

Sasanquasaponin from *Camellia oleifera* Abel. induces apoptosis via Bcl-2, Bax and caspase-3 activation in HepG2 cells

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Abstract. The present study aimed to elucidate the molecular mechanisms underlying the induction of cytotoxic effects by sasanquasaponin (SQS) in HepG2 cells. Following SQS treatment, time- and dose-dependent increases in the apoptotic rate were observed. The induction of cell death by SQS mainly occurs via programmed cell death, as indicated by Annexin V-fluorescein isothiocyanate and propidium iodide staining, where up to 30% apoptotic cells were detected following 12 h SQS treatment. Reverse transcription-polymerase chain reaction analysis demonstrated that SQS treatment upregulated B-cell lymphoma-2 (Bcl-2)-associated x protein and caspase-3 mRNA expression and downregulated Bcl-2 mRNA expression. Greater alterations in Bax, Bcl-2 and caspase-3 expression were observed with increasing treatment duration. The decrease in Bcl-2, increase in Bax and, finally, the activation of caspase-3 in HepG2 cells indicated that the apoptotic process induced by SQS was irreversible. The results of the present study therefore suggested that SQS induced HepG2 cell apoptosis via the activation of mitochondrial apoptotic pathways.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common and aggressive malignancy worldwide, accounting for >90% of all primary liver cancers and representing the third leading cause of liver cancer mortality (1). Annually, HCC is responsible for ~1 million mortalities worldwide (2). HCC is also known as adult primary liver cancer and develops from an abnormal mass of tumor nodules, metastasizing to adjoining parts of the liver and resulting in malignancy (3). An

increasing body of evidence has suggested that >0.5 million novel cases of HCC are diagnosed annually (4). Patient prognoses following surgical resection of HCC remain poor due to the high rate of recurrence and dearth of effective adjuvant therapies (5). Tumor recurrence occurs in >70% of cases within five years (6), and the five-year survival rate is just 60-70%. The clinical therapies currently available require extensive immunosuppressive pre-treatments, which frequently result in undesirable physiological side-effects (7). The identification of patients with a higher risk of recurrence and the development of more effective and targeted therapeutic strategies are required in order to improve patient prognoses. Therefore, the development of novel therapeutic strategies for the treatment of HCC, with fewer adverse effects, is urgently required for the improvement of clinical practices and HCC patient prognosis.

Camellia oleifera (*C. oleifera*) Abel. is cultivated in numerous regions throughout China (8). *C. oleifera* seeds are used in the production of oil. Sasanquasaponin (SQS) is a biologically active ingredient, which may be extracted from *C. oleifera* Abel. SQS has numerous pharmacological properties, including anti-inflammatory, anti-hyperlipidemic and anti-effusive effects (9). Studies have revealed that SQS is able to induce apoptosis in human leukemia Jurkat cells and HepG2 hepatoma cells (10,11). The present study therefore aimed to determine whether SQS was able to induce cell cycle arrest and apoptosis in the HepG2 HCC cell line, and investigate the potential mechanisms underlying these effects.

Materials and methods

Materials and reagents. RPMI-1640, fetal bovine serum (FBS), penicillin-streptomycin, Trypsin-EDTA and TRIzol were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). SuperScript II reverse transcriptase was obtained from Promega Corp. (Madison, WI, USA). B-cell lymphoma 2 (Bcl-2), Bcl-2-associated x protein Bax and β -actin primary antibodies, as well as horseradish peroxidase-conjugated secondary antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). 5-FU was obtained from Zhejiang Wanma Pharm., Co. (Hangzhou, China). All other reagents, unless otherwise stated, were obtained from Sigma-Aldrich (St. Louis, MO, USA).

