

Solanine-induced reactive oxygen species inhibit the growth of human hepatocellular carcinoma HepG2 cells

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Abstract. The aim of the present study was to investigate the effect of solanine on promoting human hepatocellular carcinoma HepG2 cells to produce reactive oxygen species (ROS), and the molecular mechanisms leading to tumor cell apoptosis. Solanine was administered to HepG2 cells *in vitro*. A selection of probes targeting various cellular localizations of ROS were used to detect ROS expression using flow cytometry. The expression levels of apoptosis-associated proteins, including apoptosis signal-regulating kinase 1 (ASK1) and thioredoxin binding protein 2 (TBP-2), and proliferation-associated proteins, including histone deacetylase 1 (HDAC1), were detected using western blotting. The percentage of cells undergoing apoptosis was measured using an Annexin V-fluorescein isothiocyanate/propidium iodide assay, and cell morphology was examined using Wright's stain followed by inverted microscopy analysis. ROS detection probes 2',7'-dichlorofluorescein diacetate and dihydrorhodamine 123 identified that abundant ROS, including hydroxyl radical (OH[•]) and hydrogen peroxide (H₂O₂), were produced in the cytoplasm and mitochondria of the solanine-treated HepG2 cells compared with the control cells (P<0.05). Superoxide anion specific probes dihydroethidium and MitoSOX™ demonstrated that there were no significant alterations in the HepG2 cells following solanine treatment compared with the control cells (P>0.05). Western blotting results revealed that solanine upregulated the expression levels of ASK1 and TBP-2 and enhanced their kinase activities, whereas solanine decreased the expression level of the proliferation-associated protein, HDAC1. The cell apoptotic rate was significantly increased (P<0.0001) in the

solanine-treated HepG2 cells compared with the control cells. (P<0.05). Overall, the study indicated that solanine induces HepG2 cells to produce ROS, mainly OH[•] and H₂O₂, in a mitochondria-dependent and -independent manner. In addition, solanine stimulates the expression of ASK1 and TBP-2, and their kinase activities, but inhibits the expression of proliferation-associated proteins, such as HDAC1, thus contributing to HepG2 cell apoptosis.

Introduction

Hepatocellular carcinoma (HCC) is a significant worldwide health issue. In developing countries, the overall incidence of HCC remains extremely high, and in the majority of developed countries, the incidence of HCC has steadily increased; this is due to the spread of hepatitis B and C (1,2). Currently, the main treatment strategies for patients with HCC consist of surgical resection, liver transplantation and local ablation therapies (3). A large number of the patients diagnosed with advanced-stage HCC may only be treated with palliative care (2,4), as there is no available effective palliative chemotherapy. Therefore, the prognosis of patients with advanced-stage HCC is poor (5). Consequently, novel effective medications for the treatment of HCC are required. Recently, the study into treatments for HCC has focused on natural products, which are pharmacologically effective, with little toxicity and few side effects.

Solanine is mainly located in the tuber of the potato plant (*Solanum tuberosum* L.) and in the whole nightshade plant (*S. nigrum* L.), which belong to the Solanaceae family (6-10). Solanine expression is relatively high in the green peel and the sprouts of a potato tuber and is the main toxic substance produced (11), with a molecular formula of C₄₅H₇₃NO₁₅ (12,13). The whole nightshade plant contains numerous steroid alkaloids, including solamargine, solasonine, solanine and saponin, and may be used for antitumor therapeutics, as it has a clear inhibitory effect on tumor growth in animal models and a toxic effect on tumor cells (14,15). The ethanol extract of nightshade is able to inhibit the growth of breast cancer and induce apoptosis in tumor cells (16).

