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Induction of Apoptosis in Lung Cancer Cells by *Viburnum grandiflorum* via Mitochondrial Pathway

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Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Lung cancer is one of the leading causes of mortality and morbidity. *Viburnum grandiflorum* is a medicinal herb known for its wide spectrum of pharmacological activities, but its anti-cancer properties against lung cancer cells have not been previously investigated. The present study elucidated the antitumor effect and associated mechanism of methanol extract of *Viburnum grandiflorum* extract (VGE) against lung cancer cells.

Material/Methods: The viability of H1650, HCC827, and H1299 cells was measured using MTT assay. Apoptosis and cell cycle progression were determined by flow cytometry using annexin-V/PI and JC-1 stains, respectively. The Lipofectamine Plus reagent (Invitrogen) was used for transfection of caspase-9 plasmid to H1650 and H1299 cells.

Results: The results showed decreased H1650, HCC827, and H1299 cell viability by VGE, which occurred in a concentration- and time-dependent manner. The VGE treatment significantly increased the rate of apoptosis in H1650 ($P<0.05$) and H1299 ($P<0.02$) cells at 48 and 72 h. Treatment of H1650 and H1299 cells with 10 μM of VGE significantly enhanced the number of cells in sub-G1 phase. The VGE treatment cleaved pro-caspase-8/-9 and-3 in H1650 and HCC827 cells at 72 h. The VGE treatment of H1650 and HCC827 cells reduced Mcl-1 protein expression. Treatment of H1650 and HCC827 cells with VGE markedly decreased the level of p-Akt. However, dominant-negative caspase-9 (caspase-9 dN) plasmid transfection prevented the viability-inhibitory effect of VGE on H1650 and HCC827 cells. Treatment of H1650 and HCC827 cells with VGE increased levels of cytochrome c in the cytosol.

Conclusions: VGE inhibited lung carcinoma cell viability by apoptosis activation through a caspase-dependent pathway. Therefore, VGE is a potent anti-cancer agent against lung cancer cells.

MeSH Keywords: Apoptosis • Molecular Conformation • Proteolysis

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Background

Lung cancer is diagnosed in more than 225 000 people every year in the United States alone and is the common cause of deaths associated with cancer [1]. The most common type of lung carcinoma is non-small cell lung cancer, which accounts for 80% of all lung cancer cases [1]. Although radiotherapy and chemotherapy employed for the treatment of lung cancer have undergone drastic improvement over the past decade, the prognosis of patients is still dismal [2]. The of 5-year survival rate for patients with lung carcinoma is around 15% [2]. Radical resection followed by administration of adjuvant chemotherapy to patients with primary tumors has been reported to induce recurrences in most cases [3–5]. There is urgent need for improvement in lung carcinoma treatment through identification of molecular targets and by discovering novel anticancer compounds.

Apoptosis is the process by which cells are killed through the activation of caspases, leading to the cleavage of proteins and ultimately to cell death [6–9]. The apoptotic stimuli catalyse efflux of mitochondrial molecules to the cytosol, where pro-apoptotic processes are initiated [8,9]. For example, secretion of cytochrome c into the cytosol causes oligomerization of Apaf-1 (adaptor protein), followed by the proteolytic cleavage of procaspases [10]. The release of apoptosis-regulatory molecules from mitochondria depends on membrane permeability, which is controlled by Bcl-2 family proteins [11]. There are pro-apoptotic (Bid and Bim) and anti-apoptotic factors 9 (Bcl-2, Bcl-xL, and Mcl-1) in the Bcl-2 family [11–13]. The unique feature of Mcl-1 anti-apoptotic protein which distinguish it from other members is that it binds to a different BH3 subset [14,15]. Mcl-1 expression has been found to inhibit ultraviolet radiation-induced apoptosis [16]. It has also been found that Mcl-1 leads to anti-apoptotic effects through targeting the expression of Bak [17]. Recently, it was found that Mcl-1 induces conformational irregularity in Bax and inhibits cytochrome c translocation [18]. Various compounds have been found to be useful for the treatment of cancers through activation of various pathways [19–21]. The present study investigated the ability of methanol extract of *Viburnum grandiflorum* to inhibit lung cancer cell growth.