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SQS drug preparation. Enrichment experiments were performed in glass columns (2.5x60 cm) packed with AB-8 (Shanghai Resin Factory Co., Ltd., Shanghai, China). *C. olifeira* was obtained from Sanming City, Fujian, China. A total of ~500 g seeds of *C. olifeira* were refluxed with 70% ethanol. The extracted solution was concentrated to 500 ml without ethanol. The ethanolic extract was adjusted to pH 5.0, and the sample flowed through the glass column at a rate of 1.0 bed volumes (BV)/h, while 0.1% NaOH solution was added via the separatory funnel, in order to elute impurities and pigment. Following clarification, the sample was sequentially eluted with distilled water to neutral, and then with 90% ethanol (Sinopharm Chemical Reagent Co., Ltd., Beijing, China) solution (containing 1% NaOH; Sinopharm Chemical Reagent Co., Ltd.) at a constant flow rate of 2.0 BV/h. Each fraction was collected and evaporated to remove the ethanol using a rotary evaporator (RE52AA; Yarong Equipment Co., Shanghai, China) under reduced pressure at 55°C. Subsequently, 500 ml acetone (Sinopharm Chemical Reagent Co., Ltd.) was added to the concentrated liquid and left to stand overnight, followed by centrifugation at 1,006 x g for 30 min. The sediment was dissolved in 70% ethanol, placed in the fridge at 4°C overnight, filtered and dried at 60°C to a constant weight (500 g) to produce high-purity SQS crystals (5 g) (12).

Cell culture and treatment. Immortalized HepG2 human hepatocellular carcinoma cells were obtained from the Live Cancer Institute of Zhongshan Hospital (Shanghai, China) and maintained in Dulbecco's modified Eagle's medium containing 10% FBS and 1% penicillin/streptomycin solution at 37°C in 5% CO₂. All experiments were performed while the cells were in an exponential growth phase (13).

Cytotoxicity assay. HepG2 cells were seeded into standard 96-well culture plates at a density of 2.0x10⁴ cells/well and cultured to ~70% confluence. Cells were maintained in medium containing various concentrations (0, 10, 20 or 30 µg/ml) of SQS and 30 µg/ml 5-FU for 6, 12 or 24 h prior to the addition of 20 µl MTT solution (5 mg/ml MTT) to each well and cultured for a further 4 h. The medium was subsequently removed and 150 µl/well dimethyl sulfoxide was added and incubated for 10 min whilst shaking gently to stop the reaction. The optical density (OD) at 570 nm was read using an ELISA reader (ELX800; BioTek Instruments, Inc., Winooski, VT, USA). Each independent experiment was performed in triplicate (14).

Cell apoptosis analysis. Annexin V staining (in the presence or absence of SQS) and cell scanning were conducted using an Annexin V-fluorescein isothiocyanate (FITC) detection kit (Beyotime Institute of Biotechnology, Haimen, China). The HepG2 cells were treated with 0, 10, 20 or 30 µg/ml SQS for 12 h. To detect apoptosis, 1x10⁵ cells were resuspended in 500 µl binding buffer in the presence of 5 µl Annexin V-FITC and 5 µl propidium iodide and incubated at room temperature for 15 min in the dark. The fluorescence was measured using a FACScan flow cytometer (BD Bioscience) and the mean fluorescent intensity of triplicate samples for each SQS concentration cohort was used to ascertain the levels of Annexin V. The data were analyzed using CellQuest software version 1.0 (BD Biosciences, Franklin Lakes, NJ, USA) (15).

Cell cycle analysis. HepG2 cells were treated with 0, 10, 20 or 30 µg/ml SQS for 12 h. Subsequently, cells were harvested, washed with solution buffer and fixed in cold 70% ethanol overnight. A DNA content detection kit (Beyotime Institute of Biotechnology, Haimen, China) was used for cell cycle analysis. The cells were collected and suspended in 100 µl solution A and incubated at room temperature for 10 min. Analogous conditions were used upon the addition of 100 µl solution B and 150 µl solution C. Cell cycle progression was analyzed using a FACScan flow cytometer (BD Biosciences). The percentages of cells in G₀/G₁, S and G₂/M phases were determined using ModFitLT software version 3.0 (Verity Software House, Topsham, ME, USA).

