VI-16, a newly synthesized flavonoid, induces apoptosis through the mitochondrial pathway in human hepatoma cells

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Abstract. VI-16, a newly synthesized flavonoid, has a hydroxy substitution at C5 position, a methoxyl substitution at C5 position, and a piperazine substitution at C7 position. Here, we firstly investigated the potential antitumor effect of VI-16 in HepG2 human hepatocarcinoma cells. The MTT assay showed that VI-16 inhibited HepG2 cell growth in a concentration- and time-dependent manner. To further investigate whether apoptosis induction contributed to the antitumor effects of VI-16, DAPI staining and Annexin-V/PI double staining were performed in our tests. The data showed that VI-16 could induce apoptotic cell death in HepG2 cells. Moreover, mechanistic studies revealed that VI-16-induced apoptosis was a caspase-dependent process by decreasing the expression of pro-caspase-3. The changes in the expression of caspase-8, capsase-9, Bax and Bcl-2 after VI-16 treatment suggested that the mitochondrial pathway was involved in the apoptosis induced by VI-16. Furthermore, VI-16 could significantly increase the loss of mitochondrial membrane potential and the expression of p53. Taken together, these results demonstrated that apoptosis induced by VI-16 might be one of the mechanisms by which VI-16 acts as a preventive antitumor drug against human hepatoma.

Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide based on statistical analysis of the number of cases. Due to the very poor prognosis, the overall survival of patients with hepatocellular carcinoma is the third lowest. Of the cases, 82% are observed in developing countries, with 55% in China alone (1). Chemotherapy has provided significant survival benefits for patients with HCC. However, increased concentrations of cytotoxic drugs and higher doses of irradiation often induce severe adverse effects hard to tolerate for these patients, resulting in a failure of chemotherapy. Thus, it is imperative to develop anticancer agents that have low normal tissue toxicity as well as high anticancer activities (2).

Apoptosis, a programmed cell death, is critical for normal development, function and homeostasis of multicellular organisms. Reduced apoptosis is thought to be a fundamental component in the pathogenesis of cancer. It is known that dysfunctions in the apoptotic pathway and uncontrolled cellular proliferation will ultimately lead to carcinogenesis and tumor progression (3). As an aim of anticancer treatment, cell apoptosis is characterized by the cell shrinkage, blebbing of the plasma membrane, and chromatin condensation that are associated with cleavage of DNA into ladders (4). However, in response to some effective therapeutic treatments, resistance to apoptosis may occur in human malignant tumor cells (5). Therefore, further development of agents that can induce or enhance the extent of apoptosis may be a promising strategy in the treatment of cancer. Apoptosis is mediated through two major pathways, the death receptor pathway and the mitochondrial pathway. In the death receptor pathway, surface receptors interact with ligands, and deliver a death signal from the extracellular microenvironment to the cytoplasm before leading to apoptosis (6).

The mitochondrial pathway is initiated by stimuli such as cytotoxic drugs and cellular distress. It is a complex course with mitochondria as central gateway controllers and the Bcl-2 family of proteins as executioners (7). The crucial step in the mitochondrial apoptotic pathway is loss of mitochondrial membrane potential (MMP), triggering the activation of the apoptotic cascade and the execution of cell death (8). In addition, mitochondria are the most important sources of cellular reactive oxygen species (ROS). The moderate increase of ROS in cancer cells has significant effects, such as stimulating cellular growth, promoting mutations, and inducing resistance to anticancer agents (9). Tumor suppressor gene p53, which plays an important role in the cell, preventing damaged or abnormal cells from becoming malignant (10), is involved in

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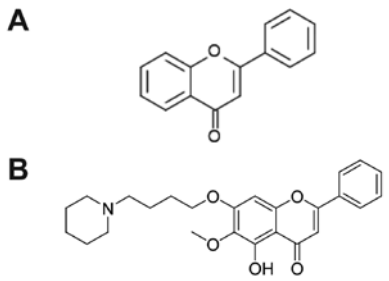

Figure 1. (A) Molecular structure of the flavone backbone; (B) molecular structure of VI-16 ($C_{25}H_{29}O_5N$, MW, 423).

the mitochondrial apoptotic pathway as well. The reaction of p53 in inducing apoptosis is tightly associated with mitochondrial and Bcl-2 family of proteins (11).

