensed and fragmented nuclei, were induced by SH. The formation of a DNA ladder pattern, a biochemical characteristic of apoptosis indicating internucleosomal DNA fragmentation, was markedly detected in a concentration-dependent manner (Fig. 3B). Correspondingly, the increase in Annexin V-positive cells, another feature of apoptosis, was also observed in the SH-treated cells. As shown in Fig. 3C, treatment with 0, 0.5, 1 and 2 μmol/ml of SH for 48 h induced 7.37, 11.72, 22.22 and 48.52% of the apoptotic cell population in the Hep3B cells and 7.45, 37.93, 49.76 and 59.90% in the SMMC7721 cells, respectively.

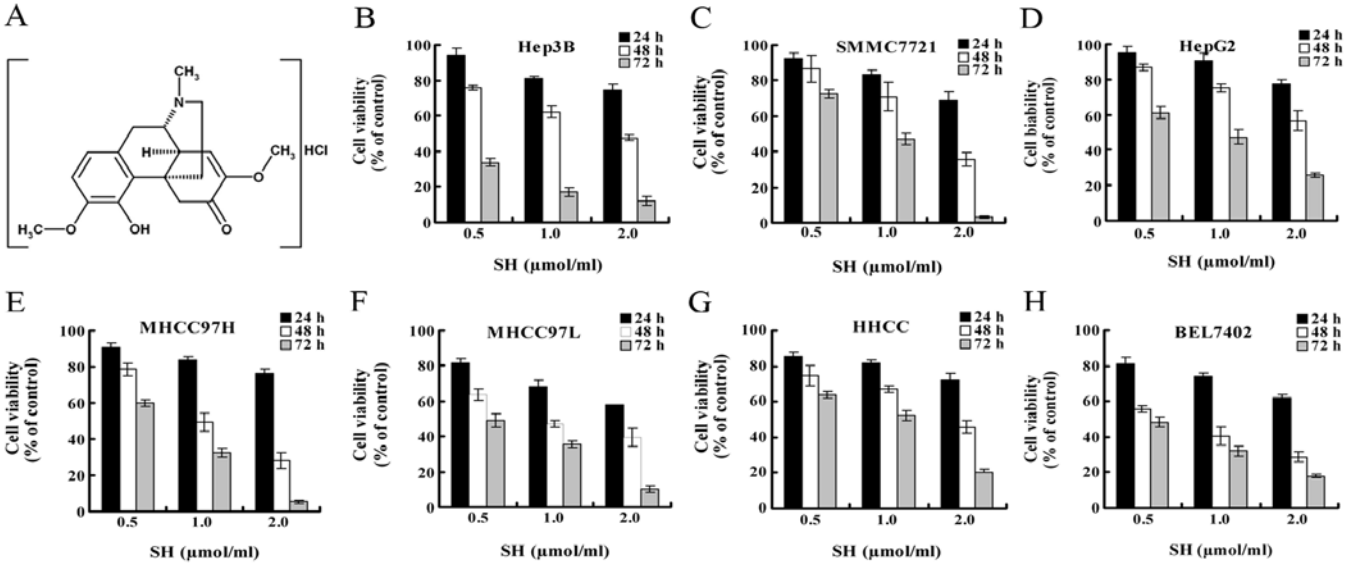

Figure 1. SH inhibits cell growth in a panel of human HCC cell lines. (A) Chemical structure of SH. (B-H) Cell viability rate after SH treatment as compared with the controls. Exponentially growing human HCC cells were seeded in 96-well plates and treated with various concentrations of SH for 24, 48 and 72 h, followed by MTT assay. Data represent the means ± SE of 3 independent measurements. SH, sinomenine hydrochloride; HCC, hepatocellular carcinoma.