Material and Methods

Preparation of plant extract

The plant material was collected in the month of September, dried in the shade, chopped, and then powdered using grinders. Identification of the plant was made by Wei Zhang and the specimen sample was deposited in the herbarium. The powder was suspended in dichloromethane for 72 h to extract the non-polar compounds present in the plant. The solvent was filtered and then evaporated using a rotatory evaporator to obtain the extract. Extraction with dichloromethane was performed 3 times to collect most of the non-polar compounds. Then, methanol was poured on the plant material so all the material was completely suspended in it. After 72 h, the methanol was filtered and evaporated using the rotatory evaporator to collect the methanol extract of *Viburnum grandiflorum*. The extract was completely dried for further experiments.

Cell culture

The H1650, HCC827, and H1299 cell lines were provided by the American Type Culture Collection (Manassas, VA, USA). All the cell lines were cultured in DMEM medium (Gibco BRL, Gaithersburg, MD, USA) mixed with 10% fetal bovine serum (FBS). The cells were incubated at 37°C in an atmosphere of 5% CO_2 and 95% air.

Cell viability assay

The evaluation of H1650, HCC827, and H1299 cell viability was performed using MTT [3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The 3 cell lines were grown for 12 h in 96-well plates and then were treated with 2, 4, 6, 8, 10, and 12 µM of VGE for 48 and 72 h. Next, 100 µl of MTT (5 mg/ml) was put into each well and cells were incubated for 2 h. Then, Me_2SO was added to the plates to dissolve the formazan crystals formed from MTT by interaction with the mitochondrial enzymes. The measurement of absorbance was carried out at 568 nm using a microplate reader (Molecular Devices, CA, USA).

Apoptosis analysis using flow cytometric assay

The H1650 and H1299 cells at a density of approximately 1×10^6 cells per well in 6-well plates were treated with 10 µM VGE for 48 and 72 h. Then, cells were suspended in PBS (100 µl) and subsequently treated with 200 µl of ethyl alcohol. Following incubation for 1 h, the cells were washed in PBS and then treated with 1.2% sodium citrate buffer (200 µl) and RNase (12.5 µg). After 45 min of incubation at 37°C, the cells were stained with 50 µg/ml solution of PI at room temperature for 40 min. The FAC Scan flow cytometer was used for determination of relative DNA content using fluorescence-activated cell sorting (FACS). The mitochondrial membrane potential was measured using JC-1 stain, which, on penetrating the mitochondria, forms monomers and emits green fluorescence when the mitochondrial membrane potential is low, whereas high membrane potential is characterised by the aggregation of JC-1 and emission of red fluorescence. The wavelength of excitation was 486 nm, while FL1 and FL2 emissions were detected at 523 nm and 576 nm, respectively.

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Cell cycle analysis

The H1650 and H1299 cells were distributed at 2×10^6 cells per well density in 60-mm plates and incubated for 48 and 72 h with 10 μM VGE. The cells were washed 2 times with PBS after trypsinization, followed by fixing with 70% ice-cold ethyl alcohol overnight. Then, cells were treated at 37°C with 20 μg/ml RNase A for 1 h and subsequently stained with 10 μg/ml PI. The distribution of cells was analyzed by flow cytometry using a FACS Calibur instrument (BD Biosciences, San Jose, CA, USA).

