Involvement of bax/bcl-2 in wogonin-induced apoptosis of human hepatoma cell line SMMC-7721

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The molecular mechanisms of wogonin-induced apoptosis of human hepatoma SMMC-7721 cells are reported. Wogonin treatment resulted in significant inhibition of SMMC-7721 cells in a time-dependent and concentration-dependent manner. Typical morphological changes and apoptotic blebbing in SMMC-7721 cells were observed after treatment with 1×10^{-4} mol/l wogonin for a period of 0–48 h. Flow cytometry and Annexin-V/propidium iodide double-staining experiments revealed a dramatic increase in the number of apoptotic and G_0/G_1 phase cells after wogonin treatment. The proapoptotic activity of wogonin is attributed to its ability to modulate the expression of bcl-2 and bax proteins. It is observed that the expression of bax protein is dramatically increased whereas the synthesis of bc1-2 protein is significantly decreased when cells are treated with wogonin. The results presented in this paper suggested an important relationship between gene regulation and wogonin-induced apoptosis, and indicated the possibility of developing naturally occurring monoflavonoids as novel anticancer agents for better management of human cancers. *Anti-Cancer Drugs* **17:797–805 © 2006 Lippincott Williams & Wilkins.**

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Keywords: apoptosis, bax, bcl-2, human hepatoma cells SMMC-7721, wogonin

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Introduction

Apoptosis or programmed cell death is a physiological process responsible for the removal of cells that are generated in excess, have already completed their specific functions or are harmful to the organism. It plays a central role in controlling cell proliferation and hence is pivotal to the prevention of tumor development [1,2]. Cell apoptosis is characterized morphologically by cytoplasmic shrinkage, plasma membrane blebbing, nuclear chromatin condensation, chromosomal DNA cleavage and fragmentation of the cells into membrane-enclosed vesicles or apoptotic bodies [3–5]. The understanding of the apoptosis process has provided the basis for novel, targeted therapies that specifically induce cell death in carcinoma cell lines or enhance the cytotoxic effects of established chemotherapeutic agents in these cells [6,7]. These novel agents include those targeting the extrinsic pathway such as tumor necrosis factor-related apoptosis-inducing ligand receptor 1 [8,9] and those targeting the intrinsic Bcl-2 family pathway such as antisense bcl-2 oligonucleotides [10]. Many pathways and proteins control the apoptosis machinery. Examples include p53 [11], the nuclear factor κ B [12], the phosphatidylinositol 3-kinase pathway [13], and the ubiquitin/proteosome pathway [14]. These can be targeted by specific modulators such as bortezomib [15], and mammalian target of rapamycin inhibitors such as CCI-779 and RAD 001 [16,17]. As these pathways may be preferentially altered in tumor cells, there is great potential for a selective effect in tumors sparing normal tissues provided an efficient and selective agent can be identified [1].

Wogonin ($C_{16}H_{12}O_5$, Fig. 1), a naturally occurring monoflavonoid extracted from *Scutellariae radix* [18], has been shown to be a promising candidate for selective and effective management of human cancers [19]. For example, wogonin-induced apoptosis has resulted in the significant inhibition of human ovarian cancer cells A2780 [20], human promyeloleukemic cells HL-60 [21,22] and human hepatocellular carcinoma cells SK-HEP-1 [22,23]. Knowledge of the molecular mechanisms of wogonin-induced apoptosis, however, is extremely scarce and remains to be delineated. Here, we demonstrate the involvement of bax/bcl-2 as the molecular mechanism of wogonin-induced apoptosis of cultured human hepatoma cell line SMMC-7721. It is observed that wogonin treatment of cell line SMMC-7721 significantly reduced the expression of bc1-2 protein while increasing the

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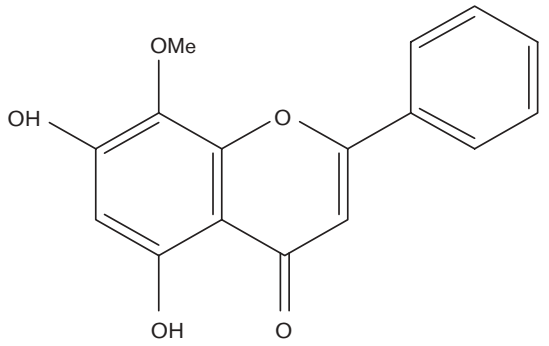
Fig. 1



Molecular structure of wogonin ($C_{16}H_{12}O_5$. MW: 284.27), Me = CH_3.

expression of bax protein in a time-dependent and concentration-dependent manner. Our results suggested that the potent anticancer activity of wogonin is mainly attributed to the modulation of the expression of two key proteins, bax/bcl-2, that are critical to the normal apoptosis of malignant cells.