Reverse transcription-polymerase chain reaction (RT-PCR) and gene expression analysis. RT-PCR was performed as described previously (16). HepG2 cells were seeded into six-well plates at a density of 1x10⁵ cells/well in 2 ml medium and treated with 0, 10, 20 or 30 µg/ml SQS for 12 h. Total RNA was isolated with TRIzol, and oligo (dT)-primed RNA (1 µg) was reverse-transcribed using SuperScript II reverse transcriptase (Promega Corp.) according to the manufacturer's instructions. The obtained cDNA was used to determine the mRNA expression levels of Bcl-2, Bax and caspase-3 by PCR analysis. GAPDH was used as an internal control. The primer sequences used for the amplification of Bcl-2, Bax, caspase-3 and GAPDH were as follows: Bcl-2 forward, 5'-TTCTCTCGTTCGCTACCGTTCGC-3' and reverse, 5'-CCTCCCCAGTTCACCCCATC-3'; Bax forward, 5'-CTTTTTGCTACAGGGTTTCA-3' and reverse, 5'-CCATGTTGTTGTCCAGTTCAT-3'; caspase-3 forward, 5'-CAGAAGATACCA GTGGAGGCC-3' and reverse, 5'-TTCCGGTTAACACGA GTGAGG-3'; GAPDH forward, 5'-CGACCACTTTGTCA AGCTCA-3' and reverse, 5'-AGGGGTCTACATGGCAA CTG-3'. All primers were synthesized by Sangon Biotech (Shanghai, China). The reaction included 1 µl cDNA, 2 µl 10X Taq Buffer, 1.2 µl 25 mM MgCl₂, 0.4 µl 10 mM dNTP, 0.8 µl 1U/µL Taq polymerase, 1 µl each primer and DEPC water upto 20 µl. The PCR conditions were as follows: 95°C for 3 min and 30 cycles at 95°C for 30 sec, 56°C for 40 sec and 72°C for 40 sec. Samples were analyzed by 1.5% agarose gel electrophoresis. The DNA bands were evaluated using a Gel Documentation system (Gel Doc 2000; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Western blot analysis. HepG2 cells were seeded in culture dishes (2x10⁵/well) and treated with 0, 10, 20 or 30 µg/ml SQS for 12 h. The cells were washed twice with phosphate-buffered saline (PBS) and lysed with lysis buffer according to the manufacturer's instructions. The protein concentration of the lysate was determined via the bicinchoninic acid assay (Pierce, Rockford, IL, USA). Samples were analyzed using 10% SDS-PAGE (Dingguo Biotech., Fuzhou, China). Following electrophoresis, the proteins were transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% skimmed milk in PBS with Tween 20 (PBST; Xilong Chemical Co., Ltd., Shantou, China) for 1.5 h and incubated with rabbit anti-Bcl-2, anti-Bax and anti-caspase-3 antibodies (1:1,000 dilution) at 4°C overnight. Following three washes with PBST buffer, the membranes were incubated

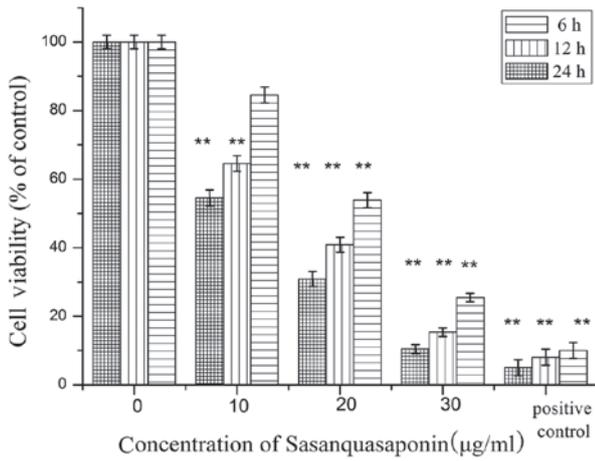


Figure 1. Determination of sasanquasaponin cytotoxicity in HepG2 cells using the MTT assay. HepG2 cells were incubated with the indicated concentrations of SQS for 6, 12 or 24 h. For the cell death-positive control, cells were incubated with 5-fluorouracil (30 µg/ml). **P<0.01 compared with the respective control.

