

# Wogonoside induces apoptosis in Bel-7402, a hepatocellular carcinoma cell line, by regulating Bax/Bcl-2

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**Abstract.** The anticancer effect of *Scutellaria baicalensis* extract has recently become a topic of interest. In this study, the anticancer effects and underlying mechanisms of wogonoside, the main constituent of *Scutellaria baicalensis*, were investigated in a human hepatocellular carcinoma (HCC) cell line *in vitro*. The effects of wogonoside on the proliferation, cell cycle progression and apoptosis of hepatocellular carcinoma cells were examined. Western blotting was employed to analyze the proteins associated with the biological effects of wogonoside. Wogonoside exerted anti-proliferation properties *in vitro*. HCC cell growth was attenuated by wogonoside (8  $\mu$ M) treatment. Cell cycle progression analysis and DNA ladder assay revealed that apoptosis was enhanced in wogonoside-treated cells and that cell cycle arrest occurred in the G2/M phase. It was also demonstrated that increased apoptosis was accompanied by increased levels of Bax protein and decreased levels of Bcl-2 protein. The results of this study suggest that wogonoside may represent a potential therapeutic agent against HCC.

## Introduction

Hepatocellular carcinoma (HCC), the most common primary malignancy of the liver, is the sixth most common type of cancer worldwide and the third leading cause of cancer-related mortality (1). The number of cases diagnosed with HCC is expected to increase worldwide, particularly in East and Southeast Asia (1). Several lines of study demonstrate that the major risk factors of HCC include chronic hepatitis B or

C virus infection, diabetes, non-alcoholic fatty liver disease, excessive alcohol consumption and dietary exposure to aflatoxin (2,3). Despite substantial and accelerated research in the area of HCC, the 5-year survival rate for advanced disease remains <10% (4). The first line of therapy consists of ablative therapy, surgical resection and transplantation (5). However, due to a shortage of donor livers, and patients' advanced tumor stage or liver dysfunction, less than 20% of HCC patients are eligible for these treatments (6). Therefore, there is a need to develop novel therapeutic approaches to improve the outcomes of patients with HCC.

Hepatocarcinogenesis is a complex, multi-step process which occurs as a result of a combination of epigenetic and genetic alterations. It has been reported that apoptosis, which triggers a range of extrinsic and intrinsic signals leading to cell shrinkage, blebbing of the plasma membrane, chromatin condensation and DNA laddering, plays a role in the development of neoplastic transformation and tumor growth (7). Accumulating evidence suggests that the dysregulation of the apoptotic process plays a crucial role in the occurrence and progression of human HCC (8). Therefore, a number of potential anticarcinogens, which induce the apoptotic process in various ways, may be applied in the management of HCC (9,10).

In recent years, traditional Chinese medicines have slowly gained considerable attention as a new source of anticancer drugs. Although their curative mechanisms remain largely unknown, certain drugs have been used to treat cancer (11). Wogonin (5,7-dihydroxy-8-methoxy-2-phenylchromen-4-one, C<sub>16</sub>H<sub>12</sub>O<sub>5</sub>), a flavonoid extracted from the root of the traditional Chinese herb *Scutellaria baicalensis*, has been considered to be responsible for the therapeutic actions of *Scutellaria baicalensis* due to its observed pharmacological actions including anti-inflammation and anticancer properties, reduction of total cholesterol level and anti-HIV activity (12-14). Due to its therapeutic potential, wogonin has been recognized as a new source of anticancer drug and a new chemotherapy adjuvant to enhance the efficacy of chemotherapy and to ameliorate the side effects of cancer chemotherapies (13-16). Wogonoside (wogonin-7-glucuronide, C<sub>22</sub>H<sub>20</sub>O<sub>11</sub>), a metabolite of wogonin, is also derived from *Scutellaria baicalensis*, sharing the same flavone backbone as wogonin (17). Accumulating evidence demonstrates that

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wogonoside is involved in various biological and pathological processes including anti-inflammation, anti-angiogenesis, cell cycle arrest and induction of autophagy (18-21). The difference between the two chemical structures is the presence and placement of 7-glucuronic acid, indicating that wogonoside and wogonin may affect similar biochemical processes. Wogonin has been reported to kill HCC cells *in vitro* and inhibit tumor growth *in vivo* in a number of mouse tumor models (15,16,22,23). All the properties of wogonoside mentioned above indicate that wogonoside may be involved in the anticancer process. However, as a metabolite of wogonin, relatively few studies have investigated the potential effects of wogonoside on HCC, and its antitumor properties have not been elucidated.