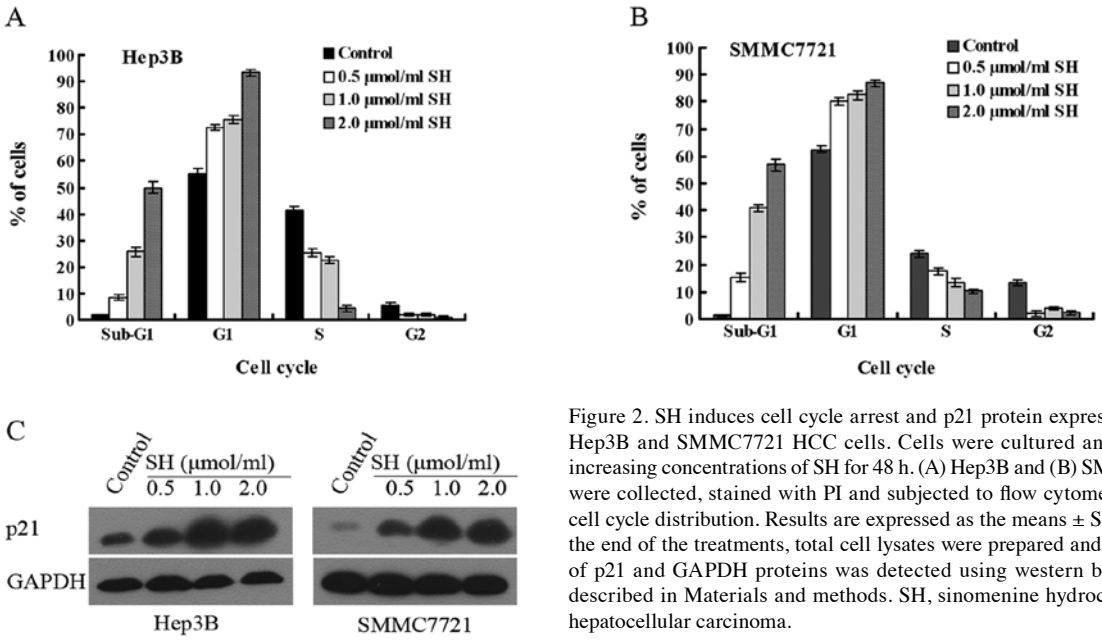

Figure 2. SH induces cell cycle arrest and p21 protein expression in human Hep3B and SMMC7721 HCC cells. Cells were cultured and treated with increasing concentrations of SH for 48 h. (A) Hep3B and (B) SMMC7721 cells were collected, stained with PI and subjected to flow cytometry to analyze cell cycle distribution. Results are expressed as the means ± SE (n=3). (C) At the end of the treatments, total cell lysates were prepared and the expression of p21 and GAPDH proteins was detected using western blot analysis as described in Materials and methods. SH, sinomenine hydrochloride; HCC, hepatocellular carcinoma.

SH disrupts $\Delta\psi_m$, promotes the release of Cyt c and Omi/HtrA2 from the mitochondria and decreases the Bcl-2/Bax ratio in Hep3B and SMMC7721 cells. As the mitochondria play a key role in propagating apoptotic signaling, we examined the effect of SH on $\Delta\psi_m$ by JC-1 staining. Quantitative analysis using flow cytometry revealed that the percentage of cells with collapse of $\Delta\psi_m$ increased following treatment with SH in a concentration-dependent manner in the Hep3B and SMMC7721 cells (Fig. 4A). Additionally, we found that SH treatment gradually increased the levels of apoptogenic Cyt *c* and Omi/HtrA2 proteins in the cytosolic fraction in a concentration-dependent manner (Fig. 4B). As the altered Bcl-2/Bax ratio is a precursor for the release of apoptogenic proteins, we further examined whether the Bcl-2/Bax ratio decreased upon SH treatment in HCC cells. As shown in Fig. 4C, SH treatment resulted in an upregulation of Bax protein and a downregulation of Bcl-2 protein expression in a concentration-dependent manner.

SH induces apoptosis through caspase-dependent pathway in human Hep3B and SMMC7721 HCC cells. Based on the above findings, we then determined whether the activation of a caspase cascade is involved in SH-induced apoptosis in Hep3B and SMMC7721 cells. Western blot analysis showed that SH treatment decreased the protein levels of precursors of caspase-8 and -10 and increased the levels of cleaved caspase-9, caspase-3 and PARP, a known substrate of caspase-3 (23,28), in a concentration-dependent manner (Fig. 5A). To gain an insight into the contribution of caspases to SH-induced apop-

