

# Apoptosis Induction in Human Lung and Colon Cancer Cells via Impeding VEGF Signaling Pathways

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## Research Article

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# Abstract

There is ample evidence to suggest that vascular endothelial growth factor (VEGF) is a potent mitogen factor in vasculogenesis and angiogenesis and that blockade of VEGF-mediated signals can also prevent tumor growth via enforcing cell apoptosis. In the current study, we assessed the suppressing effect of VGB4, a VEGF antagonist peptide with the binding ability to both VEGF receptor1 and VEGF receptor2, on VEGF-induced proliferation and migration of the human lung adenocarcinoma cell line A549 and the human colon adenocarcinoma cell line HT29 using MTT assay, colony formation assay, and Scratch-wound assay. To evaluate the apoptotic inductive effect of VGB4 on A549 and HT29 cells, apoptosis assay was carried out by flow cytometry analysis. Likewise, p53 and PTEN expression level was examined by immunofluorescence microscopy. In addition, the level of proteins involved in VEGF signaling pathways related to apoptosis was investigated using western blot analysis. Our results indicated that VGB4 markedly inhibited VEGF-induced proliferation and migration, and induced apoptosis of A549 and HT29 cells dose dependently. Encouragingly, significant downregulation of B-cell lymphoma 2 (Bcl2), X-Linked Inhibitor of Apoptosis (XIAP), Procaspase9, and procaspase3, as well as upregulation of PTEN and P53 tumor suppressors, BCL2 Associated X (BAX), Cytochrome c, cleaved caspase9, and cleaved caspase3 in VGB4-treated A549 and HT29 cells, further confirmed the profound inductive influence of VGB4 on apoptotic pathways. These findings along with the results from our previous studies show that VGB4 may be considerable for cancer therapy.

## Introduction

The phenomenon of tumorigenesis is caused due to an imbalance between cell proliferation and cell death. Neovascularization, as a result of the constant activation of the VEGF/VEGFR signaling pathways, led to the uncontrolled cell proliferation in many types of human cancers by providing nutrients [1].

Escape of apoptosis is often considered as one of the hallmarks of cancer that promotes tumor development [2]. In essence, cancer cells acquire the mechanisms to circumvent programmed cell death [3]. Some of the mechanisms engaged by cancer cells to avoid apoptosis initiation include upregulation of survival proteins (Bcl2 and IAPs), downregulation of pro-apoptotic effectors (BAX and Cytochrome c), loss of PTEN and p53 tumor suppressors, and inactivation of the final effectors of apoptosis (Caspases) [4–6].

Due to the important role of VEGF as one of the families implied in the regulation of vascular angiogenesis [7] and upregulation of VEGF and VEGF receptors in many types of cancers, therapeutic strategies against VEGF/VEGFR signaling pathways have been widely studied [8]. VEGF upregulates Bcl2 and suppresses apoptosis in human cancer cells and withdrawal of VEGF induces cell apoptosis both *in vitro* and *in vivo* [9–11].

In the former investigations, we reported a VEGF-antagonist peptide, called VGB4, and found that VGB4 has the ability to bind to both VEGFR1 and VEGFR2, which subsequently leads to the inhibition of 4T1

tumor growth and angiogenesis [12]. Also, in another study, it was found that VGB4 strongly suppresses the metastatic pathways in human breast cancer cell MDA-MB-231 and 4T1 tumor tissue [13]. In the present study, we particularly assessed the influence of VGB4 on apoptosis pathways in A549 and HT29 cell lines. Our results show that a significant reduction in AKT phosphorylation, followed by VGB4 binding to VEGFR1 and VEGFR2 and inactivation of downstream signaling pathways, down-regulates the expression of the apoptotic-related factors in VGB4-treated A549 and HT29 cells. The profound impact of VGB4 on apoptosis pathways once again confirmed that blocking both VEGFR1 and VEGFR2 is an accurate and efficient approach for impeding of tumor progression.

## Materials And Methods

### Peptide VGB4

Peptide VGB4 (2HN-KQLVIKPHGQILMIRYPSSQLEM-COOH) was synthesized by Shine Gene Biotechnology (Shanghai, China), purified by high performance liquid chromatography (HPLC) to 90% purity, analyzed by matrix-assisted laser desorption/ionization time of-flight mass spectrometry (MALDI-TOF), and approved by electrospray ionization mass spectrometry (ESI-MS) analysis (see also [12])

### Antibodies and reagents

Anti-p53 (sc-126), anti-PTEN (sc-7974), goat anti-mouse IgG-FITC (sc-2010), anti- $\beta$ -Actin (sc-47778), anti-Bcl2 (sc-509), anti-BAX (sc-20067), anti-Cytochrome c (sc-13156), anti-XIAP (sc-55551) and goat anti-mouse IgG-PE (sc-3738) were from SANTA CRUZ BIOTECHNOLOGY, INC., Santa Cruz, California, USA. Anti-AKT phospho S473 (#9271), anti-Caspase 3 (#9662) and anti-Caspase 9 (#9502) were from Cell Signaling Technology, Danvers, Massachusetts, USA; DAPI (D9542), Propidium iodide (P4170), RIPA buffer, polyvinyl difluoride (PVDF) membranes (IPVH00010) and anti-AKT (SAB4500800) was from Sigma (St Louis, MO, USA). Annexin V-FITC Apoptosis Staining / Detection Kit (ab14085) was purchased from Abcam, Cambridge, UK.