In the present study, the effects of wogonoside on cell apoptosis were evaluated in the human HCC cell line Bel-7402. The potential regulation pathway involved in its apoptotic effect was also investigated.

## Materials and methods

**Cell lines and reagents.** The human liver cancer cell line Bel-7402 was donated by the State Key Laboratory of Medical Genetics of Central South University, Changsha, China. It was cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 15% fetal bovine serum (Invitrogen Life Technologies, Carlsbad, CA, USA), 100 U/ml penicillin and 100 µg/ml streptomycin in a humidified incubator under 95% air and 5% CO<sub>2</sub> at 37°C. Wogonoside, kindly provided by the Pharmacy College of Central South University, was first dissolved in phosphate-buffered saline (PBS) to prepare 10 mg/ml store solution and then serially diluted to various concentrations prior to experiments. The present study was approved by the ethics committee of Xiangya Hospital, Central South University (Changsha, China).

**Measurement of cell viability.** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to evaluate the cell viability according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO, USA). Briefly, 1x10<sup>4</sup> cells per well were plated onto 96-well plates and incubated for 3 h. The cells were then treated with wogonoside at the indicated concentrations (1, 2, 4, 8, 16, 32, 64, 128, 256 and 512 µM, and 1 and 2 mM) for 48 h. Each experiment was performed in triplicate. MTT reagent was added. Following incubation for 4 h at 37°C, the absorbance, which is directly proportional to the number of viable cells in cultures, was measured at 570 nm using a microplate reader (Mithras LB940 multilabel reader; Berthold Technologies, Bad Wildbad, Germany). The cell viability was expressed as a percentage value of control cells cultured with medium alone. The test was run three times and the inhibition rate was calculated with the formula: Inhibition rate = 1 - [(Treatment<sub>A570</sub> - Blank<sub>A570</sub>) / (Control<sub>A570</sub> - Blank<sub>A570</sub>)] x 100% to produce an inhibition curve and derive the half maximal inhibitory concentration (IC<sub>50</sub>) of wogonoside.

DNA ladder assay was also performed as previously described (24). Briefly, Bel-7402 cells were cultured in a 25-mm<sup>2</sup> flask with 4, 8, 12 and 16 µM wogonoside at ~2x10<sup>6</sup> cells per group for DNA sample extraction. The control group were treated with 100 µM 5-fluorouracil (5-FU; Sigma-Aldrich).

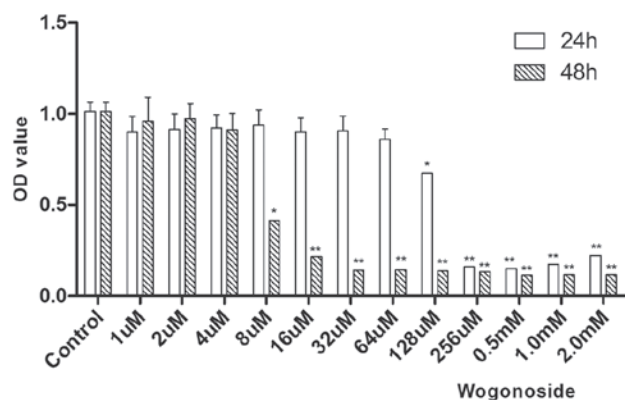


Figure 1. Effects of wogonoside on the proliferation of hepatocellular carcinoma cells. Cells were treated with various concentrations of wogonoside for the indicated times (24 and 48 h) and subjected to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Each reported value is the mean  $\pm$  standard deviation of at least three independent experiments performed in triplicate. \*P<0.05 and \*\*P<0.01 compared with control group.

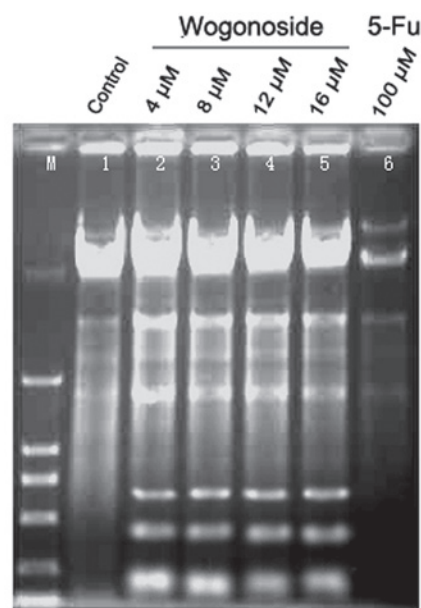


Figure 2. DNA fragmentation of Bel-7402 following wogonoside treatment for 24 h. Bel-7402 cells were treated with various concentrations (4, 8, 12, 16 µM) of wogonoside and 5-fluorouracil (5-FU; 100 µM). Integrity of DNA was assessed through a 1.2% agarose gel and visualized by staining with ethidium bromide.