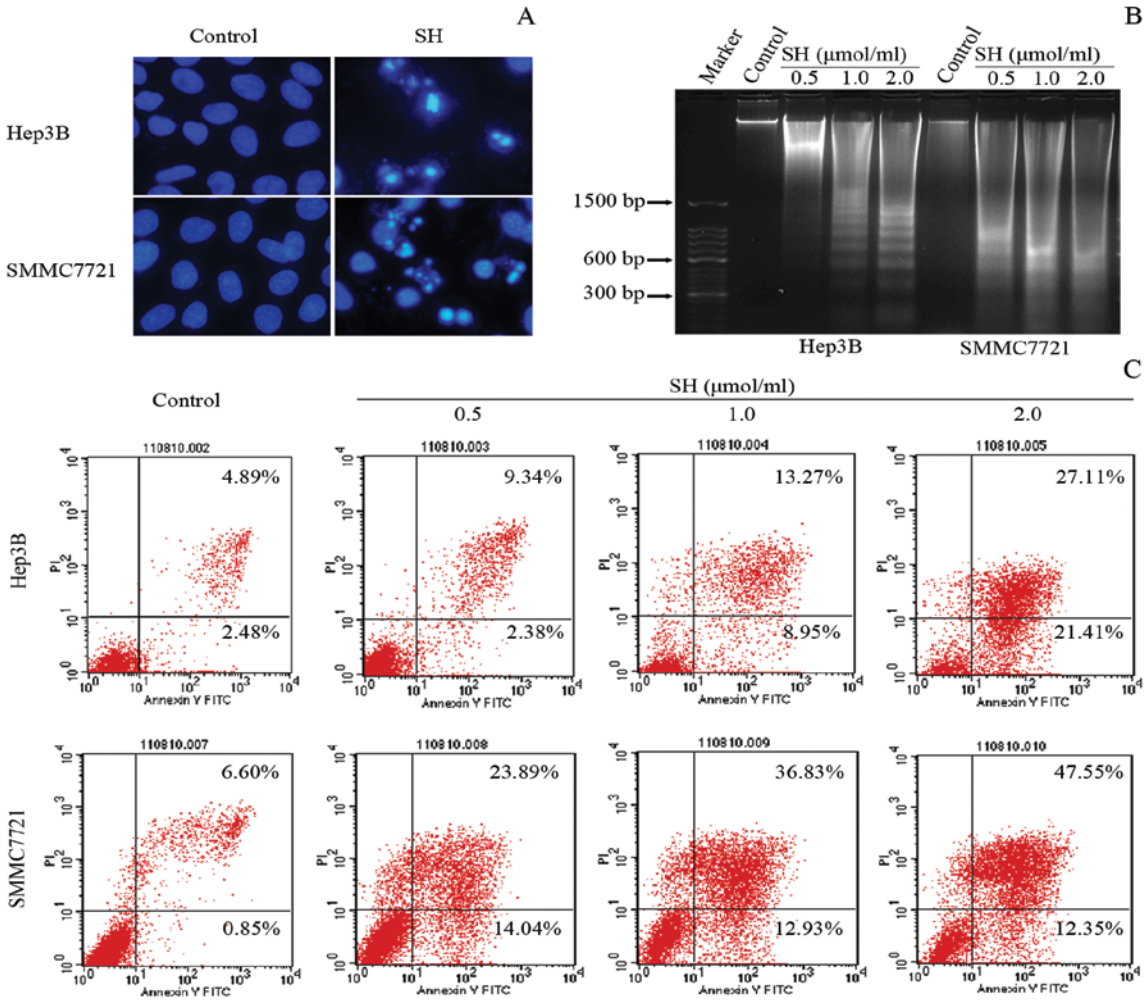

Figure 3. SH induces apoptosis in a concentration-dependent manner in human Hep3B and SMMC7721 HCC cells. Cells were cultured and treated with various concentrations of SH for 48 h. (A) Cells untreated or treated with 2 μmol/ml of SH were fixed and stained with Hoechst 33528 to detect the nuclear morphologic changes under a fluorescence microscope. Magnification, x400. (B) DNA was isolated and subjected to agarose gel analysis of DNA ladder formation. (C) Cells were collected, stained with Annexin V and PI and subjected to flow cytometry for the quantitative analysis of apoptosis. PI, propidium iodide; SH, sinomenine hydrochloride; HCC, hepatocellular carcinoma.

tosis, cells were pre-treated with a general caspase inhibitor, Z-VAD-FMK, prior to SH treatment. We confirmed that pre-treatment with Z-VAD-FMK efficiently inhibited SH-induced caspase activation, which was revealed by the reduction in caspase-3 activity (Fig. 5B) and PARP cleavage (Fig. 5C). As expected, Z-VAD-FMK efficiently suppressed SH-induced apoptosis as shown by flow cytometry analysis (Fig. 5D). These results suggest that the SH-induced apoptosis in HCC cells depends on the activation of a caspase cascade.

SH downregulates the expression levels of survivin protein. As a key regulator of mitosis and apoptosis, survivin inhibits p21 expression and caspase activation and plays an important role in HCC tumor cell proliferation and survival (29-32). We therefore anticipated that SH may decrease the expression levels of survivin in Hep3B and SMMC7721 cells. As shown in Fig. 6, compared with the untreated controls, treatment with SH for 48 h significantly caused a concentration-dependent decrease in the level of survivin protein expression.

SH attenuates growth of human HCC subcutaneous xenografts in athymic nude mice. To further evaluate the antitumor activity of SH, we conducted an *in vivo* study with an athymic nude mice xenograft model by subcutaneous inoculation of human Hep3B HCC cells. All animals survived throughout the experiment. Fig. 7A shows that SH at the doses of 50, 100 and 150 mg/kg for 24 days caused a 5.51, 25.10 ($P<0.05$) and 50.01% ($P<0.05$) inhibition of tumor volume, respectively, as compared with the controls. Similarly, the tumor weight per mouse in the 50, 100 and 150 mg/kg SH-treated groups was 4.56, 23.89 ($P<0.05$) and 43.79% ($P<0.05$) less, respectively, than that in the control group treated with the vehicle (Fig. 7B). We did not find any gross sign of toxicity following SH administration, demonstrated by no obvious changes in body weight and dietary consumption throughout the study (Fig. 7C and D).