Flavonoids are found in almost every plant, which act as pharmacologically active constituents in many plant medicines. Epidemiological studies have shown that flavonoids display a remarkable array of biochemical and pharmacological actions, including anti-oxidative, anti-viral, anti-thrombotic, anti-inflammatory, and anti-cardiovascular disease activities (12). Importantly, there have been many reports about their anticancer activity in recent years (13). As a newly synthesized flavonoid, which bears the same three-ring structure of the flavone backbone (Fig. 1A), VI-16, with OH substitutions at C5 position, OCH3 substitutions at C5 position, and a piperazine substitution at C7 position (Fig. 1B), may have anticancer properties. In the present study, we demonstrated that VI-16 possessed anticancer activity by inducing apoptosis through the mitochondria pathway in human hepatoma cell line HepG2 cells.

Materials and methods

Reagents. VI-16 ($C_{25}H_{29}O_5N$, Fig. 1B) was obtained from Z.L. (China Pharmaceutical University, China). In the experiments, samples containing 95% or higher VI-16 were used. It was dissolved in 100% dimethylsulfoxide (DMSO) as a stock solution, stored at -20˚C, and diluted with medium before each experiment. The final DMSO concentration did not exceed 0.1% throughout the study.

Antibodies to caspase-3, caspase-9, caspase-8, Bax, Bcl-2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); the antibody to p53 was from Calbiochem (Merck, Darmstadt, Germany); the antibody to β-actin was from Boster (Wuhan, China). IRDye™ 800-conjugated secondary antibodies were obtained from Rockland, Inc. (Philadelphia, PA, USA) and diluted at the ratio of 1:15,000. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenytetrazolium bromide] was obtained from Fluka Chemical Corp. (Ronkonkoma, NY) and was dissolved in 0.01 M PBS.

Cell culture. Human hepatocellular carcinoma HepG2 cells were purchased from Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in 90% RPMI-1640 medium (Gibco, Invitrogen, Inc.) supplemented with 10% heat-inactivated fetal bovine serum (Sijiqing, Hangzhou, China), 100 U/ml penicillin, and 100 μg/ml streptomycin. Exponentially growing cultures were maintained in a humidified atmosphere of 5% CO_2 at 37˚C.

Cell viability assay. The colorimetric MTT assay was used to measure the effects of VI-16 to cell viability of HepG2 cells. The logarithmic cells were seeded at a density of $5x10^3$ to $7x10^3$ cells per well into 96-well plates at 100 μl/well for 24 h at 37˚C. Then, the cells were treated with or without different concentrations of VI-16 for 24 and 48 h in 5% CO_2 incubator at 37˚C, respectively. At the end of the assay period, 20 μl of a 5 mg/ml solution of MTT (Sigma-Aldrich, St. Louis, MO) (20 μl/well) was added and incubated for 4 h at 37˚C. Then the supernatant was discarded and DMSO was added (100 μl/well). The absorbance (A) of the solution in the well was measured at 570 nm by a universal microplate reader (EL800, Bio-Tek Instruments, Inc.).

Cell morphological assessment. Human hepatoma HepG2 cells were seeded in 6-well tissue culture plates at a concentration of $1x10^5$ cells/well and treated with VI-16 (0, 20, 40 and 60 μM) for 48 h. At the end of the incubation, the morphology of the cells was monitored under an inverted light microscope. Then the cells were fixed with ice-cold 4% paraformaldehyde for 20 min, permeabilized with 0.1% Triton X-100 for 25 min and washed with ice-cold PBS. At last, the cells were stained with the fluorochrome dye DAPI (1 μg/ml) (4', 6-diamidino-2-phenylindole; Santa Cruz, CA) for 25 min in the dark and observed under a fluorescence microscope (Olympus IX51, Japan) with a peak excitation wavelength of 340 nm.