Materials and methods

Cell culture

Human hepatoma cell line SMMC-7721 was purchased from the Cell Bank of Shanghai Institute of Biochemistry & Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI-1640 medium (lot 20040904; Gibco, Carlsbad, California, USA) at 37°C in a Water Jacketed CO_2 incubator (Thermo Forma, Waltham, Massachusetts, USA) in a humidified atmosphere with 5% CO_2. The culture medium was supplemented with 10% heat-inactivated calf serum (Sijiqing, Hangzhou, China), 100 U/ml penicillin G and 100 U/ml streptomycin.

Chemicals

Wogonin was isolated from *S. radix* according to the protocols reported previously [24] with slight modifications. Samples containing 99% or higher wogonin were used in all experiments unless otherwise indicated. The stock solution of wogonin was prepared using 30 mmol/l NaOH and was kept at −20°C until needed.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) was purchased from Fluka (St Louis, Missouri, USA) and was dissolved in 0.01 mol/l phosphate-buffered saline (PBS). Primary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, California, USA) and IRDye 800-conjugated anti-goat and anti-rabbit second antibodies were obtained from Rockland (Gilbertsville, Pennsylvania, USA). CycleTest Plus DNA reagent kit was obtained from Becton Dickinson (Franklin Lakes, New Jersey, USA). Annexin V–FITC Apoptosis Detection kit was purchased from Bender Medsystems (Burlingame, California, USA).

Colorimetric MTT assay

Human hepatoma cell line SMMC-7721 was cultured in RPMI-1640 medium according to the protocols described previously [25] till mid-log phase. Cells were harvested by centrifugation at 250 *g* for 5 min and resuspended in RPMI-1640 containing 0.02% ethylene diaminetetraacetic acid (EDTA) to make a stock cell suspension containing 4.4×10^4 cells/ml. One hundred microliters of this stock cell suspension was then added to the wells of a 96-well plate. A stock solution of wogonin was prepared using 30 mmol/l NaOH. Wogonin was applied at eight different final concentrations (0.3, 0.5, 4.0, 5.0, 10, 60, 80 and 100×10^{-5} mol/l). Medium was then added to bring the total volume of each well to 200 µl. After 24, 48 and 72 h of incubation, the culture medium was removed and the cells washed twice with PBS. MTT assay was performed using a Universal Microplate Reader (EL800; Bio-Tek Instruments, Winooski, Vermont, USA). Cell inhibition ratio (*I*%) was calculated by the following equation:

$$I\% = \left(\frac{A_{\text{control}} - A_{\text{treated}}}{A_{\text{control}}}\right) \times 100$$

where A_{treated} and A_{control} are the average absorbance of three parallel experiments from treated and control groups, respectively. The IC_{50} was taken as the concentration that caused 50% inhibition of cell proliferation and was calculated by SAS statistical software (SAS Institute, Cary, North Carolina, USA).

Cell morphological assessment

Human hepatoma cell line SMMC-7721 was cultured in RPMI-1640 till mid-log phase. Wogonin (1×10^{-4} mol/l) was then added to the culture media and incubation was continued for an additional 24 or 48 h. At the end of incubation, the morphology of cells was monitored under an inverted light microscope. All floating and attached cells were harvested with 0.02% (w/v) EDTA and 0.25% (w/v) trypsinase. The cell suspension (95 µl) was mixed with 5 µl of dye mixture containing 100 mg/l acridine orange (AO) and 100 mg/l ethidium bromide (EB) in PBS. The cells were observed immediately under a fluorescence microscope (Olympus IX51; Olympus, Tokyo, Japan) with a peak excitation wavelength of 490 nm [26].

DNA content and cell cycle analyzed by flow cytometry

The logarithmic cells were dispersed with 0.02% EDTA to prepare a cell suspension containing 1×10^6 cells/ml. Cells were treated with 1×10^{-4} mol/l wogonin for 24, 48 and 72 h, then collected and washed with buffer solution, treated with solutions A and B, and stained with solution C before DNA content was determined. The buffer and solutions A, B, and C were from the CycleTest Plus DNA

reagent kit (Becton Dickinson). DNA content and cell cycle were assessed by flow cytometry (FACSCalibur, Becton Dickinson), and analyzed by the software Modfit and CellQuest (BD Biosciences, Franklin Lakes, New Jersey, USA).

Annexin-V/propidium iodide double-staining assay

Cells (1×10^6) were seeded in 50-ml flasks and incubated for 24 h at 37°C. Wogonin (5×10^{-5}, 1×10^{-4} and 2×10^{-4} mol/l) was directly added into the flasks and incubation was continued for an additional 48 h. Cells were harvested, and 1×10^6 cells were washed and resuspended with PBS. Apoptotic cells were identified by double supravital staining with recombinant fluorescein isothiocyanate (FITC)-conjugated Annexin-V and propidium iodide (PI), using the Annexin V–FITC Apoptosis Detection kit according to the manufacturer's instructions. Flow cytometric analysis was performed immediately after supravital staining. Data acquisition and analysis were performed in a Becton Dickinson FACSCalibur flow cytometer using CellQuest software. Cells not binding FITC–Annexin-V and excluding PI were classified as Annexin-V-negative [27]. Cells that bound FITC–Annexin-V [excitation wavelength (λ_{ex}) = 488 nm and emission wavelength (λ_{em}) = 520 nm] but excluded PI (λ_{ex} = 540 nm and λ_{em} = 630 nm) were termed Annexin-V-positive and cells permeant to PI (regardless of whether or not they bound FITC–Annexin-V) were deemed necrotic [28].