SH inhibits cell proliferation, induces apoptosis and modulates the expression levels of p21, cleaved caspase-3 and survivin proteins in Hep3B xenografts. The i*n vivo* anti-proliferative

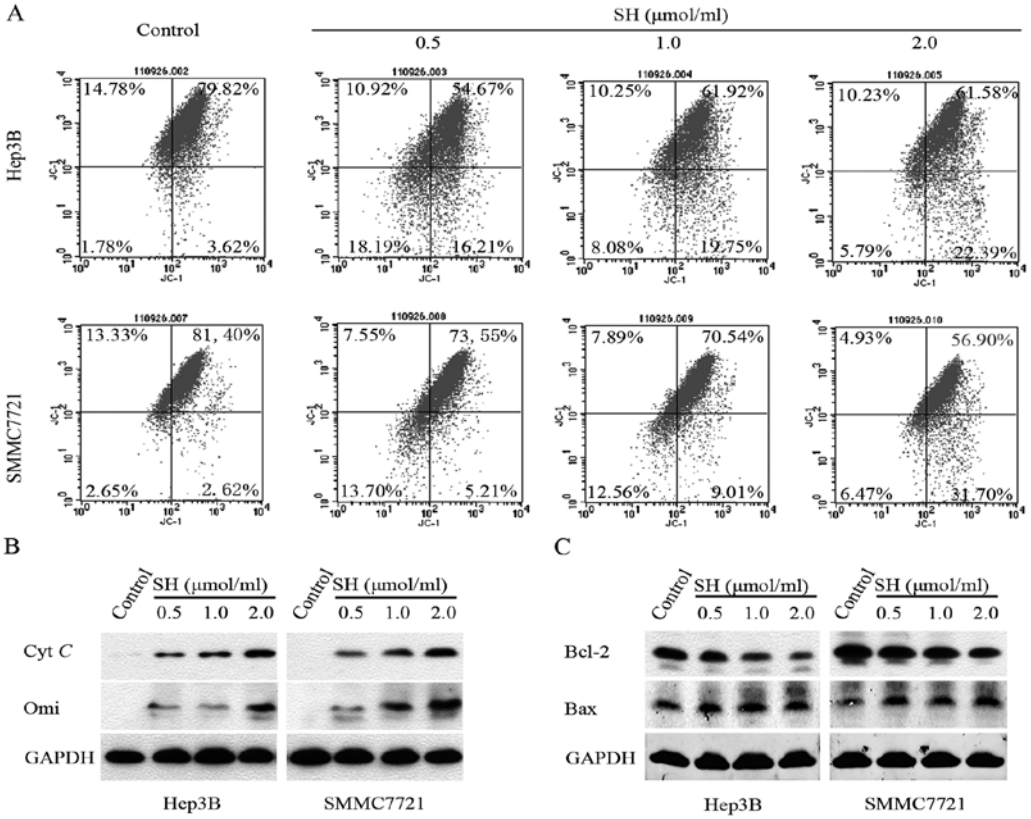

Figure 4. SH activates mitochondrial events in human Hep3B and SMMC7721 HCC cells. (A) SH disrupts. Cells were cultured, treated with SH at various concentrations for 48 h and stained with JC-1, followed by quantitative analysis using flow cytometry. (B) SH promotes the release of Cyt *c* and Omi/HtrA2 from the mitochondria into the cytoplasm. Cytosolic fraction was prepared and the expression of Cyt *c* and Omi/HtrA2 proteins was assessed using western blot analysis. GAPDH was used as the internal control. (C) SH decreases Bcl-2 and increases Bax. The expression of GAPDH, Bcl-2 and Bax proteins in total cell lysates was assessed using western blot analysis. SH, sinomenine hydrochloride; HCC, hepatocellular carcinoma; $\Delta\psi_m$, mitochondrial membrane potential.

