The regulation and mechanism of a dual PI3K/mTOR signaling inhibitor in hemangioma vascular endothelial cells in vitro

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Research article

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Abstract

Objective To observe the influence of the dual PI3K/mTOR inhibitor NVP-BEZ235 on proliferation and apoptosis of hemangioma cells in vitro and key molecules of the PI3K/Akt/mTOR signaling pathway.

Methods Hemangioma-derived endothelial cells (HeECs) were obtained by surgical resection and cultured after the explants with the trypsin-digestion method. Fourth generation cells were cultured with serum-free medium for 24 hours. Then, the intervention group cells were added to the culture medium with 0.50 μM or 1.00 μM NVP-BEZ235. Cell proliferation was detected with CCK-8 assays, apoptosis was detected by flow cytometry, and PI3K, Akt, mTOR, and p70s6k protein levels were detected by Western blots. Then, the relationship between the phenotype of hemangioma vascular endothelial cells and the four proteins was analyzed.

Results the 0.50 μM and 1.00 μM NVP-BEZ235 groups were significantly lower (0.88±0.03 and 0.59±0.05, respectively) than the control group (1.10±0.02) (P<0.01). The rate of G_0/G_1 phase cells in the 0.50 μM and 1.00 μM NVP-BEZ235 group were higher than the control group (P<0.01). The total rates of apoptotic cells in the 0.50 μM and 1.00 μM NVP-BEZ235 groups were higher than the control group (2.77±1.23)% (P<0.01). The PI3K pathway related protein levels in the NVP-BEZ235 group were lower than control group (P<0.01).

Conclusion The PI3K/Akt/mTOR signaling pathway participates in hemangioma development. NVP-BEZ235 affected hemangioma vascular endothelial cells in vitro by regulating the PI3K/Akt/mTOR signaling pathway in a dose-dependent manner.

Introduction

The PI3K/Akt/mTOR signaling pathway is abnormally activated in a variety of human tumors. Mutations in components of this pathway induce cell transformation and play an essential role in tumor cell survival, proliferation and migration. The PI3K/Akt/mTOR signaling pathway is involved in the development and progression of infantile hemangiomas[1]. Furthermore, the PI3K/Akt/mTOR signaling pathway inhibits hemangioma growth. Previous experiments in our group also confirmed that the mTOR inhibitor RAD001 inhibited the proliferation of hemangioma endothelial cells in vitro. However, RAD001 causes negative feedback activation of the PI3K/Akt and MAPK/ERK signaling pathways, which could affect its inhibition of proliferation. NVP-BEZ235, a newly developed PI3K/mTOR dual inhibitor, has been investigated for its active antitumor effects in preclinical trials of various tumors[2]. NVP-BE Z235can effectively avoid the feedback mechanism of the PI3K survival pathway caused by a mTOR inhibitor alone, which can increase the inhibitory effect of mTOR and inhibit Akt activity for a longer period[3].

In this study, the PI3K/mTOR dual inhibitor NVP-BEZ235 was used to treat endothelial cells of hemangioma in vitro. Using CCK-8, flow cytometry and Western blot techniques, hemangioma endothelial cell proliferation, apoptosis and signal molecule protein expression in PI3K signaling pathways were analyzed, elucidating the role and mechanism of PI3K/mTOR dual inhibitors in hemangioma endothelial

cells and providing an experimental reference for broadening the treatment options of infantile hemangioma.

1 Materials And Methods

1.1 Hemangioma specimen

The hemangioma specimen used in this study was taken from the abdominal hemangioma of a 5-month-old female child. The tumor was large and grew rapidly, and the family members required surgical resection. The use of the specimen was approved by the family.

1.2 Reagents

Rabbit anti-human mTOR monoclonal antibody, rabbit anti-human PI3K monoclonal antibody, goat anti-human p-Akt polyclonal antibody, rabbit anti-human p70s6k monoclonal antibody (Santa Cruz, USA), rabbit anti-coagulation factor Ⅷ (Bioss, Beijing, China), NVP-BEZ235 (Selleck, USA), and RPMI1640 medium (HyClone, USA) were used.

1.3 Primary cell culture

Hemangiomas were surgically resected from the infant. The tissue was flushed, digested with trypsin, and cut to 1-2 mm^3 to obtain tumor tissue for culture. The tumor tissue was uniformly distributed on the bottom of a culture flask at 3×4 per row. These culture bottles were inverted in a 37℃ 5% CO_2 incubator for 8 hours, then turned upside down and filled with 1 ml complete medium. The medium was replaced once daily. The tissue block was removed when many spindle or irregular cells crawled out of the cell island and occupied more than 75% of the culture bottle. Then, 4 ml complete medium was added to the culture bottles. The culture medium was replaced every other day. After cells were more than 80% confluent, subculturing was carried out.