DNA fragmentation assay

Wogonin-induced apoptosis of human hepatoma cell line SMMC-7721 was detected by DNA fragmentation assay. Cells were collected and stored at 4°C after treated with 5×10^{-5}, 1×10^{-4} and 2×10^{-4} mol/l wogonin and 1×10^{-5} mol/l As_2O_3 for 48 h. In this assay, DNA was extracted by the Genomic DNA Purification Kit (Fermentas, Hanover, Maryland, USA) according to the manufacturer's instructions, and then the products were analyzed by electrophoresis on a 1.5% agarose gel and observed by EB staining using Gel-Pro analyzer (Genegenius, Syngene, Frederick, Maryland, USA).

Western blot analysis for bax/bcl-2 proteins

After incubation with 5×10^{-5}, 1×10^{-4} and 2×10^{-4} mol/l wogonin and 1×10^{-5} mol/l As_2O_3 [29] for 48 h, proteins were isolated by lysis buffer [100 mol/l Tris–Cl, pH 6.8, 4% (m/v) sodium dodecyl sulfate, 20% (v/v) glycerol, 200 mmol/l β-mercaptoethanol, 1 mmol/l phenylmethylsulfonyl fluoride and 1 μg/ml aprotinin] and measured using the Bradford assay with BioPhotometer (BioPhotometer 6131GB/HK; Eppendorf, Westbury, New York, USA) at 595 nm. Protein samples were separated with 18% sodium dodecyl sulfate–polyacrylamide gel and transferred onto the polyvinylidine diflouride membranes (Millipore, Bedford, Massachusetts, USA). Immune complexes were formed by incubation of the proteins with primary antibodies, rabbit anti-Bax, rabbit anti-Bcl-2 and goat anti-actin overnight at 4°C. Blots were washed and incubated for 1 h with IRDye 800-conjugated anti-goat and anti-rabbit secondary antibodies. All antibodies were obtained from Santa Cruz Biotechnology. Immunoreactive protein bands were detected with an Odyssey Scanning System (LI-COR Lincoln, Nebraska USA).

Reverse transcription-polymerase chain reaction assay for mRNA levels of bcl-2/bax

After incubation with 5×10^{-5}, 1×10^{-4} and 2×10^{-4} mol/l wogonin and 1×10^{-5} mol/l As_2O_3 for 48 h, total RNA of cells was extracted by TriPure Isolation Reagent (Roche, Indianapolis, Indiana, USA). The primers for Bcl-2, Bax and glyceraldehyde-3-phosphate dehydrogenase were as follows: bcl-2 (340 bp): 5′-TTCCCATCGCTGTCCT TCG-3′, 3′- CGCTTAGATACAAATGTCCGTGTC-5′; bax (386 bp): 5′-GGATGCGTCCACCAAGAA-3′, 3′-AAA-CACCGCCCTCACG-5′; glyceraldehyde-3-phosphate dehydrogenase (450 bp): 5′-CTCAGACACCATGGGGA AGGTGA-3′, 3′-ATACTGTTGTCGGAGTTCTAGTA-5′. The following polymerase chain reaction conditions were used: at 94°C for 5 min, 1 cycle; at 94°C for 30 s, at 59°C (bcl-2) or 56°C (bax) for 30 s, at 72°C for 45 s, 35 (bcl-2) or 30 (bax) circles; at 72°C for 7 min, 1 circle. The polymerase chain reaction products were separated by electrophoresis using a 1.5% agarose gel and observed by EB staining using Gel-Pro analyzer.

Statistical evaluation

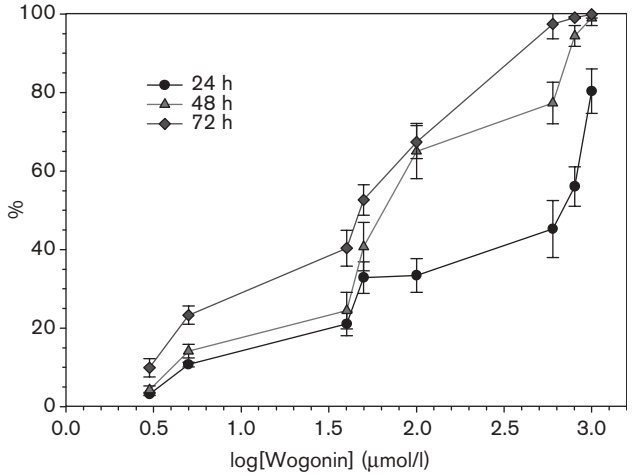
All results shown represent the means ± standard deviation from triplicate experiments performed in a parallel manner unless otherwise indicated. Statistical analyses were performed using an unpaired, two-tailed Student's *t*-test. All comparisons are made relative to untreated controls and significance of difference is indicated as $^*P < 0.05$ and $^{**}P < 0.01$.