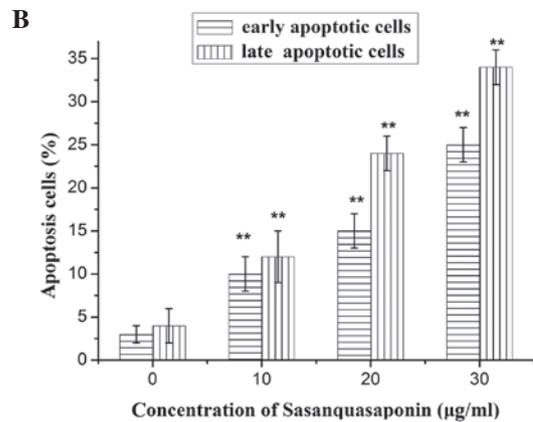
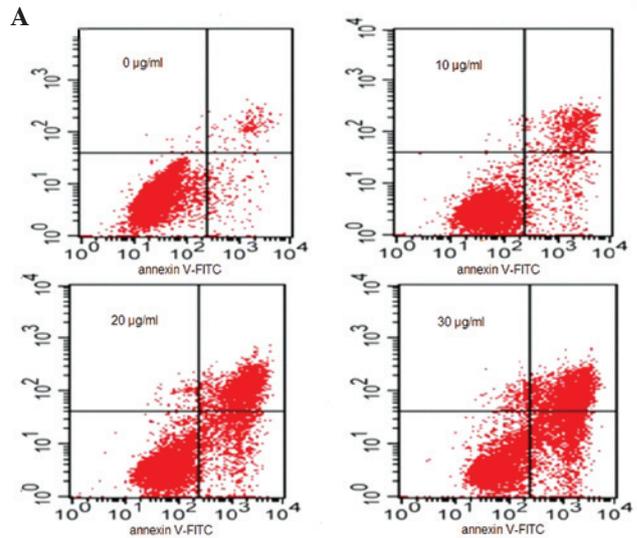


Figure 3. Induction of apoptosis in SQS-treated HepG2 hepatocellular carcinoma cells. (A) Dot plot diagrams obtained from flow cytometric analysis of Annexin V-fluorescein isothiocyanate and propidium iodide fluorescence of cells treated with 0, 10, 20 or 30 µg/ml SQS for 12 h. The upper and lower right quadrants were used to measure the percentage of apoptotic cells. The figure is representative of at least three independent experiments; (B) bar graph summarizing the percentage of apoptotic cells in each treatment group. Values are presented as the mean ± standard deviation of triplicate experiments. **P<0.05 vs. untreated cells. SQS, sasanquasaponin.

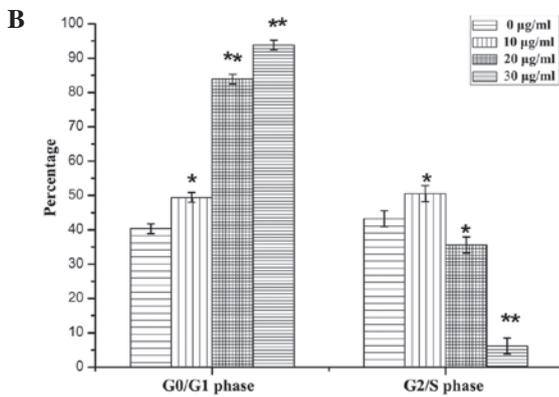
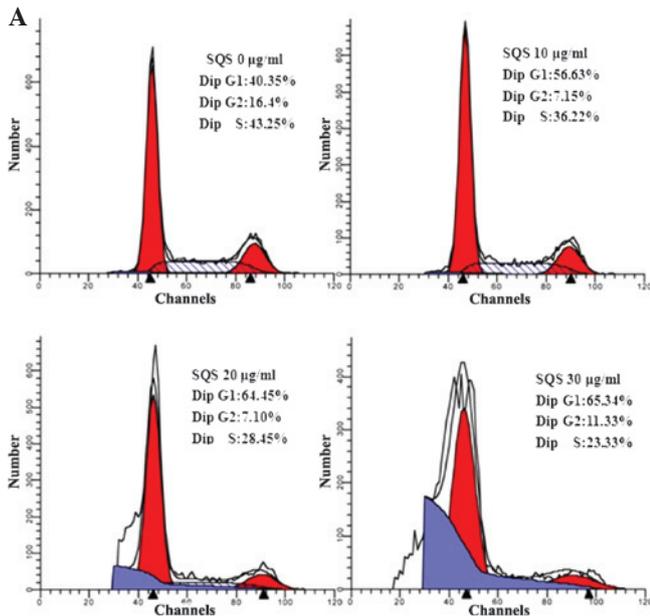