Flow cytometry assay of apoptosis. Apoptosis in the plasma membrane was identified by flow cytometry with Annexin-V/PI (propidium iodide) staining. HepG2 cells ($1x10^5$) were treated with different concentrations of VI-16 (0, 20, 40 and 60 μM) during the exponential growth phase. The cells were collected after being cultured for 48 h and washed once in ice-cold PBS. Apoptotic cells were identified by using the Annexin V-FITC Apoptosis Detection kit (KeyGen, Nanjing, China). Apoptotic cell death was examined by FACSCalibur flow cytometry (Becton-Dickinson, San Jose, CA).

Flow cytometry assay of mitochondrial membrane potential. Mitochondrial membrane potential changes were assayed with the mitochondrial membrane potential detection kit (KeyGen). After being incubated with VI-16 (0, 20, 40 and 60 μM) for 48 h, all floating and attached cells were harvested and resuspended with ice-cold PBS (2000 rpm x 5 min). Then cells were incubated in JC-1 prepared with 1X incubation buffer for 20 min at 37˚C, and detected with a FACSCalibur flow cytometer (Becton-Dickinson).

Western blot analysis. HepG2 cells were treated with VI-16 (0, 20, 40 and 60 μM) for 48 h. Cells were harvested and rinsed twice with ice-cold PBS. Proper volumes of western blotting lysing buffer (100 mM Tris-HCl, pH 6.8, 4% (m/v) SDS, 20% (v/v) glycerol, 200 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 1 g/ml aprotinin) was added to cell pellets and the mixture was sonicated on ice for 1 h. The mixture was microcentrifuged at 12,000 rpm at 4˚C for

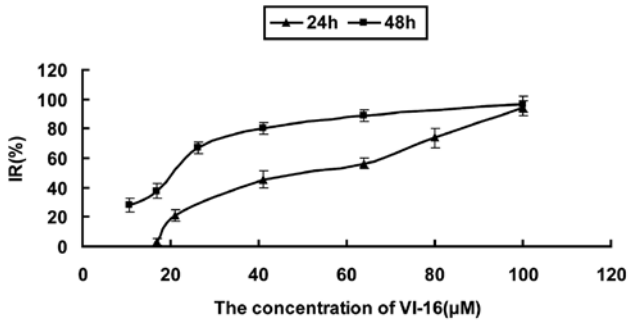

Figure 2. Growth inhibition of HepG2 cells induced by VI-16. HepG2 cells were treated with VI-16 at the concentration range of 10-100 μM for 24 and 48 h, respectively. The results shown are the mean of three parallel experiments for each concentration point. Error bars represent ± SD.

20 min and the supernatant was preserved at -20˚C for later use. The concentration of proteins was measured using the BCA assay method with a Varioskan spectrofluorometer and spectrophotometer (Thermo, Waltham, MA) at 562 nm.

Proteins were separated by 10 or 12% SDS-PAGE gel electrophoresis, and transferred to a nitrocellulose membrane. Immune complexes were formed by incubation of proteins with a 1:500 dilution of primary antibodies at 4˚C overnight followed by IRDye™ 800-conjugated second antibody for 1 h at 37˚C. Immunoreactive protein bands were detected with an Odyssey Scanning system (LI-COR Inc., Superior St. Lincoln, NE).

Statistical evaluation. Results are presented as the mean ± SD from triplicate experiments performed in a parallel manner unless otherwise indicated. Statistical analyses were performed using one-way analysis of variance (ANOVA), followed by the Bonferroni post-hoc test for multiple-group comparisons. All comparisons are made relative to untreated controls and significant differences are indicated by $P<0.05$ or $P<0.01$.