Western blotting

The H1650 and H1299 cells at approximately 1×10^5 cells per well density were treated with 10 μM VGE for 48 and 72 h. The plates were kept on ice and lysed on treatment with pre-chilled proteinase inhibitor, which consisted of phenyl methyl sulfonyl fluoride and phosphatise. The protein extraction from the cells was performed using an extraction kit (Sangon Biotech Co., Shanghai, China). The cell lysate was cleared of the debris by filtration after centrifugation. A bicinchoninic acid (BCA) kit was used for determination of protein concentration in the supernatant. The protein samples (50 μg) were separated with a 10% gel using SDS-PAGE and then transferred on polyvinylidene difluoride membranes. The membranes were blocked overnight at 4°C with 5% non-fat dry milk in phosphate-buffered saline (PBS) plus Tween-20. The membrane incubation was performed with primary antibodies against pro-caspase-8,-3, Bcl-2, PARP, and p-Akt overnight at 4°C. The membranes washed with tris-buffered saline and Tween-20 were incubated for 2 h with horseradish peroxidase-labelled goat anti-rabbit IgG secondary antibody. The protein band detection was performed using chemiluminescence and autoradiography (ChemiDoc XRS; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Determination of densitometry was carried out using Quantity One software version 4.6.2 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA extraction from H1650 and H1299 cells at 48 and 72 h of treatment with 10 μM VGE was performed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The RNA samples (2 μg) were used for synthesis of complementary DNA using the MMLV reverse transcriptase (Takara Co., Japan). The amplification of Mcl-1 mRNA was done using forward 5'-CCT TCCAAG GAT GGG TTT GT-3' and reverse 5'-TCTTCA ATC AAT GGG GAG CA-3' primers. The PCR cycling conditions used for Mcl-1 amplification consisted of: 94˚C for 30 s, 93°C for 2 s, and 58°C for 30 s for 40 cycles. The value of the average threshold for each cycle was normalized to GAPDH and the level of expression was quantified using the $2^{-\Delta\Delta Cq}$ method.

Transfection of H1650 and H1299 cells with dominant-negative caspase-9

Lipofectamine Plus reagent (Invitrogen) was used for transfection of caspase-9 (caspase-9 dN) plasmid to H1650 and H1299 cells. The cells seeded at 1×10^5 cells per well density in 6-well plates were cultured in DMEM mixed with 10% FBS, and incubation was carried out for 24 h. After washing twice with Opti-MEM medium, the cells were incubated with Opti-MEM medium and caspase-9 dN for 4 h. The medium was changed with DMEM containing 10% FBS and incubation was continued for 24 h. The pcDNA 3.1-caspase-9 plasmid or control pcDNA 3.1 vector plasmid was stably transfected to H1650 and H1299 cells using Lipofectamine.

Determination of cytochrome c release

The H1650 and H1299 cells after 48 and 72 h of treatment with 10 μM VGE were harvested and then washed in PBS. The cells were put in lysis buffer consisting of HEPES (25 mM, pH 7.5), sucrose (250 mM), EDTA (1 mM) EGTA (1 mM), DTT (1 mM), potassium chloride (10 mM), magnesium chloride (1.5 mM), PMSF (1 mM), and protease inhibitor cocktail for 15 min. The lysate obtained was subjected to centrifugation for 25 min at 4°C to collect mitochondria-free cytosolic extract and mitochondrial extract. Immunoblotting was performed to determine cytochrome c expression in the supernatants.

Statistical analysis

Data are presented are the mean±standard deviation. All experiments were carried out in triplicate. One-way analysis of variance and post hoc Geisser-Greenhouse correction were used for determination of statistically significant differences, set at $P<0.05$. SPSS v17 software (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis of the data.

Results

Viburnum grandiflorum has inhibitory effect on lung cancer cell viability

The cytotoxicity of VGE was analyzed against H1650, HCC827, and H1299 cells by MTT. The results showed decreased H1650, HCC827, and H1299 cell viability by VGE in a concentration- and time-dependent manner. VGE treatment at 10 μM for 48 h reduced viability of H1650, HCC827, and H1299 cells to 48%, 45%, and 41%, respectively (Figure 1). Treatment of H1650, HCC827, and H1299 cells with VGE at 10 μM for 72 h decreased viability to 34%, 31%, and 29%, respectively (Figure 2).