1.4 Cell identification

An immunohistochemical method was used to detect the coagulation factor Ⅷ-related antigen in hemangiomas. The coagulation factor Ⅷ antibody was diluted 1:100 by universal antibody diluent.

1.5 Cell groups

Logarithmic-phase cells were synchronously incubated with serum-free medium for 24 h. Then, 4 ml of complete medium was added to the control group, and 0.50 μMNVP-BEZ235 was added to 4 ml of culture medium containing NVP-BEZ235 at 0.5 μmol/L. The 1.00 μMNVP-BEZ235 group had 4 ml culture medium containing NVP-BEZ235 at 1.00 μmol/L.

1.6 CCK-8 detects cell proliferation

In a 96-well culture plate, cells were inoculated at 2×10^4 cells/well at 37°C in a 5% CO_2 incubator. After 24 hours, medium was removed, three washes with PBS were performed, and 10 μl samples were used to test the drugs. Among them, NVP-BEZ235 was used at 0.50 μM and 1.00 μM, while complete medium was used as the blank. Each group set up had four holes. After further incubation for 24 hours, 10 μl CCK-8 was added into each hole, and the plate was placed back into the incubator for approximately 2 hours. The OD value was detected on the enzyme labeling apparatus, and the wavelength was set at 450 nm.

1.7 Flow cytometry for detection of hemangioma endothelial cell apoptosis and cell cycle (Annexin V/PI double staining)

In the intervention group, 3 ml of 0.50 μM and 1.00 μM NVP-BEZ235 were added to the control group, and 3 ml of serum-free medium was added. Then, cells were incubated in the incubator for 24 hours, washed with PBS buffer solution once, digested with trypsin, and centrifuged at 1000 rpm for 5 minutes in a 15 ml centrifuge tube with the same volume of PBS buffer solution. The cell concentration was adjusted to 1×10^6 /ml. The supernatant was discarded, and 200 μl binding buffer was added to the mix and transferred to the flow tube. Then, 10 μl A-V was added to the icebox to avoid light for 15 minutes, and 10 μl PI was added to the mix. Five minutes later, 300 μl of binding buffer was added. Flow cytometry was used to detect the cells within 1 hour.

1.8 Western blotting

Proteins (60 μg/μl) extracted from cultured cells by RIPA lysate were separated by SDS-PAGE, transferred onto nitrocellulose membranes and incubated with the relevant antibodies overnight at 4°C. After membrane blocking, antibody incubation in 5% low fat dry milk was performed (rabbit anti-human mTOR monoclonal antibody, 1:400; rabbit anti-human PI3K monoclonal antibody, 1:400; goat anti-human p-Akt polyclonal antibody, 1:200; rabbit anti-human p70s6k monoclonal antibody, 1:400; and anti-β-actin, 1:3000). Then, the membrane was washed in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBST), incubated with goat anti-rat IgG (diluted 1:20000) for 1 hour, washed and detected by enhanced chemiluminescence (LI-COR Odyssey).

1.9 Statistical analysis

SPSS 20.00 and GraphPad Prism software were used for statistical analysis, and grouped data are presented as the mean±s.d. The difference between two experimental groups was assessed using a two-independent samples *t*-test, test level alpha=0.05.

2 Results

2.1 Endothelial hemangioma cells were cultured in vitro

The culture technique was mature, and the cells were easily cultured. Approximately 1 week later, cells moved out of the floccus around the tissue mass. Under an inverted phase difference microscope, the

endothelial cells of hemangioma appeared spindle-shaped or multilateral with a scattered distribution. At 4 weeks, cells covered more than 90% of the bottom of the bottle. Some cells grew in concentric circles and exhibited the characteristic paving stone arrangement of endothelial cells. (Figures 1, 2)

2.2 Endothelial cell identification of hemangioma

The Ⅷ clotting factor is mainly expressed in the cytoplasm of endothelial cells. Immunohistochemical results showed that the cytoplasm of hemangioma endothelial cells contained scattered brown-yellow positive particles. Combining the morphological characteristics of cultured cells, human hemangioma endothelial cells were confirmed.