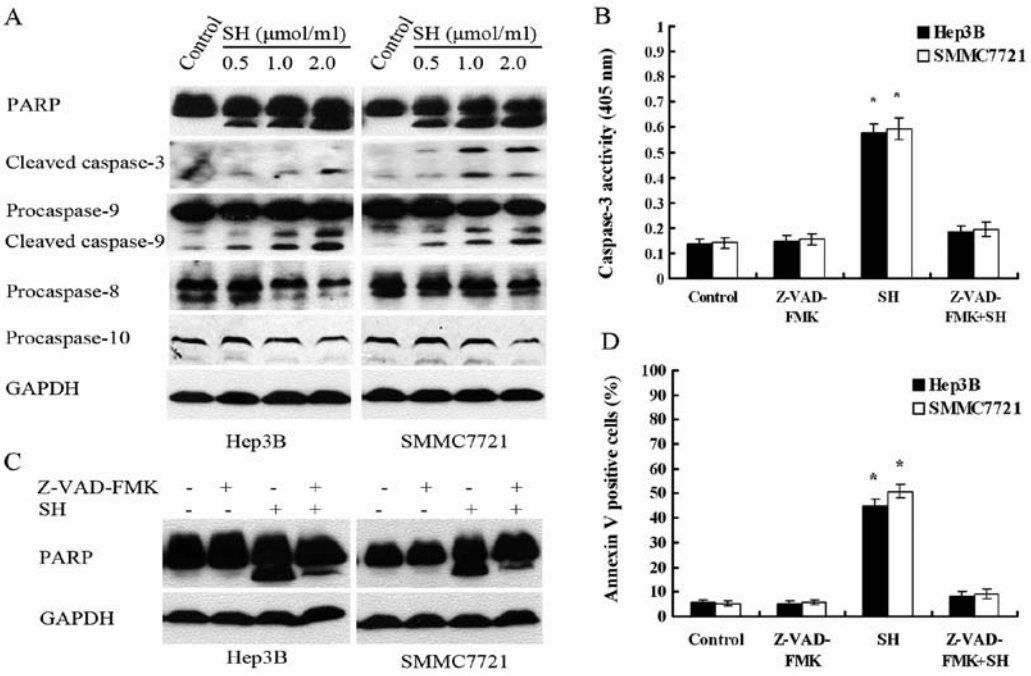

Figure 5. The activation of caspases is required for SH-induced apoptosis in human HCC Hep3B and SMMC7721 cells. (A) Cells were treated with SH at various concentrations, total cell lysates were prepared and the cleavage of PARP and caspase-3, -9, -8 and -10 were detected using western blot analysis. (B) Cells were pre-treated with 40 μmol/l of Z-VAD-FMK for 1 h, followed by exposure to 2 μmol/ml of SH for 48 h. The activity of caspase-3 in cell extracts was colorimetrically quantified as detailed in Materials and methods. (C) PARP cleavage in total cell lysates was detected using western blot analysis and (D) quantitative analysis of apoptotic cells was determined using flow cytometry. Data are expressed as the mean ± SE of 3 independent measurements. *P<0.05 compared with the control. SH, sinomenine hydrochloride; HCC, hepatocellular carcinoma.

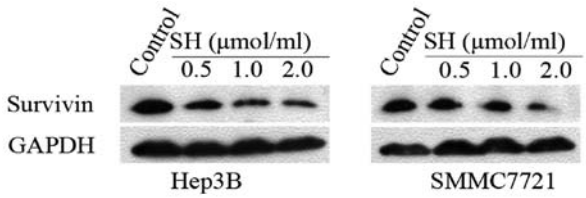

Figure 6. SH decreases survivin protein expression in human Hep3B and SMMC7721 HCC cells. Cells were cultured and treated with increasing concentrations of SH. Total cell lysates were prepared and analyzed for the level of survivin protein expression using western blot analysis. GAPDH was used as the internal control. SH, sinomenine hydrochloride; HCC, hepatocellular carcinoma.

effect of SH treatment on HCC xenografts was investigated by PCNA immunostaining. Qualitative analysis showed that the number of PCNA-positive cells decreased from 82.2% in the control group to 78.5, 57.7 ($P<0.05$) and 38.9% ($P<0.05$) in the 50, 100 and 150 mg/kg SH-treated groups, respectively (Fig. 8A-C). TUNEL staining was performed to assess the *in vivo* apoptosis induction by SH administration. The number of apoptotic cells increased from 3.2% in the control group to 4.1, 14.7 and 32.1% ($P<0.05$) in the 50, 100 and 150 mg/kg SH-treated groups, respectively (Fig. 8D-F). Correspondingly, SH treatment at the doses of 100 and 150 mg/kg increased the expression levels of p21 and cleaved caspase-3 proteins, as compared with the control and 50 mg/kg SH-treated groups (Fig. 8G-L). Additionally, as expected, the decreased number of survivin-stained cells was also observed in the tumor samples from the SH-treated groups (Fig. 8M-O). Overall, these data demonstrate the potential molecular mechanisms by which SH exerts its *in vivo* anti-neoplastic effect on HCC.