Cells were harvested at 12, 24, 36 and 48 h after treatment. DNA was electrophoresed in 1.2% agarose gels at 10 V/cm for 2 h. The analysis of DNA fragmentation was carried out using the manufacturer's apoptotic DNA ladder kit (Calbiochem, Billerica, CA, USA).

**Flow cytometry for cell cycle detection.** Cells were plated in 35-mm dishes at concentrations determined to yield 60-70% confluence within 48 h and then treated with wogonoside at the indicated concentrations (4, 8, 12 and 16 µM) for 48 h. The adherent and floating cells were harvested, and the cells were resuspended in PBS, and fixed with 70% ethanol at -20° overnight. The cells were first incubated with RNaseA (20 U/ml; Sigma-Aldrich) at 37°C for 30 min and then labeled

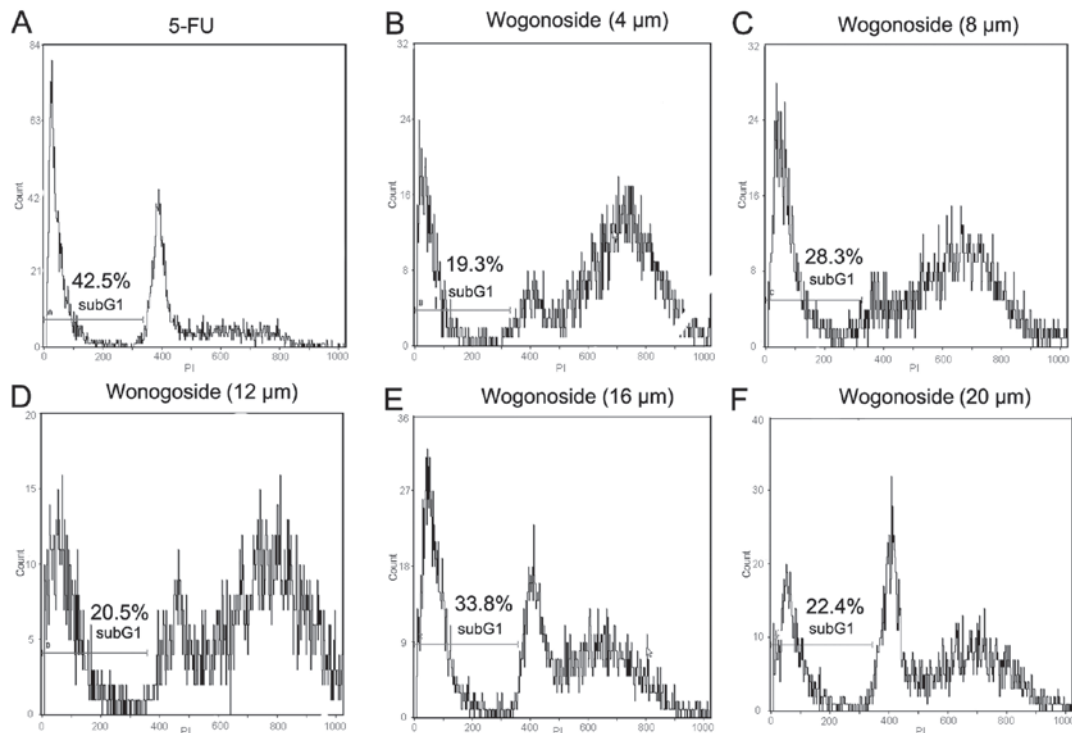


Figure 3. Effect of wogonoside on cell cycle of hepatocellular carcinoma cells. Bel-7402 cells were treated with (A) 5-fluorouracil (control) or (B-F) various concentrations (4, 8, 12, 16 and 20  $\mu$ M) of wogonoside, and the ratio of hypodiploid cells was measured by flow cytometry analysis by propidium iodide staining. Each value is presented as the mean  $\pm$  SE of three independent experiments.

with propidium iodide (50  $\mu$ g/ml) and incubated at room temperature in the dark for 30 min. DNA content was then analyzed using a FACScan instrument equipped with FACStation running CellQuest software (Becton-Dickinson, San Jose, CA, USA).