2.3 Endothelial cell growth curve of hemangioma

Hemangioma endothelial cells showed adherent growth the next day after passaging; 1-2 days after, the growth was slow, and cell division and proliferation were slower. Six days after culture, cell division and proliferation accelerated ($6.59\pm0.05\times10^4$/hole) and then gradually entered the logarithmic phase ($16.38\pm0.18\times10^4$/hole). Twelve days later, the cell growth rate was slow and was into the plateau phase ($18.58\pm0.79\times10^4$/hole). At this point, the cell growth was stable. Cell morphology was observed, and cells were quantified with inverted phase difference microscopy the next day. The growth curve was plotted. (Figure 3).

2.4 CCK-8 detection of cell proliferation

Twenty-four hours after NVP-BEZ235 treatment, CCK-8 assays showed that OD values in the 0.50 μM and 1.00 μMNVP-BEZ235 groups were 0.88±0.03 and 0.59±0.05, respectively, which were significantly different from the control group (1.10±0.02) (P<0.01). The inhibitory effect on cell proliferation of the 1.00 μM group was stronger than that of the 0.50 μM group, and the difference was significant (P<0.01). (Figures 4, 5)

2.5 Effect of NVP-BEZ235 on the endothelial cell cycle in hemangioma

Twenty-four hours after NVP-BEZ235 treatment, the proportion of G_0/G_1 phase cells in the 0.50 μM group was (60.62±0.71)% and was (65.99±2.55)% in the 1.00 μM group; both were higher than that in the control group (53.71±1.43)%, with significant differences (P<0.01). Additionally, the 1.00 μM group had a higher G_0/G_1 rate than the 0.50 μM group, and the difference was significant (P<0.05). (Figures 6, 7)

2.6 Effect of NVP-BEZ235 on apoptosis of hemangioma endothelial cells

After a 24 h NVP-BEZ235 treatment, the total apoptosis rate of endothelial cells in the 0.50 μM group was (9.20±0.75)% and (13.13±1.72)%, in the 1.00 μM group; both were higher than that in the control group (2.77±1.23)%, with significant differences (P<0.01). Moreover, the 1.00 μM group had a higher apoptosis rate than the 0.50 μM group, and the difference was significant (P<0.05). (Figures 8, 9)

2.7 Effect of NVP-BEZ235 on PI3K, p-Akt, mTOR and p70s6k protein expression in hemangioma endothelial cells

IOD values of the β-actin, PI3K, p-Akt, mTOR and p70s6k proteins were determined by software analysis after a 24 h intervention of 0.50 μM NVP-BEZ235 and 1.00 μM NVP-BEZ235 cells. Among them, the expression levels of PI3K, p-Akt, and mTOR in the 0.50 μM NVP-BEZ235 group were lower than those in the control group, and the p70s6k protein levels in the experimental group were higher than those in the control group. PI3K, p-Akt, mTOR and p70s6k in the experimental group were higher than those in the control group. Compared with the control group, PI3K, p-Akt, mTOR and p70s6k showed a significant difference (P<0.01). In the experimental group, PI3K, p-Akt, mTOR and p70s6k were significantly different from those of the control group (P<0.01). PI3K, p-Akt, and mTOR protein levels decreased significantly in the 1.00 μM NVP-BEZ235 group compared with the 0.50 μM NVP-BEZ235 group, while p70s6k protein levels increased significantly (P<0.01). (Table 1, Figure 10)

Table 1. Comparison of PI3K, p-Akt, mTOR and p70s6k expression among the 0.50 μM NVP-BEZ235 group, 1.00 μM NVP-BEZ235 group and the control group (±s, n=3).

group	PI3K	p-AKt	mTOR	p70S6K
control group	0.25±0.01	0.17±0.01	0.19±0.00	0.10±0.02
0.50 μM group	0.16±0.03[a]	0.13±0.01[a]	0.12±0.02[a]	0.18±0.01[a]
1.00 μM group	0.10±0.01[ab]	0.10±0.01[ab]	0.05±0.00[ab]	0.31±0.02[ab]

Note: a vs control group, P<0.01. b vs 0.50 μM group, P<0.01.

3 Discussion

Hemangioma is the most common benign tumor caused by abnormal blood vessels in neonatal and early childhood. Current treatments for hemangioma are unsatisfactory because the pathogenesis of hemangioma is unclear. Some studies have suggested that hemangiomas are caused by abnormal mutations of mediating molecules in cellular pathways[4]. Peng[5] found that in a mouse hemangioma endothelial cell model, Pingyangmycin inhibited the proliferation of hemangioma endothelial cells by reducing PI3K and Akt phosphorylation levels, while the effect on tumor cell activity, apoptosis, and

invasion was achieved by inhibiting a PI3K activator, insulin-like growth factor IGF-1. Thus, the PI3K/Akt signaling pathway may be involved in the antitumor effect of Pingyangmycin on the endothelial cells of hemangioma in mice. Our previous experiments also confirmed that, in the endothelial cells of hemangioma, PI3K and p-Akt, the effector molecules in the PI3K/Akt/mTOR signaling pathway, had significantly higher levels in the proliferative phase than the regression phase. In this study, the effector molecules PI3K, p-Akt, mTOR and p70s6k in the PI3K/Akt/mTOR signaling pathway were all expressed in the endothelial cells of hemangioma in vitro.