### Cell lines and cell culture

The human lung adenocarcinoma cell line A549 (NCBI code, C137) and the human colon adenocarcinoma cell line HT29 (NCBI code, C466) were purchased from the National Cell Bank, Pasteur Institute of Iran. A549 and HT29 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Life Technologies, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma, St. Louis, Missouri, USA), and 100 units/ml penicillin-streptomycin (Gibco/Invitrogen, Carlsbad, CA) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>.

### MTT cell proliferation assay

For cell growth analysis, the cells were seeded into 96-well plates at a density of  $2 \times 10^3$  cells/well and allowed to grow in DMEM medium supplemented with 10% FBS for 24 h. Subsequently, the medium was replaced with the fresh medium containing 2% FBS, 200 ng/ml VEGFA (Sigma, St. Louis, Missouri, USA),

and increasing concentrations (0.09, 0.18, 0.37, 0.55, 0.74 and 0.93  $\mu\text{M}$ ) of VGB4 or (0.93  $\mu\text{M}$ ) scr. After 24 h incubation, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) (Sigma, St. Louis, Missouri, USA) was added for 4 h at 37°C. Then, the intracellular formazan crystals were solubilized with dimethyl sulfoxide (DMSO) and the absorbance of the colored solution was read at 570 nm using ELISA plate reader (Bio Rad, USA).

## Colony formation assay

To further evaluate proliferation and efficacy of VGB4, colony formation assay was conducted on A549 and HT29 cell lines. 2000–3000 cells/well were seeded on 6-well plates and allowed to adhere overnight. The next day medium was replaced with fresh medium containing 200 ng/ml VEGFA and concentrations (0.74 and 0.93  $\mu\text{M}$ ) of VGB4 or (0.93  $\mu\text{M}$ ) scr. The cells were grown for 11 days with media changes every 3 days. The colonies were fixed with 4% formaldehyde, and stained with crystal violet (0.1%).

## Scratch-wound assay

Wound-scratch assay was performed to evaluate the migration rate of HT-29 and A549 cells treated with concentrations (0.74 and 0.93  $\mu\text{M}$ ) of VGB4 or (0.93  $\mu\text{M}$ ) scr in the presence of 200 ng/ml VEGFA. To accomplish this,  $2 \times 10^3$  cells/well were seeded in 96-well plates, and cultured until the cells reached 90 % confluence. A sterile 200- $\mu\text{l}$  pipette tip was used to create the wound in the monolayer of cells in each well. The detached cells were washed off with phosphate-buffered saline (PBS). After 24 h, the rate of wound recovery was monitored using an inverted microscope (Olympus BX-50, Japan) and a digital camera (Canon, C4742-95, Japan).

## Flow Cytometry Analysis

To quantify cell apoptosis, Annexin V/propidium iodide (PI) staining was performed on HT29 and A549 cell lines. The cells were treated with VGB4 (0.74 and 0.93  $\mu\text{M}$ ) or scr (0.93  $\mu\text{M}$ ) for 24h in the presence of 200 ng/ml VEGFA, and then scraped and digested with trypsin/EDTA. Digested cells were centrifuged at 1500 rpm for 5 min at 4 °C. Subsequently, the cells were washed two times with cold PBS, and resuspended in binding buffer at a cell density of  $1 \times 10^6$ /ml, and stained with Annexin V-fluorescein isothiocyanate (FITC) and PI for 10 min at room temperature in the dark. The cell suspensions were analyzed on a FACSCalibur flow cytometer (BD Biosciences, NJ, USA).

## Immuno fluorescence staining

$3 \times 10^4$  cells were seeded in a 12-well plate, and incubated overnight at 37°C. Then, the cells were treated with VGB4 (0.74 and 0.93  $\mu\text{M}$ ) or scr (0.93  $\mu\text{M}$ ). After 24 h of incubation, the cells were fixed with 3.7% formaldehyde for 10 min at room temperature, washed two times in PBS containing 0.05% tween 20 (PBS-T), permeabilized with 0.2% Triton X-100 for 5 min at room temperature. After two times washing in PBS-T, blocking buffer containing 1% BSA/ 10% normal goat serum/0.3 M glycine in PBS-T was added for 1 h at room temperature. The cells were reacted with anti-p53 and anti-PTEN primary antibodies overnight at 4°C, washed three times, and reacted with Fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG secondary antibody and Phycoerythrin (PE)-labeled goat anti-mouse IgG secondary

antibody, respectively, in the dark situation at 37°C for 1 h. Finally, the cells were counterstained with DAPI solution. Fluorescence was detected by a fluorescence microscope.

## Western blot

A549 or HT29 cells from VGB4-treated, scr-treated groups and control group were lysed with RIPA buffer containing protease and phosphatase inhibitor cocktail (Sigma, St. Louis, Missouri, USA). Equal volumes of cell lysates from each group were resolved by 12 % sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). After transferring the proteins to PVDF membrane and blocking with 5% non-fat milk, the membrane was incubated with the primary antibodies including anti-p53, anti-PTEN, anti-phospho-AKT, anti-total AKT, anti-Bcl2, anti-BAX, anti-Cytochrome c, anti-XIAP, anti-Caspase 3, anti-Caspase 9, and anti- $\beta$ -Actin at 4°C overnight. In the next step, the membrane was incubated with secondary HRP-conjugated anti-mouse antibody at room temperature for 1 h.  $\beta$ -Actin was used as the internal reference. The quantification of protein density was performed using Image J software (NIH Image, National Institutes of Health; online at: <https://rsbweb.nih.gov/ij/>).