Figure 2. Effects of SQS on HepG2 cell cycle progression. (A) Cells were treated with the indicated concentrations of SQS for 24 h, stained with propidium iodide and analyzed by fluorescence-activated cell sorting. Images are representative of three independent experiments. (B) The proportion of cells in G₀/G₁ and G₂/S phase was calculated using ModfitLT software. Values are presented as the mean ± standard deviation of three independent experiments. *P<0.05 vs. untreated cells, **P<0.01 vs. untreated cells. SQS, sasanquasaponin.

with goat anti-rabbit immunoglobulin G conjugated with horseradish peroxidase for 2 h at room temperature. Following three washes with PBST, the protein bands were detected using enhanced chemiluminescence using Clarity Western Substrate (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instructions and images were captured using a ChemiDoc XRS+ system (Bio-Rad). β-actin was used as the control to confirm equal loading. Densitometry index analysis of the bands was conducted using a gel imagery system (17).

Statistical analysis. Values are presented as the mean ± standard deviation. Results were analyzed by one-way analysis of variance. When significant treatment effects were detected, Tukey-Kramer's t-test was performed to make pairwise comparisons between individual means. Statistical analysis software SPSS 19.0 (International Business Machines, Armonk, NY, USA) was used to conduct statistical analyses. P<0.05 was considered to indicate a statistically significant difference.

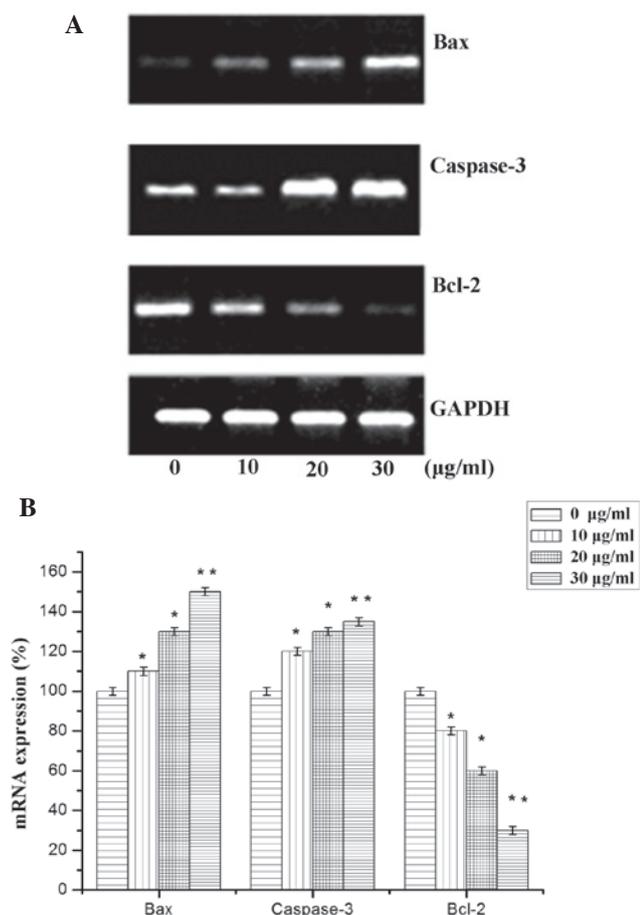


Figure 4. Effects of SQS treatment on the mRNA expression levels of Bax, Bcl-2 and caspase-3 in HepG2 cells. Cells were treated with the indicated concentrations of SQS for 12 h. (A) mRNA expression levels of Bax, Bcl-2 and caspase-3 in SQS-treated and untreated cells were determined by RT-PCR. GAPDH was used as an internal control. (B) Relative intensity expression obtained from the corresponding RT-PCR analyses. Values are expressed as the mean \pm standard deviation of at least three independent experiments. * P <0.05 and ** P <0.01 vs. untreated cells. RT-PCR, reverse transcription-polymerase chain reaction; SQS, sasanquasaponin; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated x protein.