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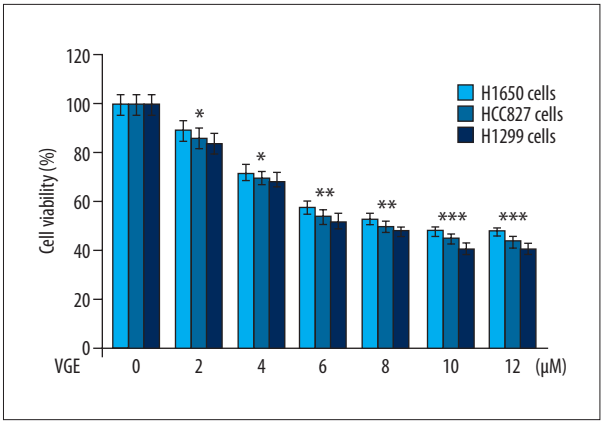

Figure 1. Effect of VGE on lung cancer cell viability. The H1650, HCC827 and H1299 cells were treated with VGE at 2, 4, 6, 8, 10, and 12 μM for 48 h. The cell viability was measured by MTT assay using dimethyl sulfoxide-treated cells as control. * P<0.05, ** P<0.02, and *** P<0.01 *vs.* control.

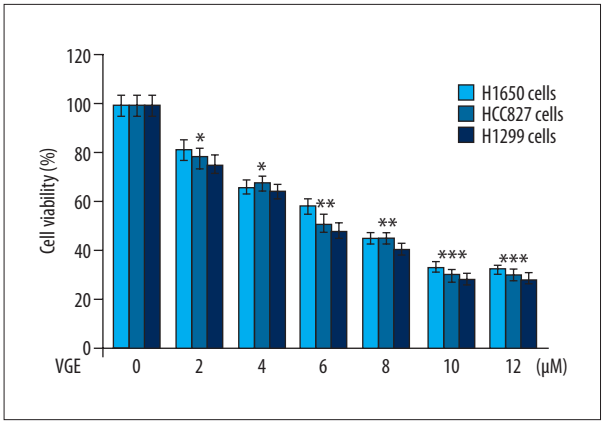

Figure 2. Effect of time of exposure of VGE on lung cancer cell viability. The H1650, HCC827, and H1299 cells were treated with VGE for 6, 12, 18, 24, 48, and 72 h. Cell viability was measured by MTT assay using dimethyl sulfoxide-treated cells as control. * P<0.05, ** P<0.02, and *** P<0.01 *vs.* control.