Results

VI-16 inhibits the growth of HepG2 cells. The MTT assay was used to investigate the inhibitory effect of VI-16 on HepG2 cells. As shown in Fig. 2, after incubation with different doses of VI-16 (from 10 to 100 μM) for different time periods (24 and 48 h), HepG2 cells presented obvious growth inhibition. The IC_{50} values at 24 and 48 h were 46.3±7.75 and 19.7±2.12 μM, respectively. These data indicate that VI-16 inhibited the viability of HepG2 cells in a time- and dose-dependent manner.

Cell morphological assessment after VI-16 treatment. Changes in the morphology of HepG2 cells with characteristic apoptotic appearance were observed after VI-16 treatment. As shown in Fig. 3A HepG2 cells were severely distorted and grew slowly after treatment with VI-16 (0, 20, 40 and 60 μM) for 48 h. Especially at the highest concentration, more than half of HepG2 cells turned to be round in shape and detached. The untreated HepG2 cells showed a normal, healthy shape with clear skeletons. Under the fluorescence microscope, the cellular nucleolus changes were observed. Untreated HepG2 cells were stained by blue fluorescence demonstrating the steady chromatin distribution in the nucleolus. On the other hand, VI-16-treated cells emitted bright fluorescence and the early phenomena of apoptosis, especially apoptotic bodies and nuclei pyknosis were observed (Fig. 3B).

VI-16 induces apoptosis in HepG2 cells. VI-16-induced apoptosis in HepG2 cells was further determined using Annexin-V/PI staining assay, after treatment with 20, 40 and 60 μM for 48 h, the percentages of early apoptotic cells were 8.6, 16.0 and 40.8%, respectively (Fig. 4). These results indicate that induction of apoptosis by VI-16 is involved in its antitumor activity.

Effects of VI-16 on the expression of apoptosis-related proteins in HepG2 cells. After treatment with VI-16 for 48 h, the expressions of pro-caspase-3 obviously decreased in a concentration-dependent manner in HepG2 cells (Fig. 5A). The result suggested that the VI-16-induced apoptosis of HepG2 cells involved a caspase-dependent apoptotic process.

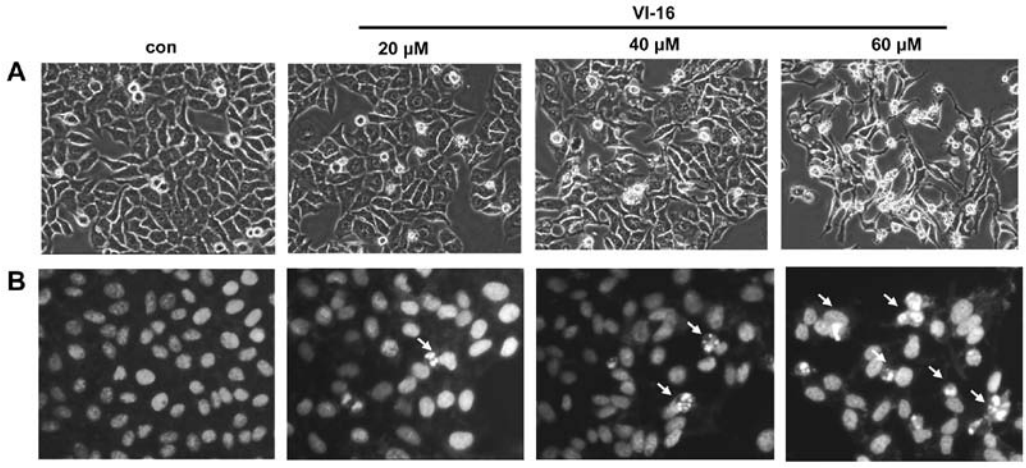

Figure 3. Morphological changes in HepG2 cells treated with VI-16 for 48 h. (A) Cell morphological changes observed under an inverted light microscope (x400). (B) Nucleolus morphological changes observed by a fluorescence microscope (x400). The white arrows indicate the apoptotic cells with nuclear fragments.