## Data analysis

Data were represented as the mean  $\pm$  standard deviation (SD). Statistical analyses were carried out by GraphPad Prism7 software. Data normality was evaluated using Kolmogorov–Smirnov test. The significant difference between groups was assessed via Unpaired Student's t-test.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

### VGB4 inhibits VEGFA-induced proliferation, migration, and colony formation of cultured A549 and HT29 cells

We initially exposed A549 and HT29 cells to different concentrations of VGB4 ranging from 0.09  $\mu$ M to 0.93  $\mu$ M in the presence of 200 ng/ml VEGFA for 24h, to determine anti-proliferative effects of VGB4 on these two types of cells. The results of MTT detection showed that the cell viability of A549 and HT29 cells was attenuated by VGB4 dose-dependently with  $IC_{50}$  value of 0.74  $\mu$ M, whereas the scrambled (Scr) peptide had no inhibitory effect on VEGFA-induced cell proliferation even at 0.93  $\mu$ M (Fig. 1A). The proliferation rate of VEGFA-treated control A549 and HT29 cells was increased by 42 % and 47 %, respectively, compared to non-treated control A549 and HT29 cells ( $P < 0.001$ ) (Fig. 1A). To further investigate the anti-proliferative potential of VGB4 on A549 and HT29 cancer cell lines, the colony formation assay was performed. Similarly, a dose dependent decrease in colony-forming ability was observed in VGB4-treated cells compared to non-treated control cells and scr-treated control cells ( $P < 0.001$ ) (Fig. 1B). Wound scratch assay was performed to assess the role of VGB4 in migratory capability of A549 and HT29 cells. As shown in Fig. 1C, VGB4 treatment inhibited dramatically VEGFA-induced A549 and HT29 cells migration ( $P < 0.001$ ). Whereas migration of A549 cell line was inhibited by 77.34 % and 87.67 % respectively at 0.74 and 0.93  $\mu$ M of VGB4, the percentages of migration inhibition of HT29 cell

line at 0.74 and 0.93  $\mu\text{M}$  of VGB4 were 93 % and 95 %, respectively, when compared to non-treated control cells and scr-treated cells ( $P < 0.001$ ) (Fig. 1C).

## **VGB4 induces apoptosis of A549 and HT29 cells**

To evaluate the occurrence of apoptosis, Annexin V/Propidium Iodide (PI) staining was conducted in VGB4-treated A549 and HT29 cells and control cells. As shown in Fig. 2A, the apoptosis proportion of A549 cells treated with 0.74  $\mu\text{M}$  and 0.93  $\mu\text{M}$  of VGB4 was increased by 25.6 % and 37.8 %, respectively, when compared to non-treated control cells and scr-treated cells ( $P < 0.001$ ). Regarding HT29 cells, the apoptosis proportion was increased by 34.6 % and 87.3 %, at 0.74  $\mu\text{M}$  and 0.93  $\mu\text{M}$  concentrations of VGB4, respectively, when compared to non-treated control cells and scr-treated cells ( $P < 0.001$ ) (Fig. 2B).

### **Immunofluorescence analysis of p53 and PTEN tumor suppressor proteins level in VGB4-treated A549 and HT29 cells**

To further assessment of VGB4-induced apoptosis, the expression level of p53 and PTEN proteins was investigated in VGB4-treated A549 and HT29 cells using immunofluorescence microscopy. For this goal, A549 and HT29 cells were incubated with 0.74  $\mu\text{M}$  and 0.93  $\mu\text{M}$  concentrations of VGB4 followed by adding anti-p53 primary antibody, anti-PTEN primary antibody, FITC-labeled goat anti-mouse IgG secondary antibody, and PE-labeled goat anti-mouse IgG secondary antibody. As shown in Fig. 3, VGB4 increased the intensity of fluorescence markedly, compared to non-treated and scr-treated control A549 and HT29 cells ( $P < 0.001$ ). These results confirm that VGB4 stimulates apoptosis in these two human cancer cell lines.

### **VGB4 downregulates the expression of the anti-apoptotic proteins Bcl2 and XIAP, and activates the pro-apoptotic proteins p53, PTEN, BAX, Cytochrome c, Caspase 3 and Caspase 9 in A549 and HT29 Cells**

Suppression of the VEGF/VEGFR signaling pathways led to induced apoptosis, the result of which is tumor cell death, while overexpression of activated protein kinase B (AKT) inversed the apoptotic effects [14]. We had earlier demonstrated that VGB4 binds to both VEGFR1 and VEGFR2 and prevents VEGF-stimulated tyrosine phosphorylation of VEGFRs [13]. AKT, as the major downstream effector of receptor tyrosine kinase (RTKs), mediates a potent anti-apoptotic signal in many human cancers [15]. Therefore, in this study, we first aimed to assess the expression level of phosphorylated-AKT in VGB4-treated A549 and HT29 cells. As shown in Fig. 4, VGB4 strongly reduced p-AKT level in A549 and HT29 cells compared to control cells ( $P < 0.001$ ). Due to the fact that many key proteins located in downstream of AKT, including pro-apoptotic and anti-apoptotic proteins, are involved in the process of apoptosis, we performed western blot analysis of the expression of some Bcl2 and IAP family members, as well as the Caspase3 and 9 activities in VGB4-treated A549 and HT29 cells. As illustrated in Fig. 4, the expression of Bcl2 and XIAP significantly decreased ( $P < 0.001$ ) and BAX, Cytochrome c, Caspase3 and Caspase9 expression markedly increased ( $P < 0.001$ ) in VGB4-treated A549 and HT29 cells compared to non-treated and scr-treated control cells. Likewise, VGB4 increased the expression of p53 and PTEN significantly, compared to non-

treated and scr-treated control A549 and HT29 cells ( $P < 0.001$ ) (Fig. 4). Our results indicate that VGB4 induces cell apoptosis through regulation of the expression of apoptosis-associated factors.