Previous studies have demonstrated that solanine affects tumor growth through several possible biological mechanisms. An *et al* (17) revealed that solanine significantly reduces the membrane fluidity and protein levels of tumor cells in xenograft

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mice with hepatoma H22 tumors. In addition, solanine effectively reduced the sialic acid level and membrane closing ability of the tumor cells. A previous study demonstrated that the mechanism by which solanine induces human hepatocellular carcinoma HepG2 cells to undergo apoptosis appeared to be mediated by the inhibition of B-cell lymphoma-2 expression (18). Furthermore, solanine has been demonstrated to increase the permeability of the mitochondrial membrane, which leads to the release of apoptosis-associated proteins and inner mitochondrial calcium ions, resulting in tumor cell apoptosis through the mitochondrial pathway (19,20). Eom *et al* (21) reported that the berberine-induced apoptosis of human glioblastoma cells was mediated by reactive oxygen species (ROS) and mitochondrial dysfunction, which indicates that ROS are important in cellular apoptosis. The present study investigated the molecular mechanism of solanine-induced HCC cell apoptosis, in particular ROS that are involved in the apoptotic pathway of tumor cells. The present study cultured HepG2 cells treated with solanine *in vitro*, and subsequently monitored the production of ROS from the mitochondria and cytoplasm of HepG2 cells using probes for ROS.

Materials and methods

Cells and solanine treatment. The human hepatocellular carcinoma HepG2 cell line was provided by the Shanghai Cancer Institute (Shanghai, China). The HepG2 cells were cultured in HyClone™ Dulbecco's modified Eagle's medium (GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% inactivated fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA) in a 5% CO₂ Forma™ humidified incubator (Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C. The cells were seeded onto a 6-well plate (4×10⁴ cells/well), allowed to adhere overnight, and subsequently treated with solanine (95% mass fraction; Sigma-Aldrich) with a final concentration of 2 mg/ml. The cells were incubated for 24–48 h. A negative control [dimethyl sulfoxide (DMSO); Sangon Biotech Co., Ltd., Shanghai, China] and a positive control (camptothecin, 0.5 mg/ml; Sigma-Aldrich) were used.

ROS detection using flow cytometry. Various concentrations of hydrogen peroxide (H₂O₂; 0.5 mM; Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) was added to each well, followed by an incubation period of 30 min prior to uniformly blending the cells in each well. The cells were washed twice with phosphate-buffered saline (PBS) and the Invitrogen™ fluorescent probes (Thermo Fisher Scientific, Inc.), 2',7'-dichlorofluorescein diacetate (DCFDA), dihydrorhodamine 123 (DHR 123), dihydroethidium (DHE) and MitoSOX™ (MitoSOX; a mitochondrial superoxide probe), were added to the cells according to the manufacturer's protocols. The cells were incubated at 37°C for 30 min in the dark. Subsequently, the cells were washed twice with PBS and analyzed using flow cytometry (BD LSR II Flow Cytometer; BD Biosciences, San Jose, CA, USA).

Western blotting. In total, 48 h after solanine treatment, total protein was extracted from the HepG2 cells using RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific, Inc.), which contained proteinase and phosphatase inhibitors, at 4°C for