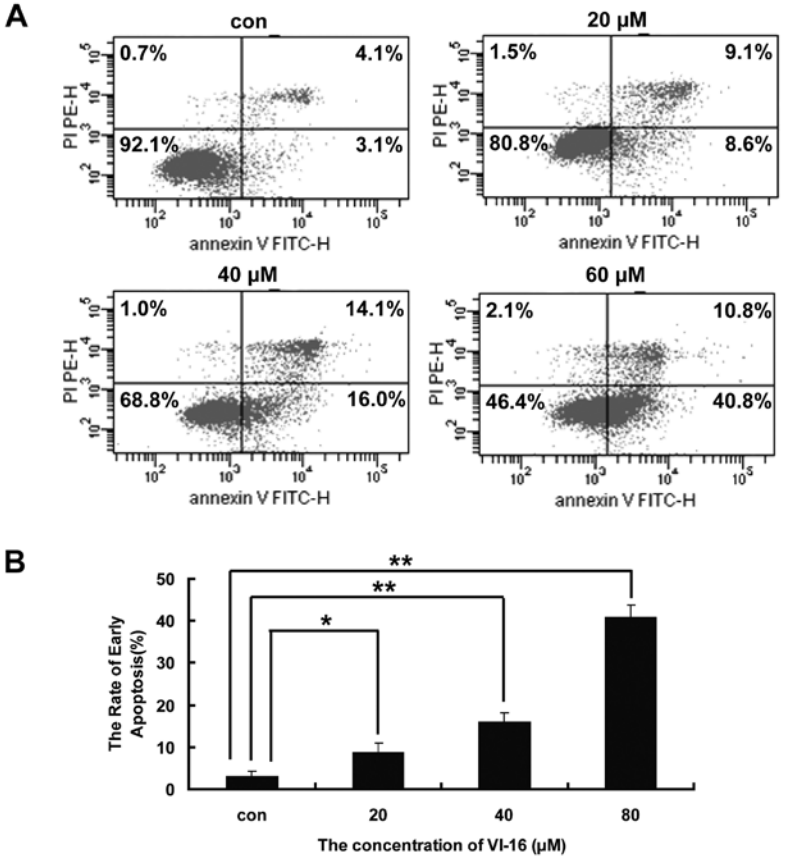

Figure 4. The apoptotic inducing effects of VI-16 were measured by Annexin-V/PI double-staining assay. (A) HepG2 cells were treated with VI-16 (0, 10, 20 and 40 μM) for 48 h, collected and incubated with Annexin-V/PI for 30 min at 37˚C, and then detected by flow cytometry. The lower left quadrant contains the vital (Annexin-V^-/PI^-) population. The lower right quadrant contains the apoptotic (Annexin-V^+/PI^-) population. The upper right quadrant contains the late apoptotic/necrotic (Annexin-V^+/PI^+) population. The upper left quadrant contains the pre-necrotic (Annexin-V^-/PI^+) population. (B) The early apoptosis rates was quantified and are presented by histogram. Error bars represent ± SD. *P<0.05 or **P<0.01 indicate significant differences compared with control.