## Discussion

Anti-VEGF/VEGFR therapy, as a novel and effective approach for cancer treatment, prevents tumor angiogenesis by blocking downstream signaling pathways of VEGFR1 or VEGFR2, which leads to the occurrence of apoptosis in tumor tissue [16–18]. Several studies have shown that blocking both VEGFR1 and VEGFR2 has a greater inhibitory effect on proliferation, migration, and invasion of 4T1 and MDA-MB 231 breast cancer cells [12, 13, 19–22]. In this study, we assessed apoptotic effects of VGB4, an antagonist peptide with the ability to bind to both VEGFR1 and VEGFR2, on human A549 lung cancer cells and HT-29 colon cancer cells. VGB4 strongly suppressed VEGF-induced A549 and HT29 cells proliferation and migration in a dose-dependent manner. In addition, marked reduction of AKT phosphorylation resulted in the activation of the intrinsic caspase-dependent apoptosis pathway in VGB4-treated A549 and HT29 cancer cells (Fig. 5).

It has been shown that continuous activation of the pro-proliferative/anti-apoptotic PI3K/AKT pathway in different cancers promotes diverse tumorigenic activities [23–25]. Noteworthy, the activated PI3K/AKT pathway protects the cancer cells against apoptosis by influencing downstream signaling molecules, which are involved in modulation of caspase- and p53-dependent apoptosis pathways [15, 26, 27]. In particular, tumor cells confer resistance to apoptosis through the increase of Bcl2 and XIAP protein expression as well as the decrease of p53, Bax, and cytochrome c protein expression following AKT phosphorylation [28–30] (Fig. 5). As evidenced by western blot analysis, VGB4 induces apoptosis of lung cancer A549 cells and colon cancer HT29 cells through caspase- and p53-dependent pathways involving BAX/Bcl2, XIAP inhibition, cytochrome c release, and caspase activation. In other words, VGB4-induced cancer cell apoptosis occurs by blocking of the PI3K/AKT/XIAP pathway and activating of the p53/BAX/cyt c/caspase 9/caspase 3 pathway.

Finally, our findings demonstrate that VGB4 can be considered as a potent anti-tumor agent due to its anti-proliferative, anti-migration, and apoptotic induction activities in A549 and HT29 cancer cell lines.

## Declarations

### Funding

No funding was received for conducting this study.

### Data Availability

All data generated or analysed during this study are included in this article.

### Author contributions

All authors contributed to the study conception and design. Material preparation, data collection, and the analyses were performed by [Maryam Farzaneh Behelgardi], [Zahra Gholami Shahvir] and [S. Mohsen Asghari]. The manuscript was written by [Maryam Farzaneh Behelgardi] and all authors read and approved the final version of the manuscript.

### **Ethics declarations**

### **Conflict of interest**

The authors declare that they have no conflict of interest.

### **Ethical approval**

This study includes only laboratory studies on stable established cell lines. No ethical or institutional approval is required.

### **Consent to Participate**

No humans were used in this study.

### **Consent to Publish**

Authors approve for submitting the publication.