**Western blotting of Bcl-2, Bax and p53 expression.** Western blotting was performed to assess Bcl-2, Bax and p53 expression as previously described (25). Briefly, a total of  $10^6$  cells were sedimented and lysed for 15 min in ice-cold lysis buffer [0.1% sodium dodecyl sulphate (SDS), 1% NP-40, 50 mM HEPES, pH 7.4, 2 mM ethylenediaminetetraacetic acid, 100 mM NaCl, 5 mM sodium orthovanadate, 40  $\mu$ M p-nitrophenyl phosphate and 1% protease inhibitor mixture set I; Calbiochem, Billerica, MA, USA]. After removing the cell debris by centrifugation at 16,200  $\times$  g for 15 min, equal amounts of proteins were separated on 12% SDS polyacrylamide gel, blotted onto a nitrocellulose membrane (GE Healthcare, Little Chalfont, UK) and blocked with 5% nonfat dry milk in PBS/Tween (0.05% Tween-20 in PBS). Bcl-2, Bax and p53 antibodies were used (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membranes were then incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (1:2000). The immunoreactive protein bands were developed by enhanced chemiluminescence.

**Statistical analysis.** Data are expressed as the means  $\pm$  standard deviation. Statistical analysis was performed using SPSS software version 17.0 (SPSS, Inc., Chicago, IL, USA). The difference between two groups was analyzed by Student's t-test.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

### *Proliferative inhibition and cell cycle arrest by wogonoside.*

To examine the effect of wogonoside on the cell viability in HCC cells, MTT assay was performed first. Cell viability was dose-dependently reduced in Bel-7402 liver cancer cells following wogonoside treatment at the indicated concentrations for 24 h (Fig. 1). Wogonoside inhibited Bel-7402 cells with an  $IC_{50}$  value of 8  $\mu$ M. The results of the DNA ladder assay revealed that cells from the control group presented an intact band of genomic DNA whereas the 5-FU and wogonoside treatment group did not (Fig. 2). The effect of wogonoside on the cell cycle progression of Bel-7402 was also examined. Cell cycle analysis revealed that exposure to wogonoside (4  $\mu$ M) for 24 h induced the accumulation of a significant proportion of cells in the G2/M phase (Fig. 3). The data above suggest that wogonoside treatment induced apoptosis in the HCC cell line.

### *Inhibition of Bcl-2 protein enhances the apoptosis-inducing activity of wogonoside.*

The potential effects of wogonoside were investigated in HCC cells. The pro-apoptotic Bax protein is among a number of key regulators of apoptosis. Therefore, the effects of wogonoside were investigated in this regulatory protein. We observed that wogonoside increased Bax expression in HCC cells in the western blotting assay (Fig. 4), which suggests that wogonoside promotes HCC cell apoptosis via the Bax protein regulatory pathway. The expression of anti-apoptotic protein Bcl-2 was also examined in this study. Bcl-2 expression was revealed to be decreased in the western blotting assay (Fig. 4). The expression of p53 protein was also

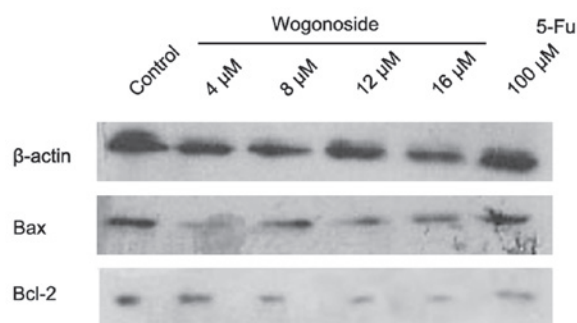


Figure 4. Effect of wogonoside on the expression of Bax, Bcl-2 in Bel-7402 cells. Bel-7402 cells were treated with various concentrations (4, 8, 12, 16  $\mu$ M) of wogonoside and 5-fluorouracil (5-FU; 100  $\mu$ M). Bax and Bcl-2 proteins were analyzed by western blotting using specific antibodies.