In recent years, with the development of molecular targeted therapy, more targeted drugs have been developed. The PI3K/Akt signaling pathway is closely related to the proliferation, differentiation, and apoptosis of tumor cells, and the development and clinical research of its inhibitors has attracted more attention. Currently, PI3K inhibitors can be roughly divided into three categories: PI3K subtype-specific inhibitors, pure pan-PI3K inhibitors and PI3K/mTOR dual inhibitors. Among them, NVP-BEZ235, as a dual inhibitor of PI3K/mTOR, was the first to enter clinical trials. NVP-BEZ235 inhibits tumor cell proliferation, induces cell cycle arrest in G_1 phase, and inhibit angiogenic activity. Additionally, while inhibiting PI3K kinase activity, it also reduces mTOR kinase activity, effectively avoiding the feedback mechanism of the PI3K survival pathway caused by the mTOR inhibitor alone, which increases the inhibitory effect of mTOR and further inhibits Akt activity for a long time[3]. By inhibiting the PI3K/Akt/mTOR signaling pathway, NVP-BEZ235 can induce cell proliferation inhibition, cycle arrest, and other antitumor effects, which have promising therapeutic prospects in many tumors[6-9]. In this study, after NVP-BEZ235 treatment, the proliferation rate of hemangioma endothelial cells in vitro was lower than that in the control group. The total apoptosis rate was increased, and the proportion of cells in G_0/G_1 phase was higher than that in the control group, suggesting that NVP-BEZ235 could inhibit the proliferation of endothelial cells in hemangioma, block the transformation of cells from G_1 phase to S phase, and promote apoptosis.

Previous studies have shown that activation of the PI3K/Akt/mTOR signaling pathway can inhibit apoptosis, promote cell proliferation, and participate in angiogenesis[10]. Previous studies of our research group found that mTOR expression was higher in the proliferative phase than in the regressive phase of pediatric hemangioma, while p70s6k-α expression was higher in the regressive phase than in the proliferative phase[11]. In a xenograft model of human hemangioma in nude mice and in vitro culture of hemangioma endothelial cells, the PI3K inhibitor LY294002 inhibited PI3K and p-Akt protein expression in the PI3K/Akt signaling pathway, inhibited PI3K activation, prevented Akt phosphorylation, and caused cultured hemangioma endothelial cells to stop at G_0/G_1 stage and induce apoptosis[12]. In this study, after NVP-BEZ235 treatment, the PI3K, p-Akt and mTOR protein expression decreased compared with the control group, while the p70s6k protein expression increased, suggesting that NVP-BEZ235 may inactivate PI3K by inhibiting PI3K activity, thereby regulating the expression of the downstream signaling molecules p-Akt, mTOR and p70s6k. NVP-BEZ235 may downregulate the expression of PI3K and inhibit the phosphorylation of Akt, which then inhibits the downstream signaling molecule mTOR and prevents the phosphorylation of p70s6k. Then, a series of signal cascade reactions are triggered, which inhibits the proliferation of hemangioma endothelial cells and promotes apoptosis. At the same time, in this

experiment, the effect of 1.00 μM NVP-BEZ235 on cell proliferation, apoptosis promotion, and cell cycle arrest was stronger than that of 0.50 μM NVP-BEZ235, and expression of PI3K, p-Akt, mTOR and p70s6k was stronger than that of 0.50 μM NVP-BEZ235, suggesting that the inhibitory effect of NVP-BEZ235 on hemangioma endothelial cells may be dose-dependent.

In summary, PI3K, p-Akt, mTOR and p70s6k protein molecules are expressed in cultured hemangioma endothelial cells in vitro. NVP-BEZ235 can inhibit the proliferation of hemangioma endothelial cells, regulate the cell cycle, and promote apoptosis by interfering with the PI3K/Akt/mTOR signaling pathway, which has antitumor effects on infant hemangioma diseases. By studying the mechanism of the signaling pathway, we will help elucidate the pathogenesis of infantile hemangioma and broaden the selection of clinical treatment programs for infantile hemangioma.