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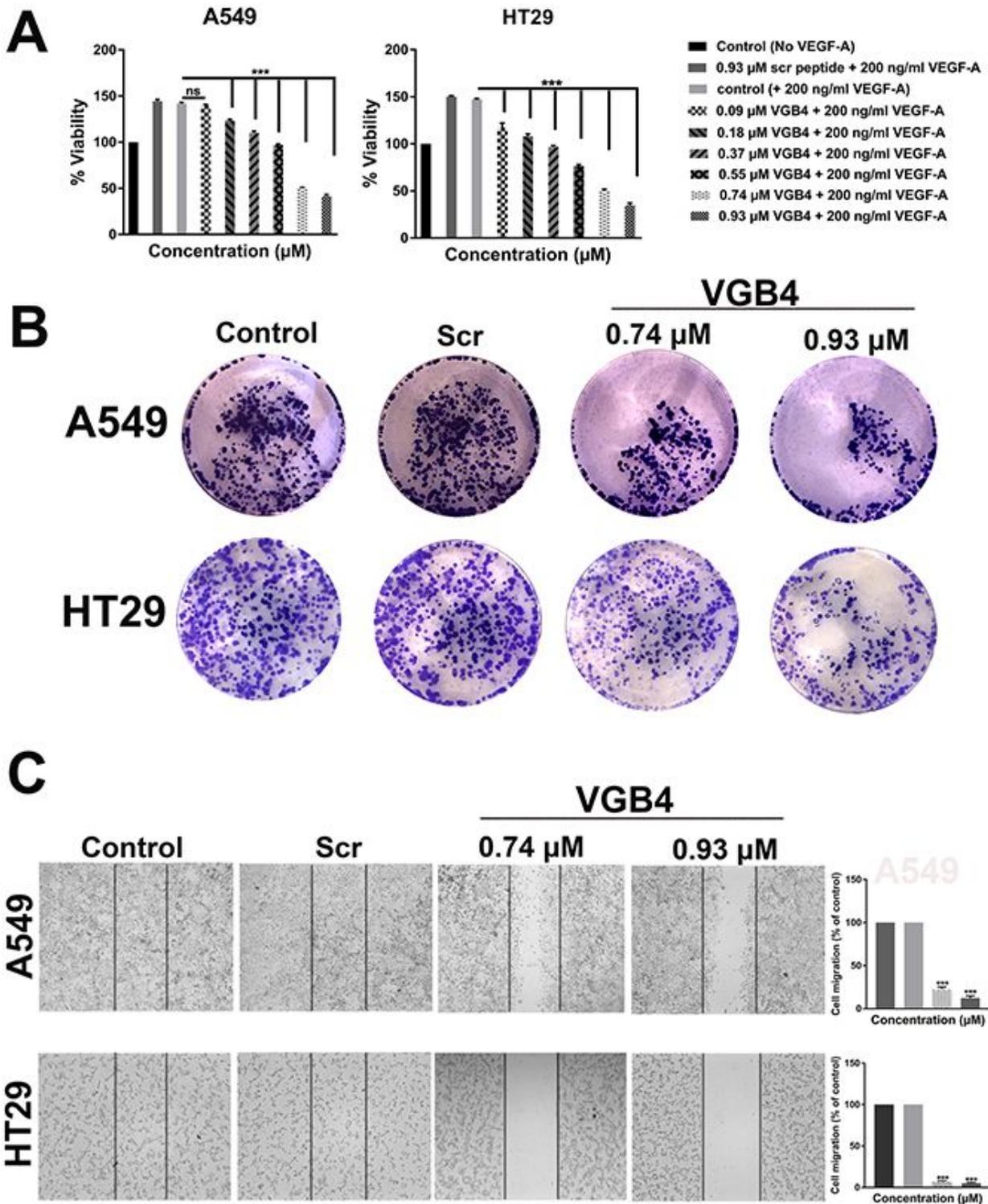
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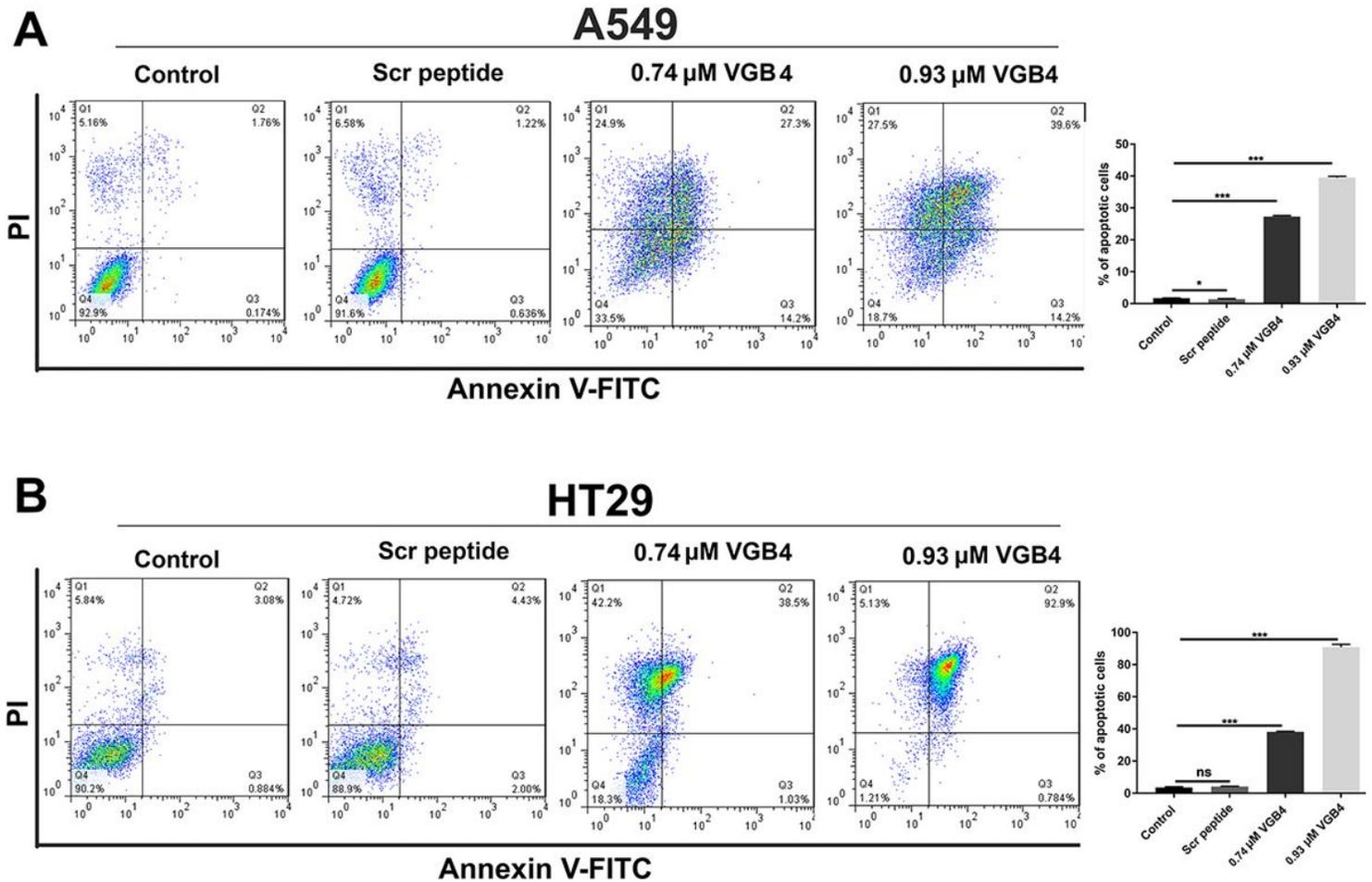
## Figures