30 min. The cell lysates were centrifuged at 12,000 × g for 20 min at 4°C. The protein concentrations of the resulting supernatants were determined using a Bicinchoninic Acid Protein Assay kit (Beyotime Institute of Biotechnology, Shanghai, China). The supernatants were mixed with 5X sodium dodecyl sulfate (SDS) loading buffer (Thermo Fisher Scientific, Inc.) and heated at 95°C for 5 min. In total, 40 µg of protein was loaded onto and separated by 12% Tris-Glycine SDS gel (Sangon Biotech Co., Ltd.), which was subsequently transferred onto polyvinylidene fluoride membranes (pore size, 0.22 µm; Merck Millipore, Darmstadt, Germany). The membranes were blocked with 5% skimmed milk in Tris-buffered saline and Tween 20 (Sangon Biotech Co., Ltd.), and incubated overnight with primary antibodies. The primary antibodies were as follows: Anti-apoptosis signal-regulating kinase 1 (ASK1; polyclonal rabbit anti-human; dilution, 1:1,000; catalog no., 3762; Cell Signaling Technology Inc., Danvers, MA, USA), anti-thioredoxin binding protein 2 (TBP-2; monoclonal mouse anti-human; dilution, 1:1,000; catalog no., K02053; MBL International Co., Woburn, MA, USA) and anti-histone deacetylase 1 (HDAC1; polyclonal rabbit anti-human; dilution, 1:1,000; catalog no., 2062; Cell Signaling Technology Inc.). Subsequently, the membranes were incubated with a horseradish peroxidase-labeled secondary antibody (dilution, 1:2,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Anti-β-actin monoclonal antibody (Santa Cruz Biotechnology, Inc.) was used as an endogenous control. The results were analyzed by AlphaView software version 3.3.0.0 (Protein-Simple, Santa Clara, CA, USA). The integral optical density (IOD) of each band was determined. The relative protein levels were used to evaluate the differences between the solanine-treated group and the control group, using the following equation: The relative protein level = IOD ratio between the target gene product bands and the β-actin protein bands in the solanine-treated group / IOD ratio between the target gene product bands and the β-actin protein bands in the control group.

Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) analysis. The HepG2 cells were harvested 48 h after solanine treatment. The percentage of cells undergoing apoptosis was detected using a FITC Annexin V Apoptosis Detection kit II (BD Pharmingen, San Diego, CA, USA) and flow cytometry (BD LSR II Flow Cytometer).

Cell counting and morphological observation. The cells were harvested at the logarithmic growth phase and counted using a Countess II FL Automated Cell Counter (Invitrogen; Thermo Fisher Scientific, Inc.). A total of 4×10⁴ cells were added to each well of a 6-well plate containing 2 ml medium, and the plate was placed into a 5% CO₂ humidified incubator at 37°C overnight (16–24 h). Subsequently, DMSO, 2 mg/ml solanine or 0.5 mg/ml camptothecin was added to the cells and incubated for an additional 24–48 h. The morphological alterations in the cells were observed using an optical microscope (Olympus IX70 Inverted Microscope; Olympus Corporation, Tokyo, Japan) following staining with Wright's stain (Sangon Biotech Co., Ltd.).

Statistical analysis. All experiments were repeated three times independently. The results are presented as the