Discussion

Available evidence suggests that some anti-inflammatory drugs, such as aspirin act as cancer preventive and therapeutic agents (33,34). As a drug with the potent anti-inflammatory and immunosuppressive efficacy against arthritis in clinical practice, SH has been found to exhibit the *in vitro* anti-neoplastic activities against a variety of tumor cell lines (12-15). However, to date, its effect on HCC remains unknown. In the present study, our findings demonstrated that SH treatment caused the growth inhibition of human HCC cell lines in culture *in vitro* in a concentration- and time-dependent manner, as well as that of xenografts *in vivo* in nude mice, associated with the arrest of cell cycle progression and the induction of apoptosis. Furthermore, the detailed molecular mechanisms may involve the increased protein expression of p21, the activation of mitochondrial events and a caspase cascade, as well as the downregulation of survivin.

p21, a key member of CDK inhibitors, has been proven to cause cell cycle arrest by universally binding to the CDK-cyclin complexes. Consistent with this fact, we found that SH promoted cell cycle arrest by increasing the protein expression of p21 in HCC cells. p21 is mainly regulated by the p53 tumor suppressor

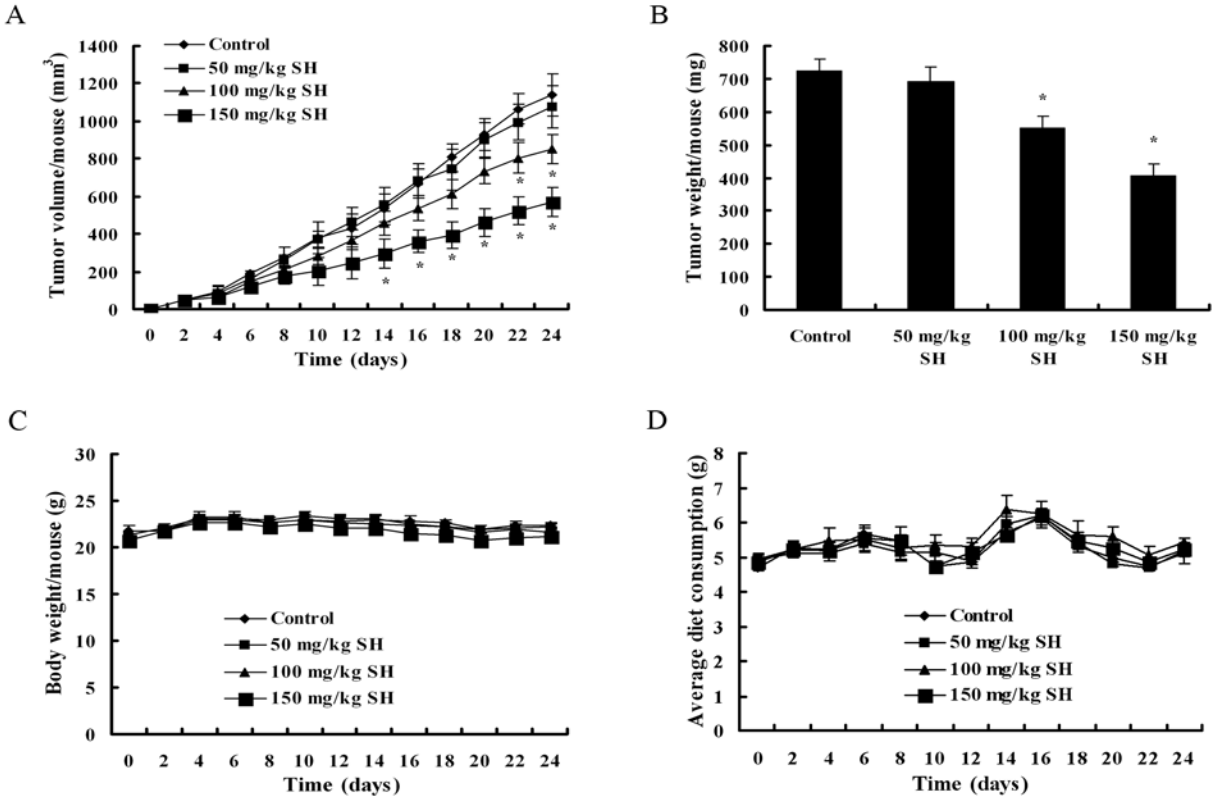

Figure 7. SH exerts *in vivo* anticancer effect on HCC xenograft model. Human Hep3B HCC cells ($5x10^6$) were subcutaneously injected in the right hind legs of athymic nude mice. Saline (control) or various doses of SH (50, 100 and 150 mg/kg) were administered by intraperitoneal (i.p.) injection daily for 24 days. Effects of SH on (A) tumor volume, (B) tumor weight, (C) body weight and (D) dietary consumption were recorded as described in Materials and methods. Data shown are the means of mice in each group. *$P<0.05$ compared with the control group. Bars, SEs; SH, sinomenine hydrochloride; HCC, hepatocellular carcinoma.