**Figure 1**

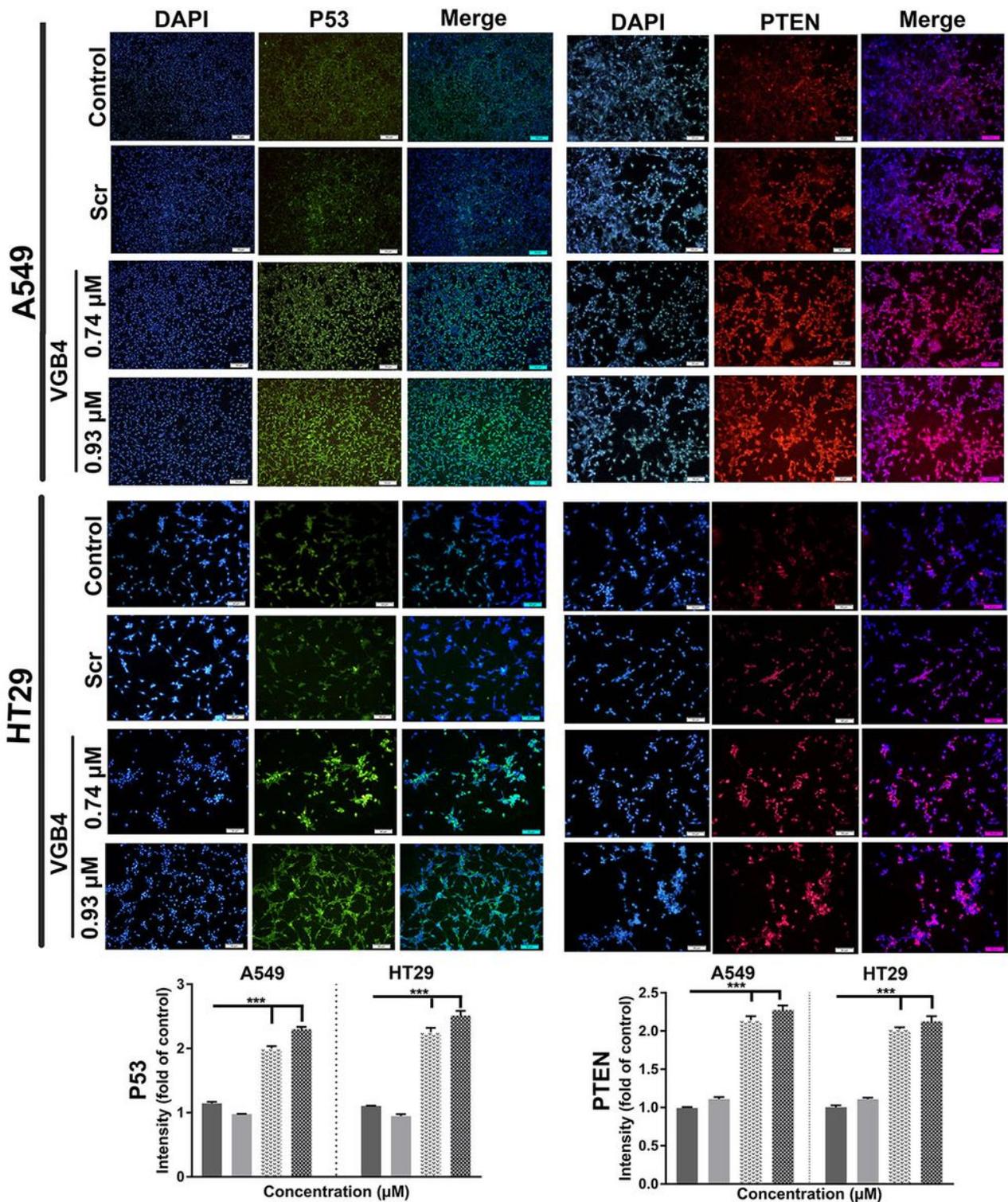
The inhibitory effect of VGB4 on cell proliferation and migration. **A** To assess the anti proliferative effect of VGB4, A549 and HT29 cells were treated with different concentrations of VGB4 (0.09, 0.18, 0.37, 0.55, 0.74 and 0.93 µM) or (0.93 µM) scr for 24 h in the presence of 200 ng/ml VEGFA. **B** To further investigate the anti proliferative effect of VGB4, cells were seeded and allowed to adhere overnight. After replacing the media with fresh medium containing 200 ng/ml VEGFA and concentrations (0.74 and 0.93 µM) of

VGB4 or (0.93  $\mu$ M) scr, cells were grown for 11 days with media changes every 3 days. The colonies were fixed with 4% formaldehyde, and stained with crystal violet (0.1%). C To examine the migration rate of HT-29 and A549 cells treated with concentrations (0.74 and 0.93  $\mu$ M) of VGB4 or (0.93  $\mu$ M) scr in the presence of 200 ng/ml VEGFA, cells were cultured until 90 % confluency then the sterile 200- $\mu$ l pipette tip was used to create the wound in the monolayer of cells. After 24 h, the rate of wound healing was monitored using an inverted microscope. Data were represented as mean  $\pm$  SD, n=3, \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001 versus control.