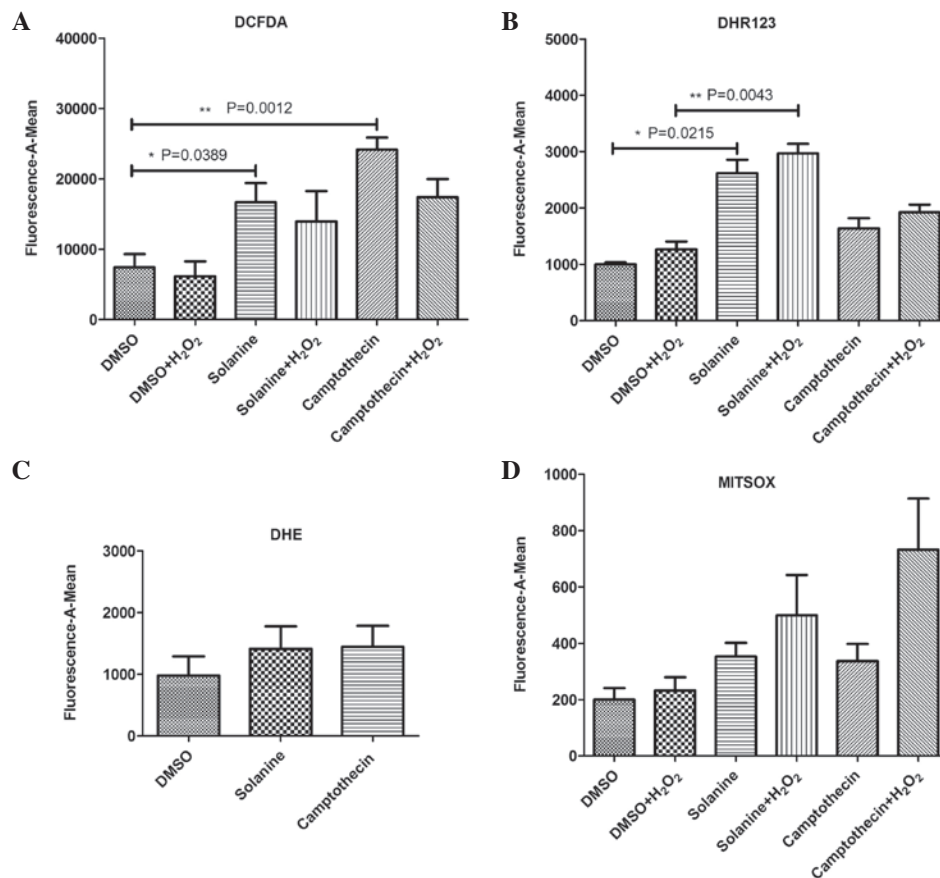


Figure 1. Various probes were used to detect reactive oxygen species produced by solanine-induced human hepatocellular carcinoma HepG2 cells. (A) DCFDA probe was used to detect OH^\cdot and H_2O_2 in the cytoplasm, (B) DHR 123 probe was used to detect OH^\cdot and H_2O_2 in the mitochondria, (C) DHE probe was used to detect O_2^\cdot in the cytoplasm, and (D) MITSOX probe was used to detect O_2^\cdot in the mitochondria. DCFDA, 2',7'-dichlorofluorescein diacetate; DHR 123, dihydrorhodamine 123; DHE, dihydroethidium; MITSOX, MitoSOX™ Red Mitochondrial Superoxide Indicator; OH^\cdot , hydroxyl radical; H_2O_2 , hydrogen peroxide; O_2^\cdot , superoxide anion; DMSO, dimethyl sulfoxide.

mean \pm standard deviation. One-way analysis of variance was performed using SPSS version 11.0 software (SPSS, Inc., Chicago, IL, USA) in order to detect significant differences in measured variables between groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Solanine induces the production of ROS in the cytoplasm and mitochondria of HepG2 cells. The HepG2 cells were treated with various concentrations of H_2O_2 for the same amount of time (30 min), and separately with the same concentration (0.5 mmol/l) for various time periods. The cells were incubated with DCFDA for 30 min. The results demonstrated that the optimal concentration and effective time for H_2O_2 stimulation of the cells were 0.5 mM and 30 min, respectively.

Following solanine pretreatment, the HepG2 cells were stimulated by H_2O_2 according to the pretested parameters, and various probes were used that were specific to certain ROS. The results revealed that ROS probes DCFDA and DHR 123 detected abundant ROS, including hydroxyl radical (OH^\cdot) and H_2O_2 , in the cytoplasm and mitochondria of the HepG2 cells pretreated with solanine compared with the control group [DCFDA (n=5), $P=0.0389$; DHR 123 (n=5), $P=0.0215$]. The amount of ROS produced by the solanine-treated cells was

decreased compared with the ROS produced by camptothecin-treated cells, which was observed using the DCFDA probe. By contrast, the amount of ROS produced by the solanine-treated cells was increased compared with the ROS produced by the camptothecin-treated cells, as determined using the DHR123 probe. This suggests that ROS production in various cellular locations may be by differential mechanisms. Increased levels of ROS production were also observed post- H_2O_2 stimulation, as detected by the DHR123 probe (n=5; $P=0.0043$). Superoxide anion (O_2^\cdot) ROS, which was generated by solanine pretreatment, was also detected by O_2^\cdot -specific DHE and MITSOX probes. However, there was no significant difference between the solanine-treated and control groups [DHE (n=5), $P=0.606$; MITSOX (n=5), $P=0.107$] (Fig. 1).