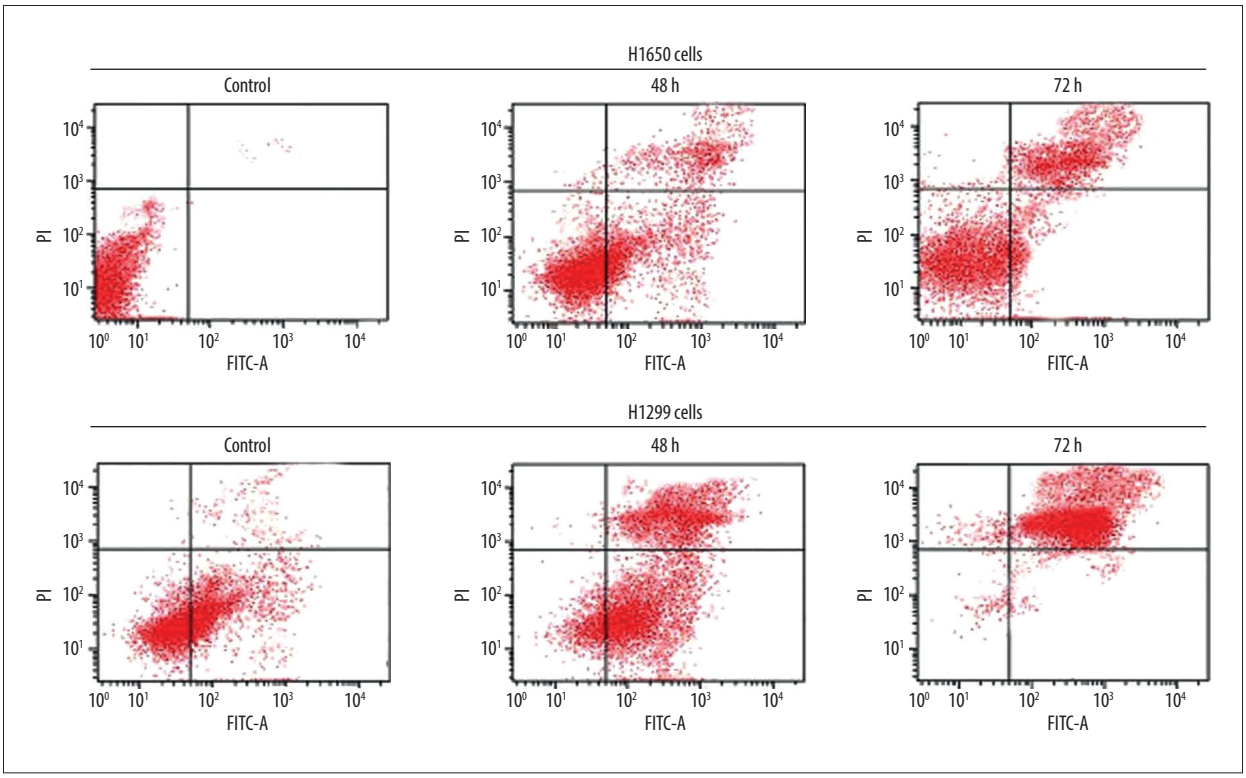

Figure 3. Effect of VGE on apoptosis induction in H1650 and H1299 cells. The H1650 and H1299 cells were treated with VGE at 10 μM for 48 and 72 h. The apoptosis induction by VGE was measured at 48 and 72 h by flow cytometry using dimethyl sulfoxide-treated cells as control. * P<0.02 and ** P<0.01 *vs.* control.

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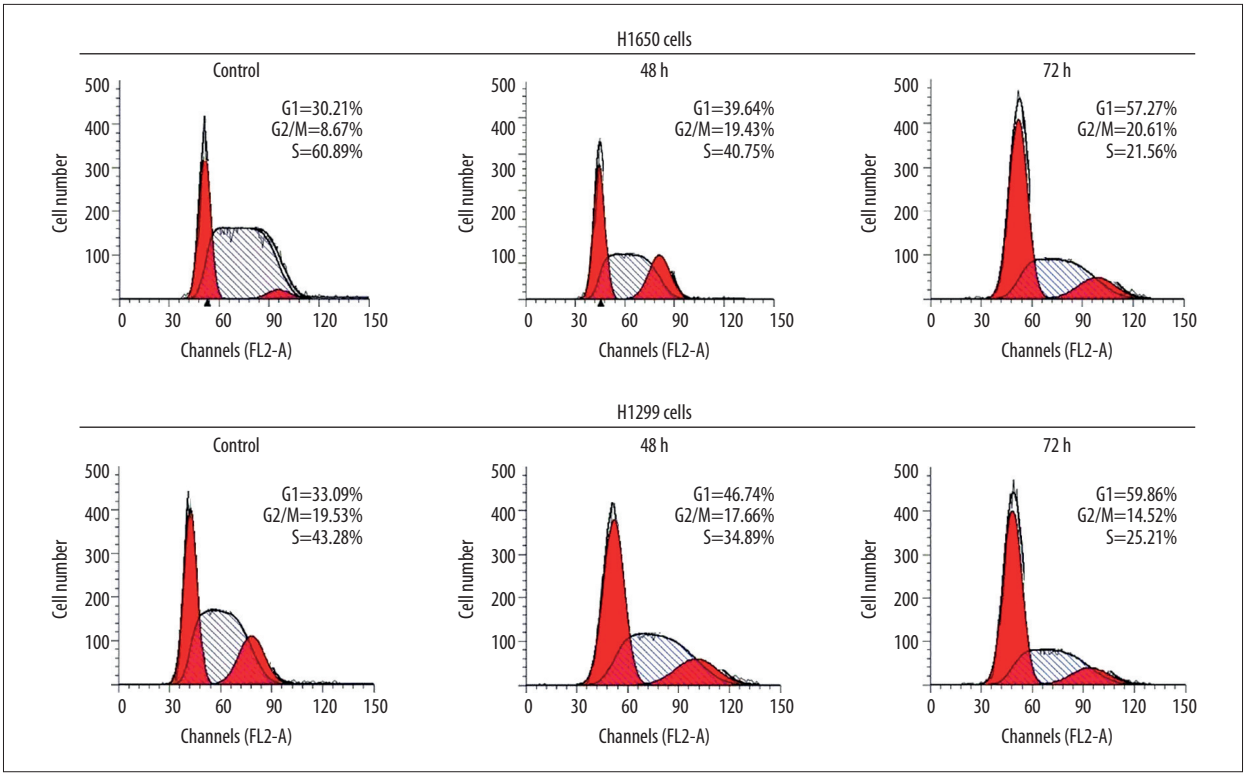