**Figure 2**

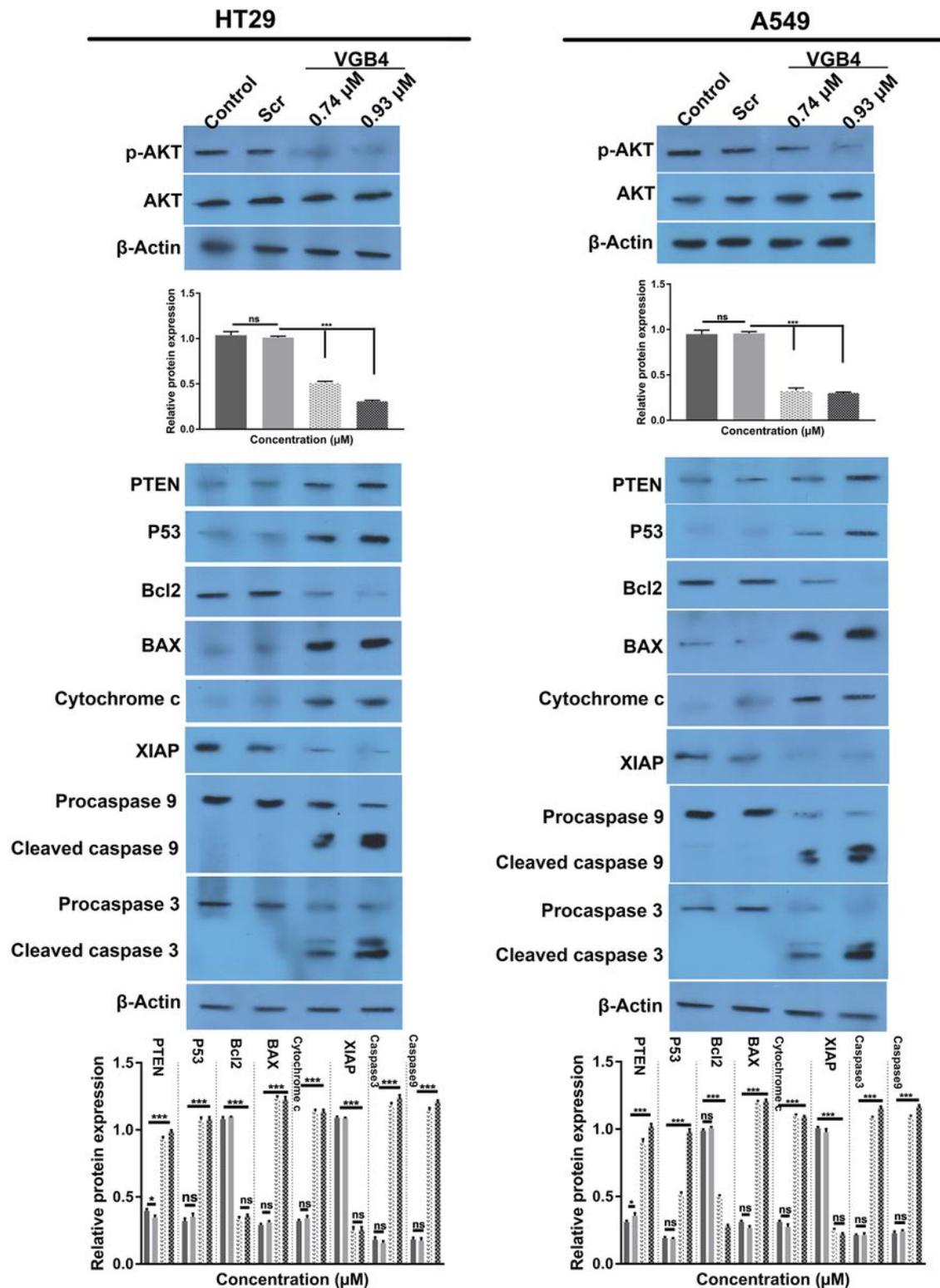
Effects of VGB4 on induction of apoptosis in (A) A549 and (B) HT-29 cells. The cells were treated with VGB4 (0.74 and 0.93  $\mu$ M) or scr (0.93  $\mu$ M) for 24h in the presence of 200 ng/ml VEGFA, labelled with FITC Annexin V and PI. All data were represented as mean  $\pm$  SD, n=3, \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001 compared to the control.