Solanine induces the upregulation of ASK1 and TBP-2, and the downregulation of HDAC1. It has previously been suggested that ASK1 is an important protein involved in regulating apoptosis (22). Another apoptosis-associated protein is TBP-2, also termed vitamin D-upregulated protein 1, which effectively combines with thioredoxin, eventually leading to cellular apoptosis (23). The increased expression of HDAC1 increases the proliferation of tumor cells and alters the extracellular matrix; therefore, significantly promoting tumor cell migration and aggression (24). In addition, HDAC1 removes

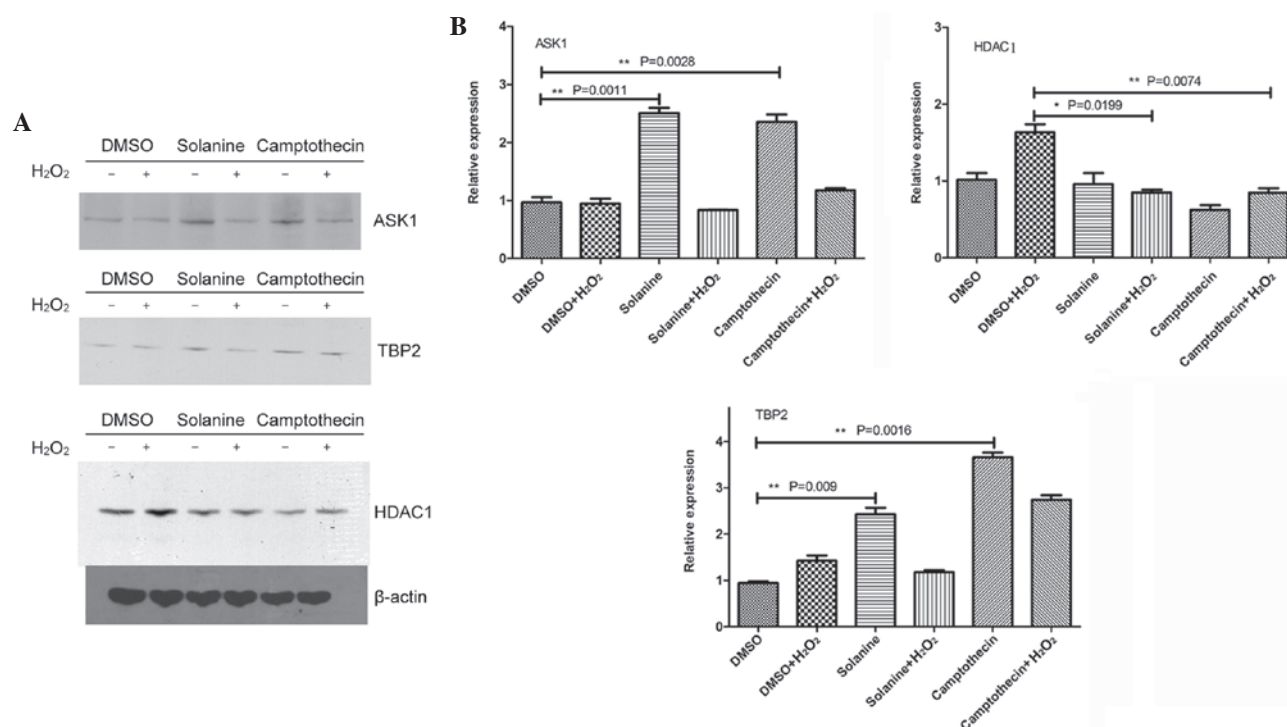


Figure 2. Western blot analysis of ASK1, TBP-2 and HDAC1 expression in human hepatocellular carcinoma HepG2 cells. (A) A representative western blot analysis. (B) Relative protein expression levels of ASK1, TBP-2 and HDAC1 were assessed by calculating the IOD values. IOD values were normalized to those of β -actin protein. ** $P < 0.001$. Results are representative of 3 experiments. ASK-1, apoptosis signal-regulating kinase 1; TBP-2, thioredoxin binding protein 2; HDAC1, histone deacetylase 1; IOD, integral optical density; DMSO, dimethyl sulfoxide; H_2O_2 , hydrogen peroxide.