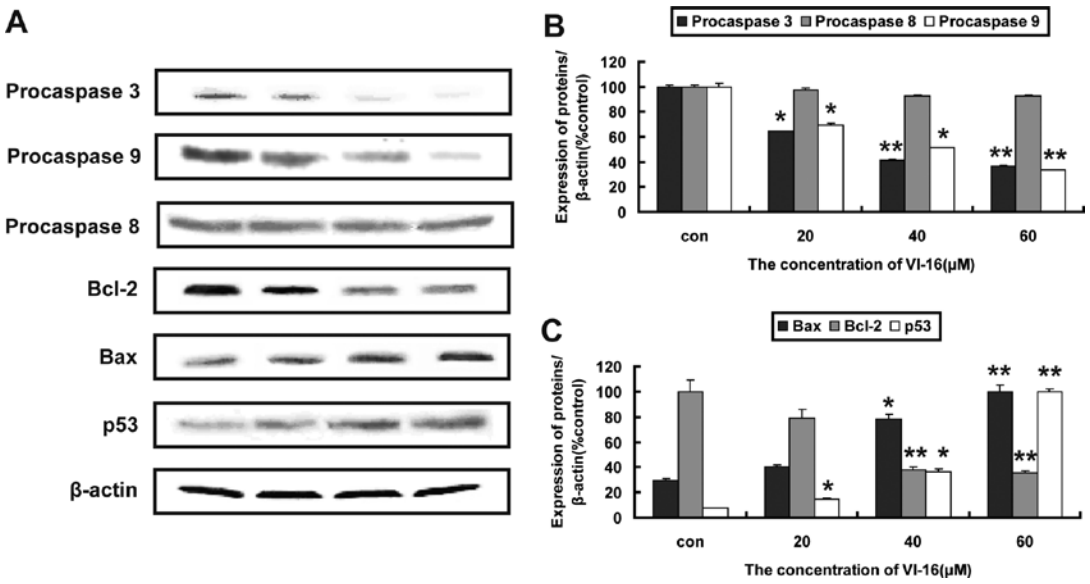

Figure 5. Protein expression of the apoptosis-related proteins in HepG2 cells. (A) Western blot assays were used to examine expressions of procaspase-3, procaspase-8, procaspase-9, Bax, Bcl-2 and p53. (B) Densitometric analysis gave the relative ratios of procaspase-3, Bax and Bcl-2 compared with control. Error bars represent ± SD. *P<0.05 or **P<0.01 indicate significant differences compared with the control.

To investigate whether a death receptor-mediated pathway or a mitochondria-mediated pathway were involved in VI-16-induced apoptosis in HepG2 cells, we measured the expressions of caspase-8, caspase-9, Bax and Bcl-2, in which

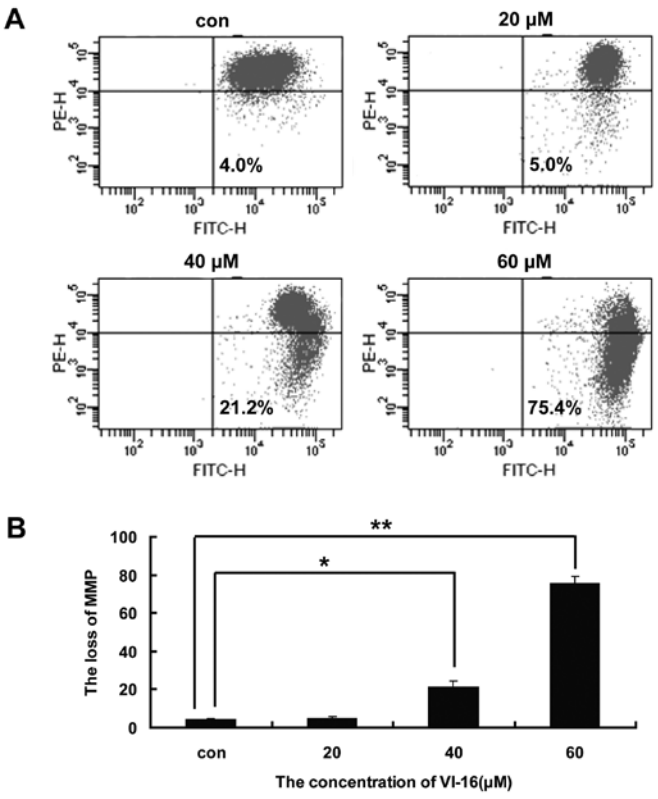

Figure 6. VI-16 increased the loss of mitochondrial membrane potential (MMP) in HepG2 cells. (A) Cells treated with VI-16 for 48 h were analysed by flow cytometry. The uptake of JC-1 analyzed by flow cytometry. Cells with high and low fluorescence (MMP) are found in the upper and lower right quadrants, respectively. The percentage of each profile represents the percentage of total cells with low fluorescence intensity. (B) The percentage of the loss of MMP in HepG2 cells and L02 cells was quantified. Error bars represent ± SD. *P<0.05 or **P<0.01 indicate significant differences compared with control.