Figure 4. VGE enhanced the numbers of H1650 and H1299 cells in sub-G1 phase. The H1650 and H1299 cells were treated with VGE at 10 μM for 48 and 72 h. The changes in cell cycle progression caused by VGE were determined by flow cytometry using dimethyl sulfoxide-treated cells as control. * P<0.02 and ** P<0.01 *vs.* control.

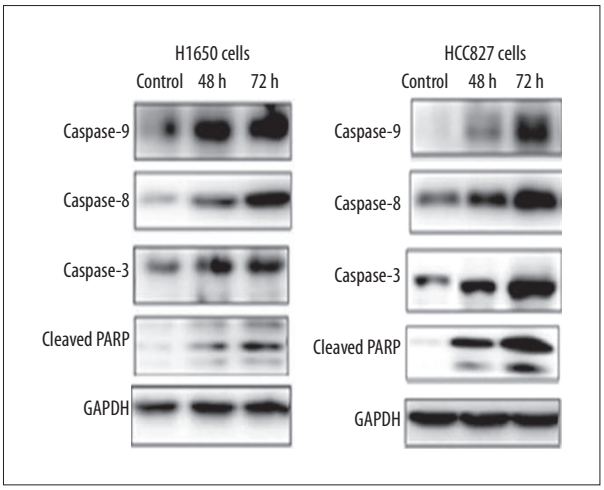

Figure 5. Effect of VGE on activation of caspases in lung cancer cells. In H1650 and HCC827 cells, activation of caspase-8/-9/-3 and PARP was assessed by Western blotting. The β-actin level was taken as a loading control.

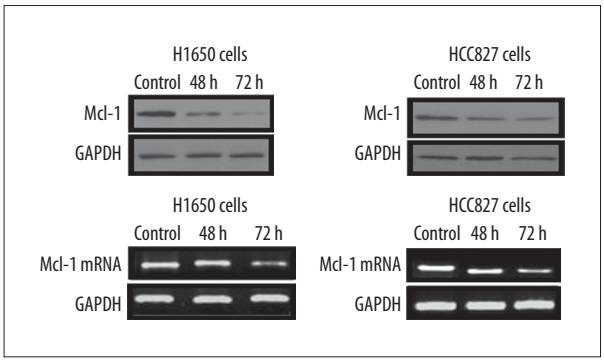

Figure 6. Effect of VGE on level of Bcl-2 proteins. The H1650 and HCC827 cells treated with VGE at 10 μM for 72 h were assessed by Western blot assay. β-actin was used as a loading control. The Mcl-1 mRNA level was determined by RT-PCR assay.