Results

Cell growth inhibition assessed by colorimetric MTT assay

Human hepatoma cell line SMMC-7721 was cultured in the absence and presence of varying concentrations of wogonin. The effects of wogonin on cell growth were assessed by the commonly used MTT assay. No significant cell inhibition was observed when wogonin was applied at concentrations lower than 0.3×10^{-5} mol/l (data not shown). Potent cytotoxicity against SMMC-7721 cells, however, was observed when wogonin was applied at higher concentrations ($P < 0.01$, unpaired *t*-test) as shown in Fig. 2. It was also observed that the degree of inhibition was directly correlated with exposure time at a given wogonin concentration. For example, approximately 30% inhibition was observed when 1.0×10^{-4} mol/l wogonin was applied for a period of 24 h. Increasing the contact time to 72 h with the same concentration of wogonin, the inhibition rate increased

Fig. 2



Inhibitory effects of wogonin on the proliferation of a human hepatoma cell line SMMC-7721. The results shown are the mean of three parallel experiments for each concentration point.

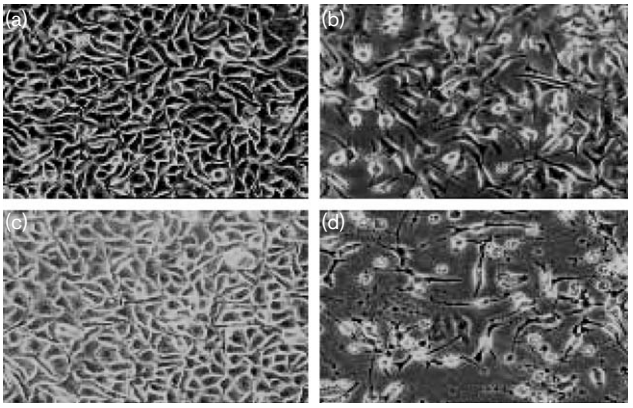
to about 65% as shown in Fig. 2. The IC_{50} of wogonin against SMMC-7721 was derived from a statistical analysis (SAS software) of the data shown in Fig. 2. This parameter is found to be time dependent, and values of 30.70 ± 0.21, 5.63 ± 0.13 and $2.46 \pm 0.06 \times 10^{-5}$ mol/l were obtained for 24, 48 and 72 h treatment, respectively.

Cell morphological assessment

Inverted microscopy demonstrated that the majority of SMMC-7721 cells were severely distorted (elongated), forming long filaments, whereas some cells turned round in shape after treatment with 1×10^{-4} mol/l wogonin for 24 h as shown in Fig. 3(b). Extending the incubation period to 48 h significantly increased the number of cells that underwent necrosis as evidenced by the round-shaped morphology of the cells (Fig. 3d). The untreated cells displayed normal, healthy growth as demonstrated by the clear skeletons observed by inverted microscopy (Fig. 3a and c).

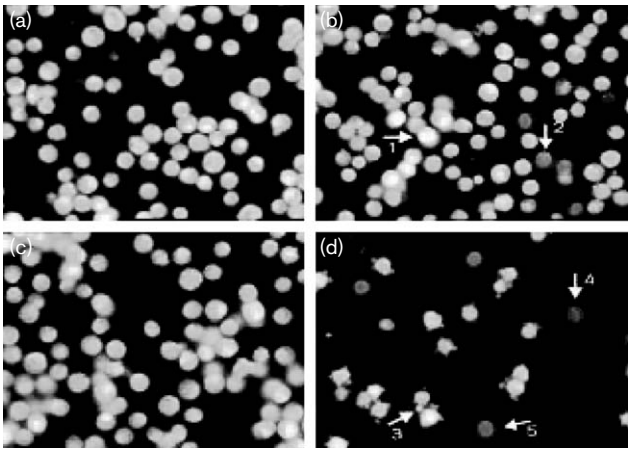
Consistent with the results from inverted microscopic studies, fluorescence microscopic analysis also showed clear morphological changes typical of early apoptosis when treated with wogonin (1×10^{-4} mol/l) for a period of 24 h as shown in Fig. 4(b). The bright condensed chromatin identified by AO staining (arrow 1 in Fig. 4b) is a clear indication of early apoptosis. Typical apoptotic blebbing required 48 h incubation of the cells with wogonin (Fig. 4d). Late apoptotic cells were observed by EB staining of the cells after 48 h treatment with wogonin (Fig. 4d). Similar to what was observed with inverted microscopy, untreated cells displayed normal, healthy growth as reflected by the clear skeletons of the cells (Fig. 4a and c).