caspase-8 was associated with the death receptor pathway and the others could control mitochondrial pathway. As shown in Fig. 5, VI-16 did not influence the expression of procaspase-8 in HepG2 cells. The expression of procaspase-9 decreased concentration dependently in HepG2 cells, showing the activation of caspase-9 under VI-16 treatment. After incubation with VI-16 for 48 h, the expression of Bcl-2 protein decreased dramatically, and the expression of Bax protein increased significantly. Besides, VI-16 could also increase the expression of p53 significantly in a concentration-dependent manner.

To determine the signaling pathway responsible for apoptosis induction of VI-16, the expression of caspase-3, -9, was examined (Fig. 6). Compared to the control group, caspase-9 and caspase-3 were activated significantly, whereas caspase-8 expression showed no significant change after VI-16 treatment for 48 h in HepG2 cells. Moreover, the apoptotic protein Bax increased while Bcl-2 decreased. Thus, the ratio of Bax/Bcl-2, which is crucial for activation of mitochondrial apoptotic pathway, increased in cells treated with VI-16. Besides, VI-16 could also increase the expression of p53 significantly in a concentration-dependent manner. Taken together, these results suggest that the mitochondrial apoptotic pathway, not the death-receptor apoptotic pathway, was involved in VI-16-induced apoptosis.

VI-16 alters the mitochondria membrane potential (MMP) of HepG2 cells. Since the mitochondrial apoptotic pathway was involved in VI-16-induced apoptosis, we detected the decline in MMP, which is another marker of early apoptosis. Intact mitochondrial membrane allows accumulation of JC-1 in the mitochondria, where it will generate red fluorescence light when a critical concentration is reached. As loss of MMP occurs, JC-1 cannot accumulation in the mitochondria and will remain as a monomer in the cytosol and fluoresce green. As shown in Fig. 6, the mitochondrial membrane potential of cells dissipated after VI-16 A treatment for 48 h. The loss of MMP reached 5.0, 21.2 and 75.4% when cells were incubated with 20, 40 and 60 μM VI-16, respectively.

Discussion

Apoptosis induction of cancer cells is one of the most important and direct ways to control the development of cancer and eliminate tumors. Though many therapeutic regimens that appear to be effective have been used in the clinic against cancer, resistance to apoptosis, caused by increased concentrations of cytotoxic drugs and higher doses of irradiation, results in a failure of chemotherapy. Therefore, efforts have increased to develop novel antitumor drugs with high efficacy in inducing apoptosis of cancer cells and low toxicity to normal cells.

In recent years, more and more attention has been paid on favonoids, which are significantly associated with a reduced risk of cancer, inflammation, and heart disease (14). Some favonoids, such as fisetin (15), silmyarin (16) and wogonin (17), have been reported to possess anticancer activity by inducing apoptosis of cancer cells. In this study, we reported the anticancer activity of VI-16, a synthetic new flavonoid, *in vitro* for the first time. VI-16 could inhibit the growth and induce apoptosis of the human hepatoma cell line HepG2 cells (Figs. 2 and 4). To investigate the mechanism of VI-16-induced apoptosis, we assayed the expressions of some key apoptotic proteins, including caspase-3, caspase-8, caspase-9, Bax, Bcl-2 and p53. Caspase-8 acts as an initiator caspase in the death receptor (extrinsic) apoptotic pathway, while caspase-9 is activated to initiate the mitochondrial (intrinsic) apoptotic pathway (18). In the process, cytochrome c (Cyt-c) is released from mitochondria in response to a variety of apoptotic stimuli, binds to APAF-1 in the presence of ATP in the cytosol, and activates caspase-9. As shown in Fig. 5, the decreased expression of procaspase-3 suggested that VI-16-induced apoptosis was involved in a caspase-dependent apoptotic process. Furthermore, the fact that VI-16 had no influences on the expression of caspase-8 turned our focal point to the mitochondrial-mediated apoptotic pathway. The data showed that the expression of procaspase-9 was decreased by VI-16 treatment. Besides, the increased expression of Bax protein and decreased expression of Bcl-2 protein were significantly induced by VI-16 in HepG2 cells. They are both key proteins in Bcl-2 protein family and participate in the regulation of the mitochondrial pathway. The main mechanism by which Bcl-2 family members initiate or prevent apoptosis is by controlling the release of Cyt-c and other pro-apoptotic factors from the mitochondria (19). Anti-apoptotic family members bind to pro-apoptotic Bcl-2 family members and thereby impair the