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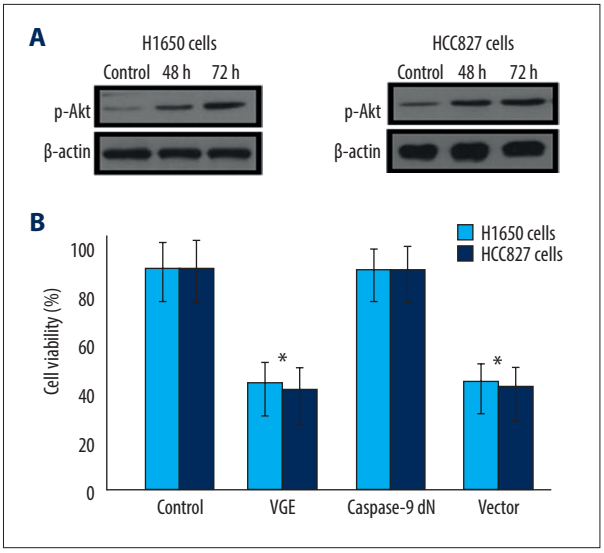

Figure 7. Effect of VGE on PI3K/Akt signalling. (**A**) The H1650 and HCC827 cells treated with VGE were analyzed by Western blot assay for p-Akt expression. β-actin was used as a loading control. (**B**) The cells transfected with caspase-9 dN or vector were assessed by MTT assay for changes in viability. * P<0.02 and ** P<0.01 *vs.* control.

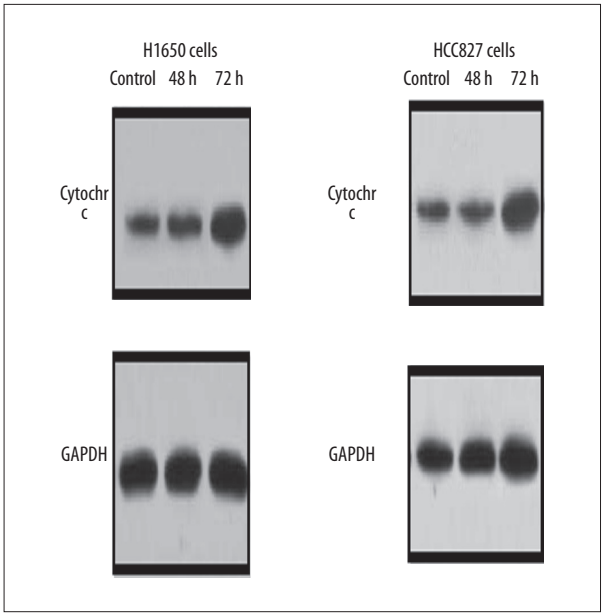

Figure 8. Effect of VGE on the mitochondrial apoptotic pathway. Cells at 72 h of VGE treatment were analyzed by Western blot analysis for cytochrome c release.

Viburnum grandiflorum induces apoptosis in lung cancer cells

Induction apoptosis by VGE treatment was assessed in H1650 and H1299 cells by flow cytometric analysis (Figure 3). The VGE treatment significantly increased the rate of apoptosis in H1650 ($P<0.05$) and H1299 ($P<0.02$) cells at 48 and 72 h. However, the data showed that the percentage of apoptotic cells was markedly higher at 72 h of treatment with VGE.

Viburnum grandiflorum increased the sub-G1 phase cell population

VGE treatment of H1650 and H1299 cells significantly enhanced the number of cells in sub-G1 phase at 10 μM (Figure 4). The increase in sub-G1 phase cell numbers was much higher at 72 h than at 48 h treatment. The percentage of H1650 and H1299 cells in S and G2/M phases was reduced after treatment with VGE for 48 and 72 h.

Viburnum grandiflorum activated caspases

The changes in caspase activation by VGE in H1650 and HCC827 cells were assessed by Western blot assay (Figure 5). Treatment with VGE cleaved pro-caspase-8 and-9 in H1650 and HCC827 cells at 72 h. The cleavage of caspase-3 was also promoted by VGE treatment in H1650 and HCC827 cells. The cleavage of PARP in H1650 and HCC827 cells was promoted by treatment with 10 μM of VGE.

Effect of *Viburnum grandiflorum* on Bcl-2 proteins in lung cancer cells

The VGE treatment of H1650 and HCC827 cells reduced the expression of Mcl-1 protein at 72 h (Figure 6). The level of Mcl-1 mRNA was also lower in H1650 and HCC827 cells after treatment with 10 μM of VGE (Figure 6).