Fig. 3



Wogonin (1×10^{-4} mol/l)-induced morphology change of SMMC-7721 cells observed with an inverted light microscope ($\times 200$). (a) Control (24 h), (b) with wogonin (24 h), (c) control (48 h) and (d) with wogonin (48 h).

Fig. 4



Wogonin-induced morphological change of SMMC-7721 detected with a fluorescence microscope ($\times 200$). (a) Control (24 h) (b) with and 1×10^{-4} mol/l wogonin (24 h), the early apoptotic cells are observed: the cells were stained green with acridine orange (AO), their membranes were still intact and the nuclei exhibited bright condensed chromatin (arrow 1); dead cells were stained aequalis red with ethidium bromide (EB) (arrow 2). (c) Control (48 h) and (d) with 1×10^{-4} mol/l wogonin (48 h), typical apoptotic blebbing (arrow 3) is obvious; the late apoptotic cells were observed: their nuclei exhibited condensed chromatin and they were stained red with EB (arrow 4); dead cells were stained aequalis red with EB (arrow 5).

DNA content and cell cycle analyzed by flow cytometry

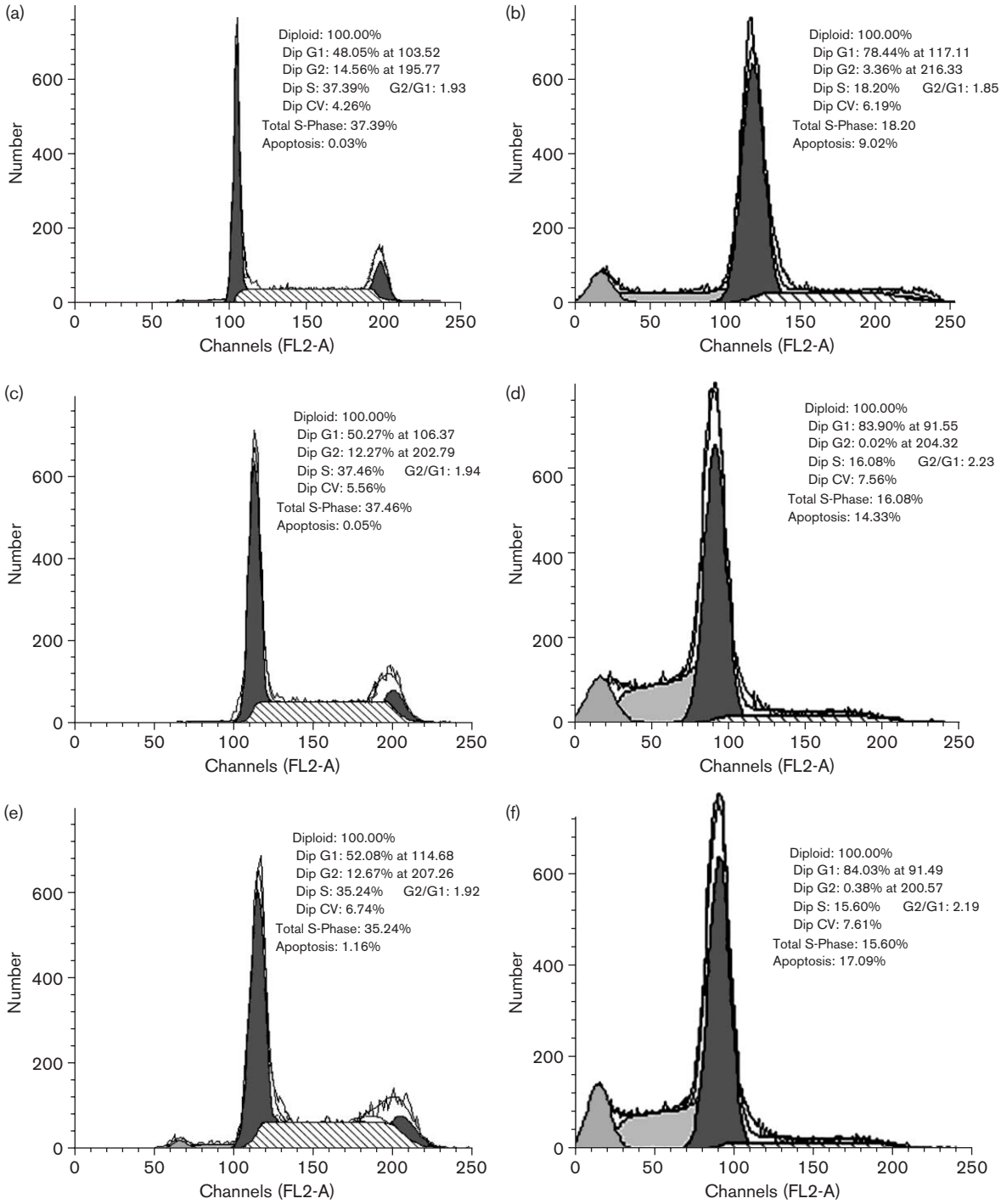
Incubation of SMMC-7721 cells with 1×10^{-4} mol/l wogonin for 24, 48 and 72 h resulted in the apoptosis of 9.02, 14.33 and 17.09%, respectively, of cultured cells, significantly higher than the rate observed in the control groups (0.03, 0.05 and 1.16% for 24, 48 and 72 h treatment, respectively). The proportion of G_0/G_1 phase