apoptotic activity of these proteins. Therefore, the results suggested that the mitochondrial apoptotic pathway was involved in the VI-16-incuced apoptosis.

The fate of cells succumbing to the mitochondrial apoptotic pathway was triggered by the loss of MMP (20). VI-16 could diminish MMP significantly in HepG2 cells (Fig. 6). The disruption of the mitochondrial membranes and mitochondrial dysfunction are regulated by Bcl-2 family proteins (21,22). Bax is located in cytoplasm in regular state. However in response to apoptotic stress, the protein undergoes conformational changes and mitochondrial translocation, leading to the formation of oligomers and perhaps higher order structures as a channel to promote the release of Cyt-c and other apoptosis-related factors (23,24). Bax channels and the release of various compounds from liposomes are blocked by the anti-apoptotic proteins Bcl-2 and Bcl-xL (25).

Interestingly, in this study we found that VI-16 increased the expression of p53 significantly. It is long been known that p53 acts as a transcription factor of Bax to promote apoptosis (26). Moreover, the other two p53 target genes are particularly important in the apoptosis induction of p53: these are PUMA and NOXA, BH-3 only members of the Bcl-2 family (27). More recently, p53 has also been shown to play an important role in the mitochondrial apoptotic pathway in a transcription-independent fashion by regulating the activity of Bcl-2 family members (28). p53 physically interacts with the effector protein BAK, causing release of BAK from the inhibitory protein MCL-1. Then a change in BAK conformation occurs, resulting in oligomerization of this protein and subsequent release of Cyt-c from mitochondria (29). To interact with pro-apoptotic Bcl-2 family members, p53 also binds and inhibits the anti-apoptotic family members Bcl-2 and Bcl-xL (30).

As a newly synthesized flavonoid, our study on the anticancer effects of VI-16 is still ongoing. As shown in Fig. 1B, VI-16 has OH substitutions at the C5 position in its structure, which is known to contribute to the apoptosis properties of flavonoids (31). The water solubility of most flavonoids are poor, which may somewhat limit their practical pharmacological use in the clinic. However, VI-16 has a piperazine substitution at C7 position that should increase the hydrophilicity of this new compound, which may possibly improve its water solubility by combining it with an acid-like hydrochloric acid to form the salt. The better hydrophilicity may decrease the solubility restriction and improve its pharmaceutical properties. For further understanding of the features of this new compound, we will continue to investigate its characteristics and compare it with other flavonoids to analyze its special advantageous activities. In addition, p53-mediated apoptotic signaling still remains an attractive target mechanism in effective chemotherapeutic drug development. The finding that VI-16 could improve p53 functions suggests that the effects of the VI-16 on p53 pathway should be further elucidated.

In summary, we provided evidence that VI-16 possesses anticancer activity by inducing apoptosis in human hepatoma HepG2 cells. The mechanism of VI-16 induced apoptosis is involved with the mitochondria pathway and p53. VI-16 is a competitive candidate of novel anticancer drugs.

Acknowledgements

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