Results

SQS reduces HepG2 cell viability. The MTT assay suggested that cytotoxic effects occurred 6, 12 and 24 h following incubation with SQS, even at doses as low as 20 μ g/ml SQS. As indicated in Fig. 1, the degree of cytotoxicity was directly proportional to the concentration of SQS, suggesting a dose-dependent effect. The results of the MTT test indicated that following 6 h of incubation with 10, 20 or 30 μ g/ml SQS, cell survival was reduced to 84.56, 53.86 and 25.25%, respectively.

SQS alters HepG2 cell cycle progression. The cell cycle has a key function in HepG2 cells, inducing their division and duplication. In addition, the G_1/S -phase transition represents one of the major checkpoints used in the regulation of cell cycle progression. Once the cell cycle passes this checkpoint late in G_1 phase, progression through the cell cycle continues with little or no extracellular stimuli (18). As indicated in Fig. 2, the proportion of cells in G_0/G_1 phase following treatment

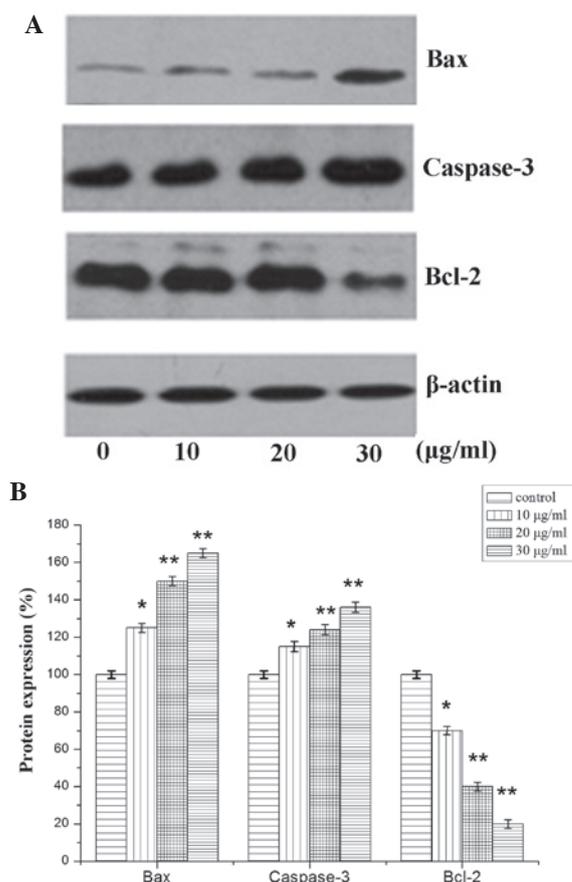


Figure 5. Effects of SQS treatment on the protein expression levels of Bax, Bcl-2 and caspase-3 in HepG2 cells. Western blot analyses of HepG2 cells treated with 0, 10, 20 or 30 μ g/ml sasanquasaponin for 12 h. HepG2 cell extracts were obtained and subjected to SDS-PAGE and western blot analysis for caspase-3, Bax and Bcl-2. (A) Western blot representative of three independent experiments. β -actin was used to verify uniform protein loading. (B) Relative intensity expression obtained from the corresponding western blot analyses. Values are expressed as the mean \pm standard deviation of at least three independent experiments. * P <0.05 and ** P <0.01 vs. untreated cells. Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated x protein.

with 10, 20 or 30 μ g/ml SQS was 56.63 \pm 1.76, 64.45 \pm 1.47 and 65.34 \pm 1.67%, a significantly higher proportion than that of untreated cells (40.35 \pm 1.33%; P <0.05). Concurrently, the percentage of cells in S phase following SQS treatment demonstrated the opposite trend. It was therefore suggested that SQS treatment may arrest cell cycle progression of HepG2 cells by inhibiting the G_1 to S phase transition.