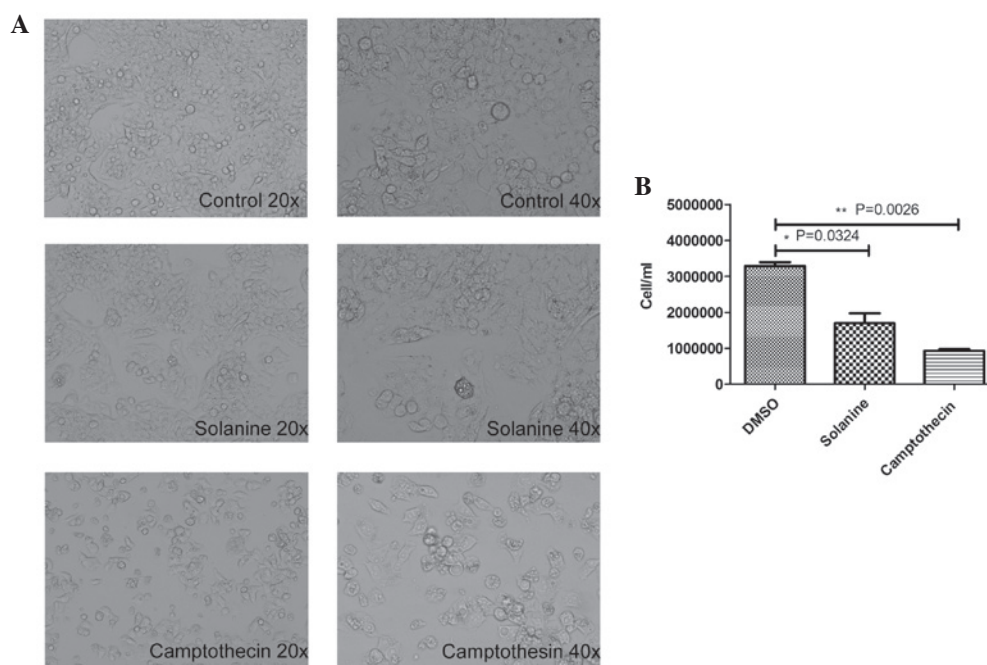


Figure 3. (A) The morphology alterations observed in human hepatocellular carcinoma HepG2 cells following treatment with solanine and camptothecin. (B) Quantification of cell counting. DMSO, dimethyl sulfoxide.

the acetyl-groups on histones, a more advanced chromosome structure, and inhibits basic transcription complex assembly; therefore, inhibiting transcription (25). The present results demonstrated that solanine and camptothecin increased the expression of ASK1 and TBP-2, but reduced the expression of HDAC1 (Fig. 2).

Solanine significantly inhibits the growth of HepG2 cells and triggers cell apoptosis. Next, the present study investigated the effect of solanine on the growth and apoptosis of the HepG2 cells. As revealed by Fig. 3A, the HepG2 cells treated with solanine exhibited notable morphological alterations in contrast to the control cells, which exhibited no alterations in