cells increased dramatically after treatment with 1×10^{-4} mol/l wogonin as shown in Fig. 5. No significant change in G_2/G_1 ratio was observed with wogonin treatment in our experiments.

Apoptosis assessment by Annexin-V and propidium iodide double-staining assay

For each sample, viable cells, necrotic cells and early apoptotic cells were obtained separately by Annexin-V

Fig. 5



Cell cycle analysis after treatment with wogonin (1×10^{-4} mol/l) for 24, 48 and 72 h. (a) Control (24 h), (b) with wogonin for 24 h, (c) control (48 h), (d) with wogonin for 48 h, (e) control (72 h) and (f) with wogonin for 72 h. The proportions of G_0/G_1 phase cells in wogonin-treated groups were 78.44, 83.90 and 84.03%, whereas those in the control groups they were 48.05, 50.27 and 52.08% after 24, 48 and 72 h incubation, respectively. Results shown are from one of the three experiments performed.

and PI double-staining assay. The early apoptotic cells were divided from the necrotic cells (including late apoptotic cells) and the viable cells. Those labeled with Annexin-V^+/PI^- cells were early apoptotic cells. Treatment of SMMC-7721cells with wogonin for a period of 48 h significantly increased the percentages of apoptotic cells from 0.76% (Fig. 6a) in the control group to 5.62 (Fig. 6b), 11.38 (Fig. 6c) and 17.32% (Fig. 6d) for 5×10^{-5}, 1×10^{-4} and 2×10^{-4} mol/l wogonin, respectively. Apoptosis was further confirmed from our DNA fragmentation experiments (data not shown).

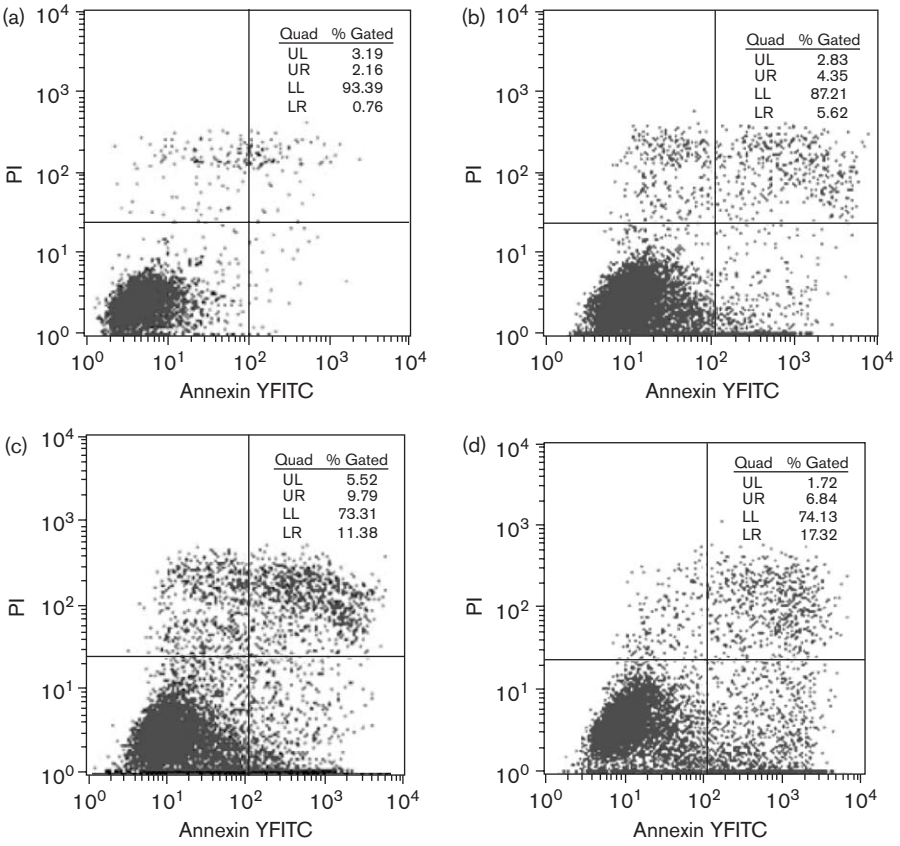
Expressions of bcl-2 protein and bax protein and synthesis of mRNA