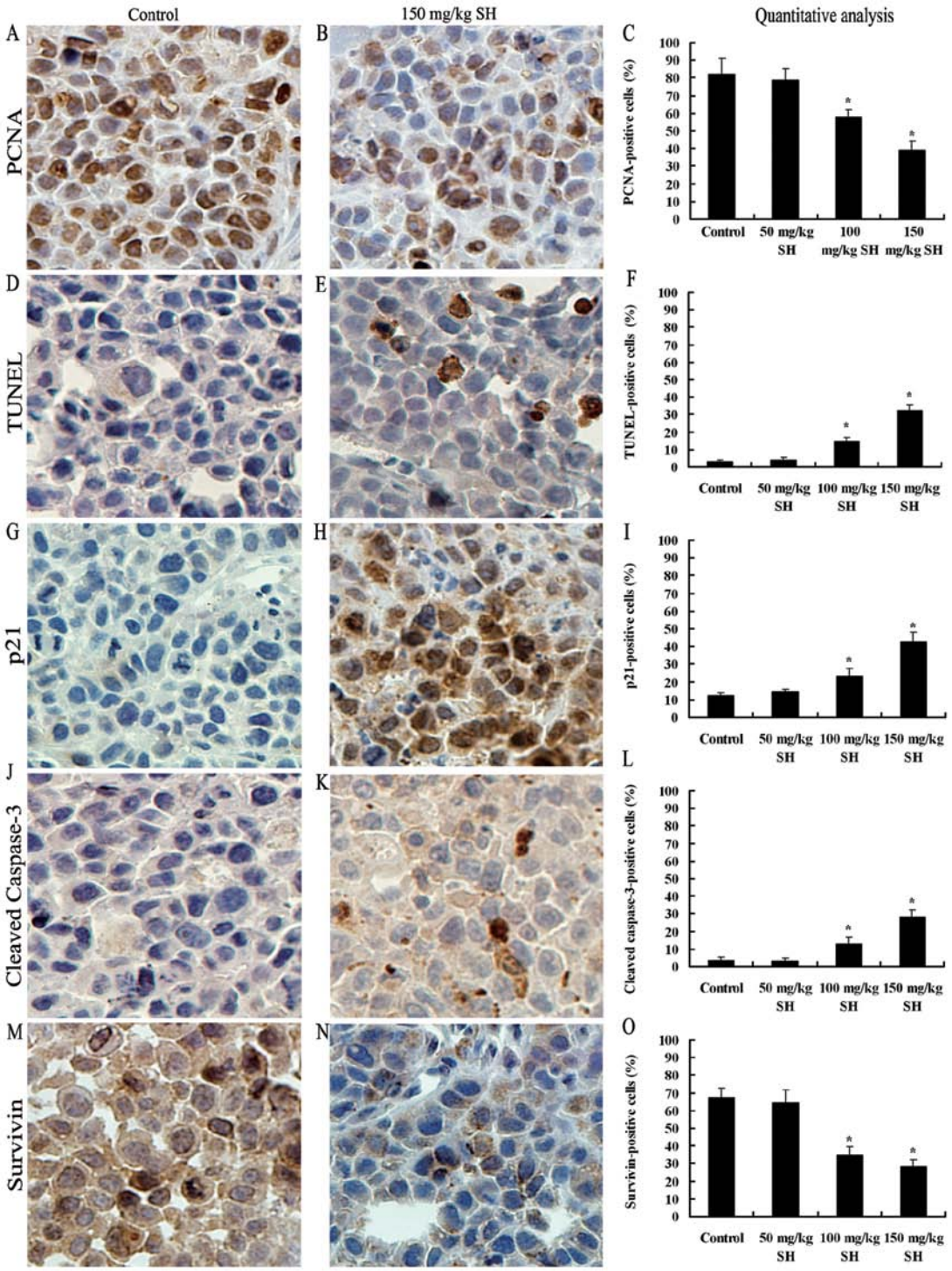

Figure 8. SH inhibits cell proliferation, induces apoptosis and modulates the protein expression of p21, cleaved caspase-3 and survivin in Hep3B tumor xenografts. Tumors were cut into sections and immunohistochemistry staining was performed. Representative images of PCNA, TUNEL, p21, cleaved caspase-3 and survivin staining at x400 magnification from the control and 150 mg/kg SH-treate4d groups are shown in (A and B), (D and E), (G and H), (J and K) and (M and N), respectively. The average percentage of (C) PCNA-positive cells, (F) TUNEL-positive cells, (I) p21-positive cells, (L) caspase-3-positive cells and (O) survivin-positive cells was counted at x400 magnification in 5 randomly selected areas in each tumor sample. Data are expressed as the means ± SE of mice in each group. *P<0.05 compared with the control group. Bars, SEs; SH, sinomenine hydrochloride; HCC, hepatocellular carcinoma.

protein. However, approximately 50% of human cancers, including HCC harbor p53 mutations (35,36). Our data suggest that the SH-induced p21 upregulation involves the p53-independent pathway, as Hep3B cells are deficient in functional p53 (37) and no increase in p53 protein expression was observed in the SH-treated SMMC7721 cells in which the *p53* gene is wild (data not shown).