**Figure 3**

Effect of VGB4 on p53 and PTEN protein expression in A549 and HT29 cells. The cells were treated with VGB4 (0.74 and 0.93 μM) or scr (0.93 μM) for 24 h, fixed with 3.7% formaldehyde for 10 min at room temperature, washed two times in PBS-T, permeabilized with 0.2% Triton X-100 for 5 min at room temperature. After washing in PBS-T and adding blocking buffer, the cells were reacted with anti-p53 and anti-PTEN primary antibodies overnight at 4 °C, and reacted with Fluorescein isothiocyanate (FITC)-

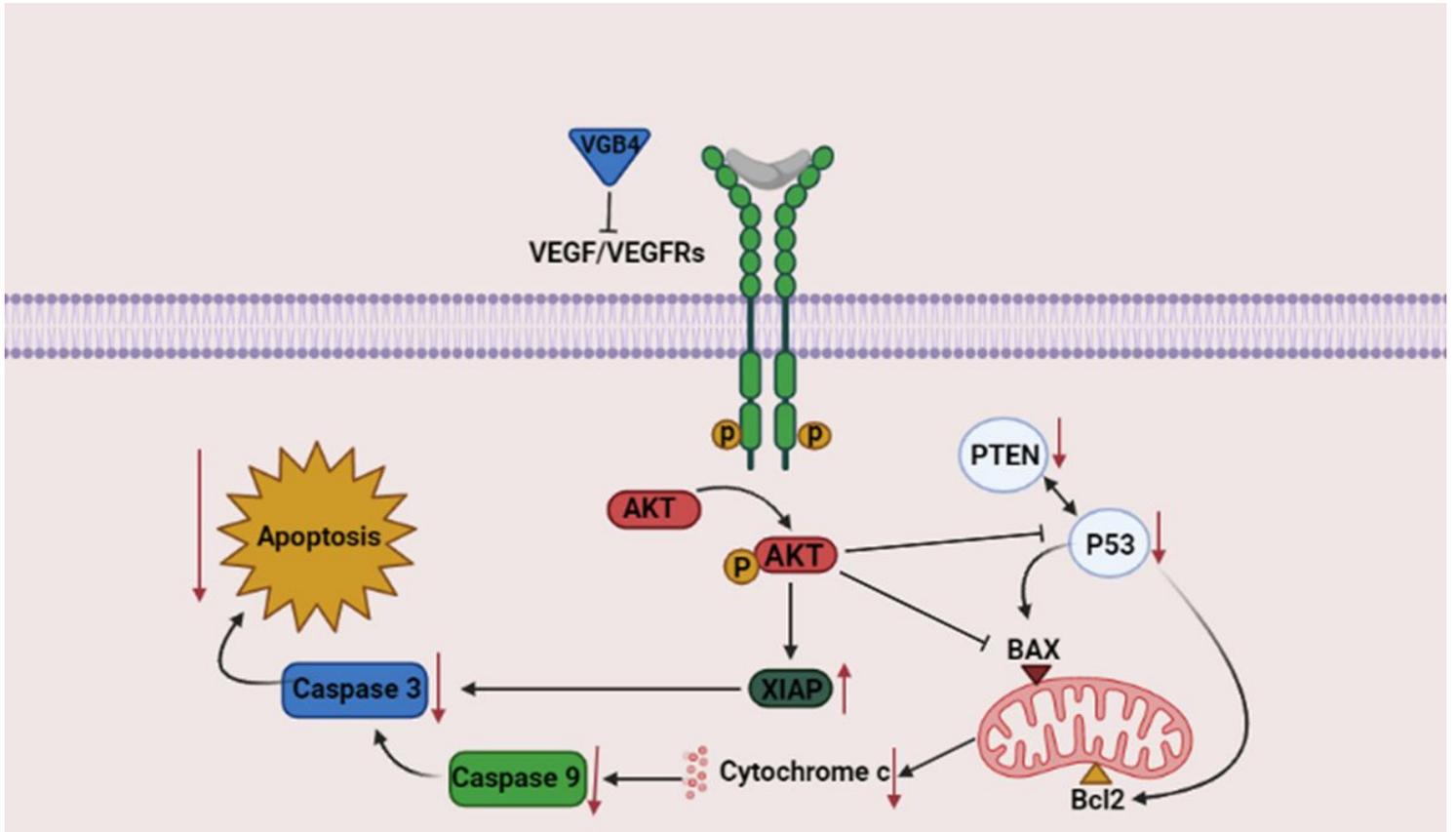
labeled goat anti-mouse IgG secondary antibody and Phycoerythrin (PE)-labeled goat anti-mouse IgG secondary antibody, respectively. The cells were counterstained with DAPI solution. Data were represented as mean  $\pm$  SD,  $n=3$ , \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  versus control.



**Figure 4**

The suppressing effect of VGB4 on VEGF signaling pathways related to apoptosis. A549 or HT29 cells were treated with different concentrations of VGB4 (0.74 and 0.93  $\mu$ M) or scr (0.93  $\mu$ M) in the presence of

VEGF-A (200 ng/ml). Then the cells were lysed with RIPA buffer, and subjected for western blot to analyze the expression level of p53, PTEN, phospho-AKT, total AKT, Bcl2, BAX, cytochrome c, XIAP, caspase 3 and caspase 9.  $\beta$ -Actin was used as reference to quantify the protein bands by densitometry using ImageJ software. Data were represented as mean  $\pm$  SD, n=3, \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001 compared to control.



**Figure 5**

Schematic representation of VEGF downstream signaling pathways related to apoptosis. VGB4 due to its ability to bind to VEGFRs, disrupts VEGF / VEGFR interaction leading to the activation of p53/BAX/cyt c/caspase 9/caspase 3 pathway and inactivation of PI3K/AKT/XIAP pathway.