After incubation with 5×10^{-5}, 1×10^{-4} and 2×10^{-4} mol/l wogonin and 1×10^{-5} mol/l As_2O_3 for 48 h, the expression of bcl-2 protein decreased dramatically, whereas the expression of bax protein increased significantly. The levels of bcl-2 and bax protein expression in SMMC-7721 were clearly correlated to the concentration of wogonin applied as shown in Fig. 7(a). When wogonin was applied at a given concentration of 1×10^{-4} mol/l, the levels of bcl-2 and bax protein expression in SMMC-7721 were clearly correlated with the duration of wogonin treatment as shown in Fig. 7(b). Similar treatments resulted in a decrease in the mRNA level of bcl-2 (Fig. 7c). The mRNA level of bax, however, increased noticeably in wogonin-treated cells as shown in Fig. 7(c). It is worth mentioning that in addition to bax/bcl-2, wogonin treatment also influenced the expression of other apoptosis proteins, such as caspase-3 (data not shown).

Discussion

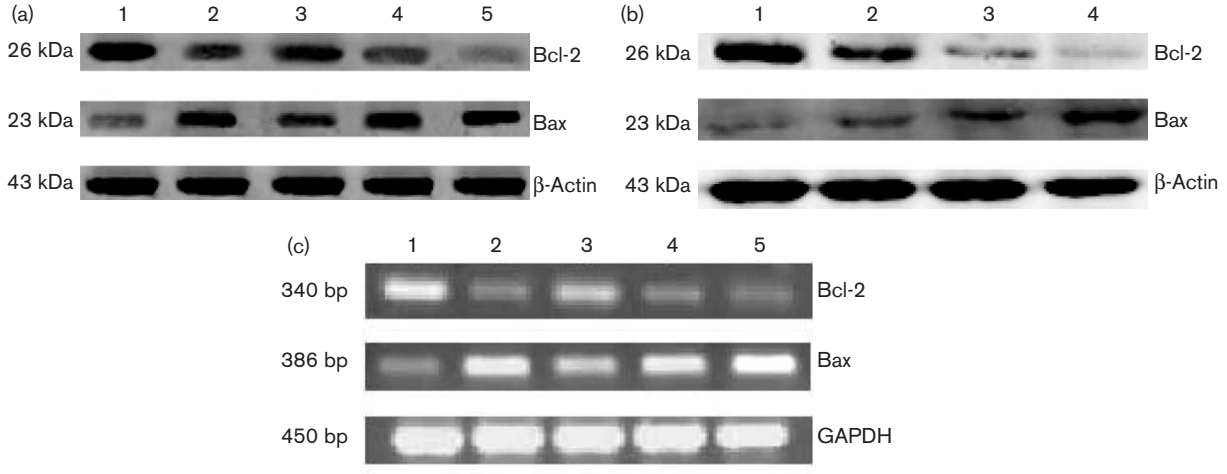
Wogonin isolated from *S. radix* has been reported to have antiproliferative effects, and to inhibit various malignant cell lines including human ovarian cancer cell A2780 [20], human promyeloleukemic cell HL-60 [21,22] and human hepatocellular carcinoma cells SK-HEP-1 [22,23]. Knowledge of the molecular mechanisms behind wogonin's potent apoptotic activity, however, is extremely limited

Fig. 6



Fluorescence-activated cell sorter analysis for Annexin-V and propidium iodide (PI) staining of SMMC-7721 cells incubated with wogonin at different concentrations for 48 h. (a) Control, (b) 5×10^{-5} mol/l, (c) 1×10^{-4} mol/l and (d) 2×10^{-4} mol/l. Upper right: necrotic cells and late apoptotic cells labeled with PI and Annexin-V–fluorescein isothiocyanate (FITC). Lower left: fully viable cells. Lower right: early apoptotic cells labeled with Annexin-V–FITC but not with PI. After exposure to wogonin, the cells of the lower right had increased from 0.76 (control) to 5.62 (5×10^{-5}), 11.38 (1×10^{-4}) and 17.32% (2×10^{-4} mol/l).

Fig. 7



Determination of bcl-2/bax protein and mRNA in SMMC-7721 cells. (a) The dose–response, (b) the time course of bcl-2/bax protein expression, and (c) the synthesis of Bax and Bcl-2 mRNA levels in wogonin-treated SMMC-7721 cells. Gels shown are from one of the triplicate experiments. In (a) and (c), lane 1: control; lane 2: 1×10^{-5} mol/l As_2O_3; lane 3: 5×10^{-5} mol/l; lane 4: 1×10^{-4} mol/l; lane 5: 2×10^{-4} mol/l wogonin, respectively. It is obvious that wogonin treatment increased the expression of bax, whereas it decreased the synthesis of bcl-2 protein in SMMC-7721 cells. In (b) cells were treated with a fixed concentration of wogonin (1×10^{-4} mol/l) for varying periods of time. Lane 1: control (no wogonin applied); lane 2: 24 h; lane 3: 48 h; lane 4: 72 h.