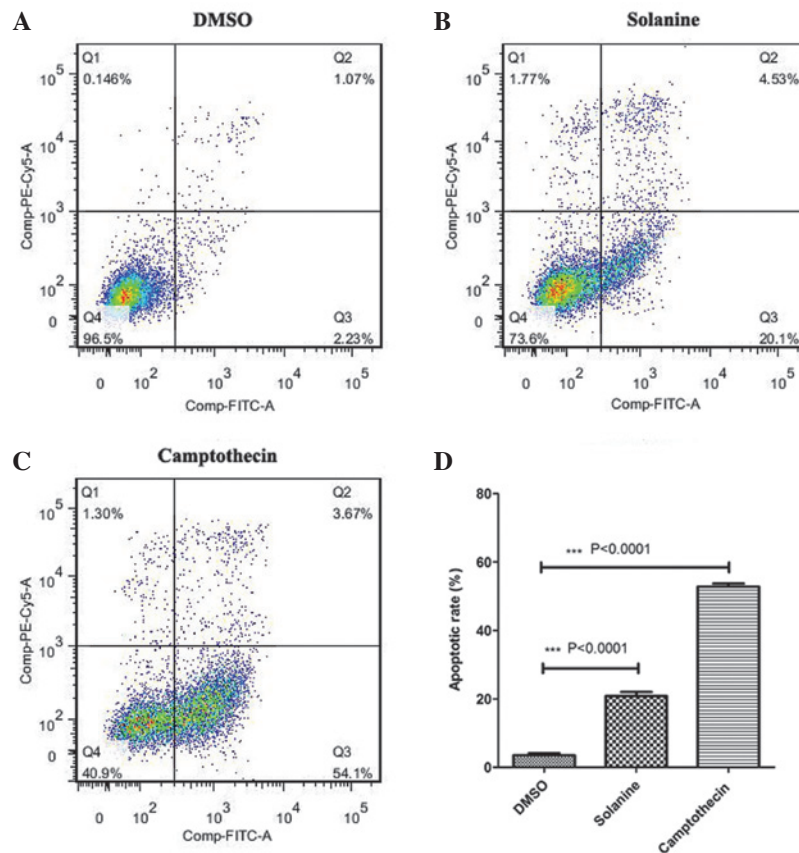


Figure 4. Cells treated with solanine or camptothecin promote the apoptosis of human hepatocellular carcinoma HepG2 cells. (A-C) Annexin V-FITC/propidium iodide staining and flow cytometric analysis of cells undergoing apoptosis with (A) DMSO, (B) solanine and (C) camptothecin treatment. The lower-right quadrant of each plot indicates early apoptotic cells. (D) Flow cytometry analysis of the percentage of apoptotic cells in each treatment group. *** $P < 0.0001$. DMSO, dimethyl sulfoxide; FITC, fluorescein isothiocyanate.

morphology. The solanine-treated cells exhibited numerous cytoplasmic vacuoles, and the cells were round, shrunken and detached. The number of cells was significantly decreased in the solanine-treated ($n=3$; $P=0.0324$) and camptothecin-treated groups ($n=3$; $P=0.0026$) compared with the control group (Fig. 3B).

Consistent with the cell counting results, flow cytometry analysis confirmed that there was an increased percentage of cells undergoing apoptosis in the solanine and camptothecin-treated groups compared with the control group (Fig. 4). This indicates that following treatment with solanine, the reduced cell growth observed may be due to enhanced cellular apoptosis.

Discussion

ROS, including O_2^- , H_2O_2 and OH^\cdot , are products of aerobic respiration in eukaryotic cells. ROS have a cytotoxic effect and are also intracellular signal transduction and gene expression regulation molecules, with key roles in the regulation of cell growth, survival and apoptosis (26-28). The mitochondria of a eukaryotic cell are the main site for the production of ROS, and a large amount of ROS may be produced in the process of oxidative phosphorylation. The impact of reactive oxygen on the mitochondria results in the dissipation of the mitochondrial membrane potential, the release of cytochrome *c* and causes the mitochondrial membrane permeability transition pore

(PTP) to open (29). A variety of proapoptotic signals, including physical damage, radiation, chemotherapy, excitatory amino acids and death ligands, may cause an increase in cellular endogenous or exogenous ROS, or alter the redox equilibrium, and these signals may trigger cellular apoptosis. Once an apoptotic pathway is activated, an increase in the expression of ROS may accelerate the apoptosis process (30-32). Therefore, the release of ROS and the process of apoptosis are mutually affected.