Viburnum grandiflorum reduced Akt activation in lung cancer cells

H1650 and HCC827 cells treated with VGE at 10 μM had markedly reduced levels of p-Akt at 72 h (Figure 7A). Transfection of H1650 and HCC827 cells with dominant-negative caspase-9 (caspase-9 dN) plasmid prevented the viability-inhibitory effect of VGE (Figure 7B). VGE treatment significantly reduced the viability of vector-transfected cells.

Viburnum grandiflorum activated apoptosis through mitochondrial pathway

Treatment of H1650 and HCC827 cells with VGE increased cytochrome *c* in the cytosol (Figure 8). The level of cytochrome c in control H1650 and HCC827 cells was markedly lower in comparison to the VGE-treated cells.

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Discussion

The present study demonstrated the role of VGE in inhibiting the viability of lung carcinoma cells. The data showed that VGE reduced lung cancer cell viability by activating apoptosis through the involvement of various factors like caspases, Akt, and Mcl-1.

Cellular apoptosis in several kinds of carcinoma cells is blocked by the PI3K/Akt pathway [22]. The expression of PI3K/Akt has been found to be much higher in cancer cells and is believed to impart resistance against anti-cancer compounds [23]. Targeting of the PI3K/Akt pathway leads to apoptosis activation in myelogenous leukemia cells, which are resistant to drugs [24]. In multiple myeloma cells that are resistant to dexamethasone, apoptosis induction by PS-341 is promoted by triptolide by targeting the PI3K/Akt/NF-κB pathway [25]. In the present study, VGE reduced H1650, HCC827, and H1299 cell viability in a time- and concentration-dependent manner. The decrease inH1650, HCC827, and H1299 cell viability by VGE was greatest after treatment with 10 μM at 72 h. The mechanistic study revealed that VGE treatment activated the apoptotic pathway for inhibition of H1650 and H1299 cell viability. The expression of the pro-survival protein p-Akt was reduced in H1650 and H1299 cells after treatment with VGE. The essential event for apoptosis induction is the release of cytochrome c, the intermembrane protein, into the cytosol [26,27]. Cytochrome c is involved in the formation of apoptosomes and activates caspases in the cytosol [26,27]. In the present study, VGE treatment increased cytochrome c release in the cytosol of H1650 and H1299 cells. Apoptosis induction is dependent on the expression of various proteins like caspases, which actually execute cell death following apoptotic stimuli [28]. The present study assessed the effect of VGE on expression of caspase-8/-9/-3 and cleaved PARP in H1650 and H1299 cells. The data from Western blotting revealed that VGE treatment of H1650 and H1299 cells promoted activation of caspase-8/-9/-3 and cleavage of PARP. In order to confirm that VGE leads to lung carcinoma cell apoptosis through the caspase-dependent pathway, H1650 and H1299 cells were transfected with caspase-9 dN. The data showed that caspase-9 dN transfection of H1650 and H1299 cells prevented VGE-induced viability reduction. These findings suggest that VGE activated apoptosis in H1650 and H1299 cells through a caspase-dependent pathway.

Bcl-2 family proteins like Mcl-1 and Bcl-2 downregulate the process of apoptosis and inhibit activation of caspases by regulating the integrity of mitochondrial membranes [29–31]. It was reported that overexpression of Mcl-1 inhibits cytochrome c release, cleavage of PARP, and caspase activation in HeLa cells irradiated with UV-radiations [16]. Mcl-1 has been found to inhibit the ability of Bid to promote cytochrome c secretion and induce apoptosis [32]. In the present study, expression of Mcl-1 was reduced in H1650 and H1299 cells after treatment with VGE. VGE treatment also leads to mitochondrial membrane potential loss in H1650 and H1299 cells.

Conclusions

VGE inhibited lung carcinoma cell viability by activation of apoptosis. The mechanism of VGE-induced apoptosis involved caspase activation, downregulation of Mcl-1, and inhibition of the Akt pathway. Therefore, VGE is a potent anti-cancer agent against lung cancer cells.

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