and remains to be uncovered. In this study, we report the involvement of bax/bcl-2 as the molecular mechanism of wogonin-induced apoptosis of a cultured human hepatoma cell line, SMMC-7721. Our results demonstrated that the extent of inhibition depends on both the length of reaction time and the concentration of wogonin applied. The value of IC_{50} at 48 h was determined to be 5.63×10^{-5} mol/l. SMMC-7721 cells incubated with wogonin displayed typical apoptotic characteristics (Figs 3 and 4) including cytoplasmic shrinkage, plasma membrane blebbing, nuclear chromatin condensation, chromosomal DNA cleavage and fragmentation of the cells into membrane-enclosed vesicles or apoptotic bodies [3–5]. The results of flow cytometry further confirmed the results of microscopic observations as evaluated by the increased proportion of G_0/G_1 phase cells with wogonin treatment (Fig. 5).

Phosphatidylserine (PS), normally confined in the inner cytoplasmic leaflet of the plasma membrane in healthy cells, was translocated and exposed to the outer leaflet at the beginning of apoptosis, therefore providing a 'molecular marker' on apoptotic cells [30]. Annexin-V has a high affinity for PS and binds to cells with exposed PS. Use of Annexin-V in combination with PI allowed the distinction of early apoptotic and necrotic cells from viable cells. Our results revealed that wogonin efficiently induced apoptosis of SMMC-7721 cells when applied at appropriate concentrations. The apoptotic rate reached 17.32% after incubation with 2×10^{-4} mol/l wogonin. Most significantly, our studies showed, for the first time, that wogonin treatment resulted in an obvious decrease in bcl-2 protein expression and a notable increase in bax protein production in SMMC-7721 cells. It is therefore postulated that wogonin's efficient inhibitory effect on tumor cell growth is achieved by its ability to modulate the expression of bcl-2/bax proteins that are involved in the regulation of normal cell apoptosis, similar to retinoid-initiated apoptosis in pancreatic cancer cells [31].

It is now well established that apoptosis is a complex biological process involving many pathways. The recent evidence suggests the presence of two types of genes that control apoptosis: the proapopototic genes including WTp53, EIA, *ced3* and *ced4*, and Fas, and the antiapoptotic genes including mutant p53, *bcl-2*, c-*fos* and c-*myc* [32]. Of all these regulatory factors, the proteins of the bcl-2 family are of special importance to normal apoptosis [33]. The bcl-2 family includes proapoptotic members such as bax, bak, bad, bcl-x_S, bid, bik, bim and hrk, and antiapoptotic members such as bcl-2, bcl-x_L, bcl-w, bfl-1 and mcl-1 [34]. Antiapoptotic protein bcl-2 acts as a repressor of apoptosis [35], whereas proapoptotic protein bax acts as a promoter [36]. Interestingly, these effects are more dependent on the ratio between bcl-2 and bax than on the quantity of bcl-2 alone [31,37,38]. Sato and coworkers [39] have proposed two possibilities for the observed relationship between bcl-2 and bax proteins: (i) bcl-2 induced a pathway that actively maintained cell survival, with bax serving as a negative regulator of bcl-2 and (ii) bax directly or indirectly generated cell death signals, bcl-2 being the dominant inhibitor of bax. Thus, the ratio of bc1-2/bax

might be a critical factor of a cell's threshold for apoptosis. The high apoptosis rate of SMMC-7721 observed in this study is therefore attributed to wogonin's ability to lower the ratio of bc1-2/bax in SMMC-7721 cells.

Chen *et al.* [23] reported that wogonin-induced apoptosis in hepatocellular carcinoma cells SK-HEP-1 proceeded through the induction of p53 protein, activation of caspase-3 cascade and alternative expression of p21. It is well known that (p53)–(bax)–(Apaf-1)–(Caspase Cascade) was an efficient apoptotic pathway. The induction of p53, the upstream protein, resulted in the upregulation of bax protein and the activation of caspase-3 protein. In addition, p21 protein of the cyclin kinase inhibitor family could inhibit cyclin-dependent kinase cyclin compounds, especially CDK4/6–cyclinD compounds, therefore, blocking cell cycle in G_0/G_1 phase. The results presented in this paper also showed that SMMC-7721 cell cycle was blocked in G_0/G_1 phase after treatment with wogonin (Fig. 6). The mechanism for the accumulation of G_0/G_1 phase cells remains undefined from the present study and is currently under investigation in our laboratories.

In conclusion, we have demonstrated that wogonin induces apoptosis in human hepatoma cell line SMMC-7721 via the modulation of bcl-2/bax protein expression. This finding reveals an interesting correlation between gene regulation and wogonin-induced apoptosis, and provides a molecular basis for the development of naturally occurring monoflavonoids as novel anticancer agents for better management of human cancers.

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