The disturbance in the balance between cell proliferation and apoptosis is responsible for cancer development (16). As a result, chemotherapeutic agents mostly exert anticancer

effects by the induction of apoptosis (21). In the present study, SH induced-apoptosis was confirmed by the characteristic morphological changes, DNA ladder formation and increase in Annexin V-labeled cells in a concentration-dependent manner in human Hep3B and SMMC7721 HCC cells.

The transduction of an apoptotic signal into cells may alter the permeability of the membranes of the mitochondria and $\Delta\psi_m$, which results in the translocation of apoptogenic proteins, such as Cyt *c* and Omi/HtrA2 from the intra-membrane space into the cytoplasm (38). Cytosolic Cyt *c* activates caspase-9, which subsequently leads to the activation of caspase-3 (19). As an antagonist of inhibitors of apoptosis (IAPs), Omi/HtrA2 can competitively displace caspases from IAPs and abolish the caspase-inhibitory effect of IAPs (39,40). In the present study, SH treatment disrupted $\Delta\psi_m$, which was measured as the fluorescence intensity ratio of JC-1 red J-aggregates/green monomers and caused a concomitant increase in Cyt *c* and Omi/HtrA2 levels in the cytosolic fraction. Furthermore, mitochondrial apoptosis induced by pro-apoptotic Bax can be prevented by anti-apoptotic Bcl-2 by inhibiting the release of Cyt *c* (22). We found that SH treatment decreased Bcl-2 and increased Bax, which further demonstrate the role of the mitochondria in SH-induced apoptosis.

The cleavage of caspase-9, caspase-3 and PARP was observed in this study. Moreover, procaspase-8 and -10 levels were decreased following SH treatment, indicating the activation of caspase-8 and -10. These results suggest that the extrinsic death receptor and intrinsic mitochondrial pathways participate in SH-induced apoptosis. It would be of interest to investigate whether the activation of caspase-8 and -10 is responsible for the activation of the mitochondrial pathway in SH-induced apoptosis and to examine whether SH affects Fas-associated proteins. Our results further demonstrate that the SH-induced apoptosis depends on caspase activation since the general caspase inhibitor, Z-VAD-FMK, at a concentration that blocks the activation of caspase-3 and the cleavage of PARP, prevents SH-induced apoptosis.

As a member of the IAP family, survivin is almost absent in normal adult tissues, but abundantly overexpressed in the majority of human cancers including HCC, and is associated with tumor progression, treatment failure and poor prognosis (29,30). Survivin can inhibit the function of p21 and the activation of caspases and is a target for certain anticancer drugs, such as silibinin and silymarin (26,30-32,41,42). We found that SH decreased the expression level of survivin protein, which might contribute to the increase of p21 and activation of caspases induced by SH.

A number of studies have shown that the histamine-releasing properties of sinomenine are possibly responsible for its anti-rheumatic effect (5). Of note, Yang *et al* recently reported that histamine decarboxylase-knockout mice exhibit a significantly increased rate of colon and skin carcinogenesis, which is attributed to the increase in immature myeloid cells (IMCs). IMCs can promote the growth of cancer xenografts and exogenous histamine can induce the differentiation of IMCs to inhibit tumor growth (43). Additionally, it has been reported that SH inhibits angiogenesis *in vitro* and *in vivo* (44). Therefore, the histamine release and anti-angiogenic effects induced by SH may also contribute to its inhibitory effect on the growth of HCC xenografts in nude mice in our present study. It would of interest to investigate whether SH induces its chemopreventive and chemotherapeutic effects by promoting the release of histamine and suppressing angiogenesis and to elucidate the correlation between SH-released histamine and the signaling molecules involved in SH-induced apoptosis in this study.

In conclusion, SH suppresses the growth of human HCC cells *in vitro* and *in vivo*, which may be possibly attributed to cell cycle arrest and apoptosis induction. SH upregulates p21, downregulates the Bcl-2/Bax ratio, promotes the release of Cyt *c* and Omi/HtrA2 from the mitochondria into the cytoplasm, disrupts $\Delta\psi_m$ and induces the cleavage of caspases. Additionally, survivin is a potential molecular target of SH. The unique properties of SH may render it a promising candidate in the prevention and therapy of HCC. However, multidisciplinary approaches are required to improve its anticancer activity.

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