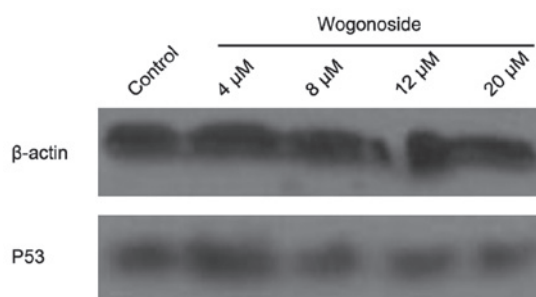


Figure 5. Effect of wogonoside on the expression of p53 in Bel-7402 cells. Bel-7402 cells were treated with various concentrations (4, 8, 12, 20  $\mu$ M) of wogonoside and the p53 protein were analyzed by western blotting.

detected in the study (Fig. 5). Western blotting assay did not reveal any significant change in p53 expression.

## Discussion

Herbs have been used successfully in traditional Chinese medicine for centuries; however, their therapeutic mechanism remains unknown. The present study demonstrates that wogonoside, a major constituent of *Scutellaria baicalensis*, attenuated *in vitro* proliferation of the HCC cell line Bel-7402 by inducing apoptosis and downregulating Bax/Bcl-2 signaling pathways. These results reveal that an adjuvant therapy of wogonoside may have potential therapeutic benefits for HCC.

*Scutellaria baicalensis* is often used for the treatment of inflammation, cardiovascular disease, and respiratory and gastrointestinal infection (26). The anticancer effect of *Scutellaria baicalensis* extract has recently become a topic of interest. Moreover, a number of *in vitro* studies suggest that the extract of *Scutellaria baicalensis* inhibits the growth of various cancer cell lines through specific biological signaling pathways, including apoptosis, angiogenesis and inhibition of the androgen receptor (15,16,18-23).

Increasing evidence has demonstrated that wogonoside, a major extract of *Scutellaria baicalensis*, induces autophagy in MDA-MB-231 cells by regulating the MAPK-mTOR pathway (19). It also participates in cell cycle arrest and differentiation by regulating the expression and subcellular localization of PLSCR1 in AML cells (20). Moreover, wogonoside induces the

inhibition of lipopolysaccharide-induced angiogenesis *in vitro* and *in vivo* via toll-like receptor 4 signal transduction (21). The present study confirmed that wogonoside possesses the ability to induce cycle arrest and apoptosis in HCC cells, indicating that the glucuronide metabolite wogonoside, like its aglycone wogonin, possesses biological activity.

Apoptosis represents a physiological means of eliminating excess cells during liver development and regeneration (27). Apoptotic cell death is initialized through the extrinsic or intrinsic signaling pathways that are ultimately coupled to the activation of the effect of caspases. The extrinsic pathway is largely controlled by the pro-apoptotic (e.g. Bax, Bad, Bid and Bak) and anti-apoptotic (e.g. Bcl-2 and Bcl-xL) Bcl-2 family proteins. It has been reported that the induction of mitochondrial apoptosis requires the involvement of the Bcl-2 family (28). The Bcl-2 family is comprised of proteins that share a Bcl-2 homology region and undergo heterodimerization or homodimerization (29). The ratio between anti- and pro-apoptotic proteins is considered a determinant for tissue homeostasis since it influences the sensitivity of cells to inducers of apoptosis (30).

In this study, it was demonstrated that the induction of apoptosis by wogonoside in HCC cells is associated with increased expression of the pro-apoptotic protein Bax and decreased expression of the anti-apoptotic protein Bcl-2. A wogonin-induced decreased ratio of Bcl-2 to Bax and increased expression of cleaved caspase 3 and 9 has been demonstrated in human breast cancer cells (31). Wogonoside, derived from *Scutellaria baicalensis*, shares the same flavone backbone as wogonin. Similar results were obtained in our study. Therefore, it is reasonable to assume that wogonoside-induced apoptosis of HCC cells is mediated by the mitochondrial pathway by altering the ratio of Bcl-2 to Bax.

The p53 protein, which acts as a guardian of the genome, is one of the key factors controlling cell proliferation, suppressing the growth and transformation of cells. Mutations in the p53 tumor suppressor gene are among the most common alterations observed in HCC (10). Various chemotherapeutic agents require p53 to induce apoptosis. Thus, we investigated p53 protein expression induced by wogonoside in HCC cells. There was no significant alteration observed in the western blotting assay. The results revealed that there may not be a correlation between wogonoside-induced apoptosis and the p53 pathway in HCC cells.

In conclusion, wogonoside may act as an effective drug with anti-proliferative and apoptotic activity in HCC cells. This study also presented a molecular mechanism responsible for the effects; namely, downregulation of the Bcl-2/Bax signaling pathway in wogonoside-induced apoptosis. The results of this study suggest that wogonoside may represent a potential therapeutic agent against HCC.

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