In recent years, solanine has become notable due to its antitumor properties. The main components of solanine that have these antitumor properties are the alkaloids, which lead to cytotoxicity and anti-nuclear splitting in tumor cells (33). Previous studies have demonstrated that solanine has a clear cytotoxic effect on human HepG2 cells through the direct inhibition of the activity of matrix metalloproteinase (MMP)-2 and MMP-9 and tumor angiogenesis; therefore, inhibiting tumor metastasis (34,35). In addition, solanine reduces the mitochondrial membrane potential, by increasing the opening of the PTP. Opening of the PTP leads to an increase in calcium ions, which initiates cell apoptosis resulting in an antitumor effect (36).

The present results demonstrated that solanine induces HepG2 cells to significantly produce ROS, including OH^\cdot and H_2O_2 , in the cytoplasm and mitochondria of the cell, to a similar level as the positive control, compared with the negative control ($P < 0.05$). In the present study, the specific probes

targeting differential-localized ROS identified that ROS components remained in the cytoplasm and mitochondria of the cells; however, the main location for the production of ROS was observed to be in the mitochondria. The ROS produced in the mitochondria may directly diffuse to the cytoplasm due to the increasing permeability of the mitochondrial membrane. However, the increased expression of ROS was not detected by the DCFDA and DHR123 probes, specific for OH^- and H_2O_2 , respectively, post- H_2O_2 stimulation. This may be due to the following possible explanations: Apoptosis, induced by antitumor drugs, may have a different apoptosis response compared with non-chemotherapy-induced apoptosis due to cell cycle sensitivity (37,38). The permeability of the membrane of the HepG2 cells remains relatively high when exposed to antitumor drugs, indicating a potential possibility of an intracellular pattern of diffusion as H_2O_2 enters the cell. Previous findings have demonstrated that growth of tumor cells may be inhibited when they are pretreated with chemotherapeutics. Subsequent simulation by H_2O_2 may therefore result in tumor shedding and necrosis, which has a lower fluorescence signal. In addition, the present study identified that there was no difference in the expression of O_2^- with specific probes DHE and MITSOX in the solanine, camptothecin and control cell groups ($P>0.05$). This suggests that solanine may induce the cytoplasm and mitochondria of HepG2 cells to produce ROS, which consists of OH^- and H_2O_2 , but not O_2^- . Therefore, the effects of solanine on tumor cells was generated by the production of OH^- and H_2O_2 .

In addition, the present study investigated the alterations in the expression of certain apoptosis- and proliferation-associated proteins in solanine-induced cellular apoptosis. The present results demonstrated that an increase in the expression levels of ASK1 and TBP-2, which activate downstream signaling pathways, including c-Jun N-terminal kinases (JNK) and p38 mitogen-activated protein kinases (p38), may be the main mechanism through which the HepG2 cells underwent apoptosis. Solanine also significantly inhibited the expression of the proliferation-associated protein HDAC1, which is another possible mechanism of the antitumor effects of solanine treatment. To additionally elucidate the molecular mechanisms of solanine-induced cellular apoptosis, the specific inhibition of mitochondrial O_2^- should be performed to investigate whether solanine-induced apoptosis is associated with mitochondrial O_2^- . Studies are ongoing to investigate this hypothesis.

In conclusion, solanine induces HepG2 cells to produce ROS, mainly OH^- and H_2O_2 , and increases the expression and activity of the apoptosis-associated proteins ASK1 and TBP-2, which activate downstream signaling pathways, including JNK and p38, eventually leading to cell apoptosis. Therefore, solanine is a potential anticancer drug, which may have a clinical application in the future.

Acknowledgements

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