SQS induces apoptosis of HepG2 cells. To determine the mechanisms underlying the effects of SQS treatment on HepG2 cells, membrane alterations were analyzed by flow cytometry and double staining with Annexin V-FITC and PI. A progressive increase in the percentage of apoptotic cells was observed with increasing SQS concentration, which demonstrated the dose-dependent cytotoxicity of SQS to HepG2 cells (Fig. 3).

Expression levels of Bcl-2, Bax and caspase-3. Based on the aforementioned results, the association between the expression of apoptotic factors and SQS-induced apoptosis of HepG2 cells was investigated. The mRNA and protein expression levels of

apoptosis-associated factors Bcl-2, Bax and caspase-3 were therefore evaluated (19). A significant reduction in Bcl-2 mRNA expression levels was observed following treatment with 10, 20 and 30 $\mu\text{g/ml}$ SQS, compared with those in untreated cells (Fig. 4). Conversely, a significant increase in caspase 3 and Bax mRNA expression was observed. An analogous trend was observed in the protein expression levels of caspase-3, Bcl-2 and Bax following SQS treatment (Fig. 5).

Discussion

The results of the present study revealed that SQS inhibited human HepG2 hepatocellular carcinoma cell growth, indicated by a decrease in cell density following SQS treatment. Furthermore, the mRNA and protein expression levels of key apoptotic regulators Bax, Bcl-2 and caspase-3 were found to be altered following SQS treatment (20).

It has previously been demonstrated that the intracellular signaling pathways influenced by SQS and its metabolites are involved in the regulation of numerous cell signaling pathways, which contribute to the activation or inhibition of various cellular processes (21). SQS influences not only the transduction of signals from the extracellular space, but also functions as a mediator and modulator of intercellular and intracellular signaling networks (22-24).

The results of the present study indicated that pre-treatment of HepG2 cells with SQS resulted in Annexin V-positive staining. These results suggested that SQS may block the signal transduction pathway required for cell survival and induce HepG2 cell apoptosis. The mRNA and protein expression levels of Bcl-2, caspase-3 and Bax were therefore examined. Bax is a pro-apoptotic Bcl-2 protein, which contains BH1, BH2 and BH3 domains and initiates apoptotic signaling. The expression of Bax is upregulated by the tumor suppressor protein p53, and Bax has been shown to be involved in p53-mediated apoptosis. The results of the present study demonstrated that SQS treatment increased Bax expression in HepG2 cells. Caspase-3 is encoded by the *CASP3* gene and interacts with caspase-8 and -9. In apoptotic cells, caspase-3 activation may occur via extrinsic (death ligand) or intrinsic (mitochondrial) pathways. Of note, Bax is involved in the intrinsic pathway. Activated caspase-3 induces caspase-8 activation and subsequently activates Bax, which stimulates cytochrome C and induces apoptosis (25). An identical signaling pathway was identified in the HepG2 cells evaluated in the present study. Caspase-3 activation was previously demonstrated to be increased following treatment of cells with SQS (26,27).

SQS interacts with mitochondrial membranes and alters their permeability by opening transition pores and decreasing the mitochondrial membrane potential (28). SQS therefore induces structural changes and increases mitochondrial membrane unsaturation.

These mitochondrial alteration may influence the activity of pro- and anti-apoptotic proteins of the Bcl-2 family. It was previously demonstrated that SQS contributes to the down-regulation of Bcl-2 expression, a well-known anti-apoptotic molecule, and blocks lipid peroxidation; therefore, inhibiting the induction of apoptosis (29). The results of the present study indicated that SQS suppressed Bcl-2 expression.

Based on previously published studies and the results of the present study, it was hypothesized that SQS induces human hepatocellular carcinoma cell apoptosis. The signal transduction occurs via the caspase-3 signaling pathway. Further study on this signaling pathway is required to elucidate the function of SQS in tumor invasion and survival.

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