Declarations

Compliance with Ethical Standards

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Conflict of Interest: All of the authors declares that they have no conflict of interest.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Contributions

Authors' contributions

WSL, YY, JL, HH, HL, MY, HYT, NW, MN, XSH and SY conceived, designed, or planned the study. WSL, YY, JL, HH, HL and SY analyzed the data. MN and XSH acquired data. HH, HL, MY, HYT, NW, MN, XSH and SY helped interpret the results. WSL and SY provided study materials or patients. WSL, YY and SY drafted the manuscript. All authors revised and reviewed this work, and all authors gave their final approval of the submitted manuscript.

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Correspondence to Yu Song.

Ethics declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

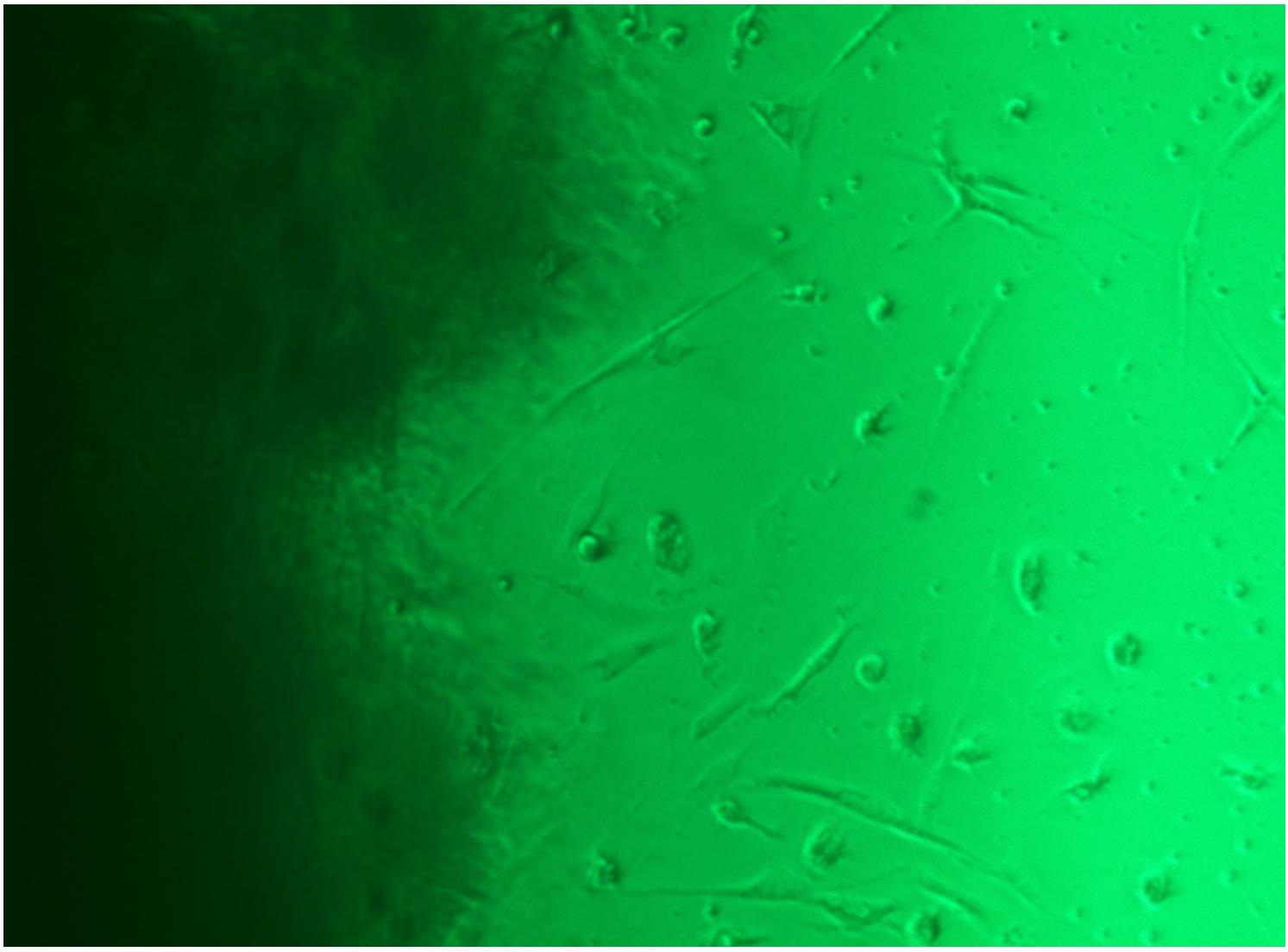


Figure 1

After 1 week of culture, hemangioma cells scattered and crawled out (× 100)

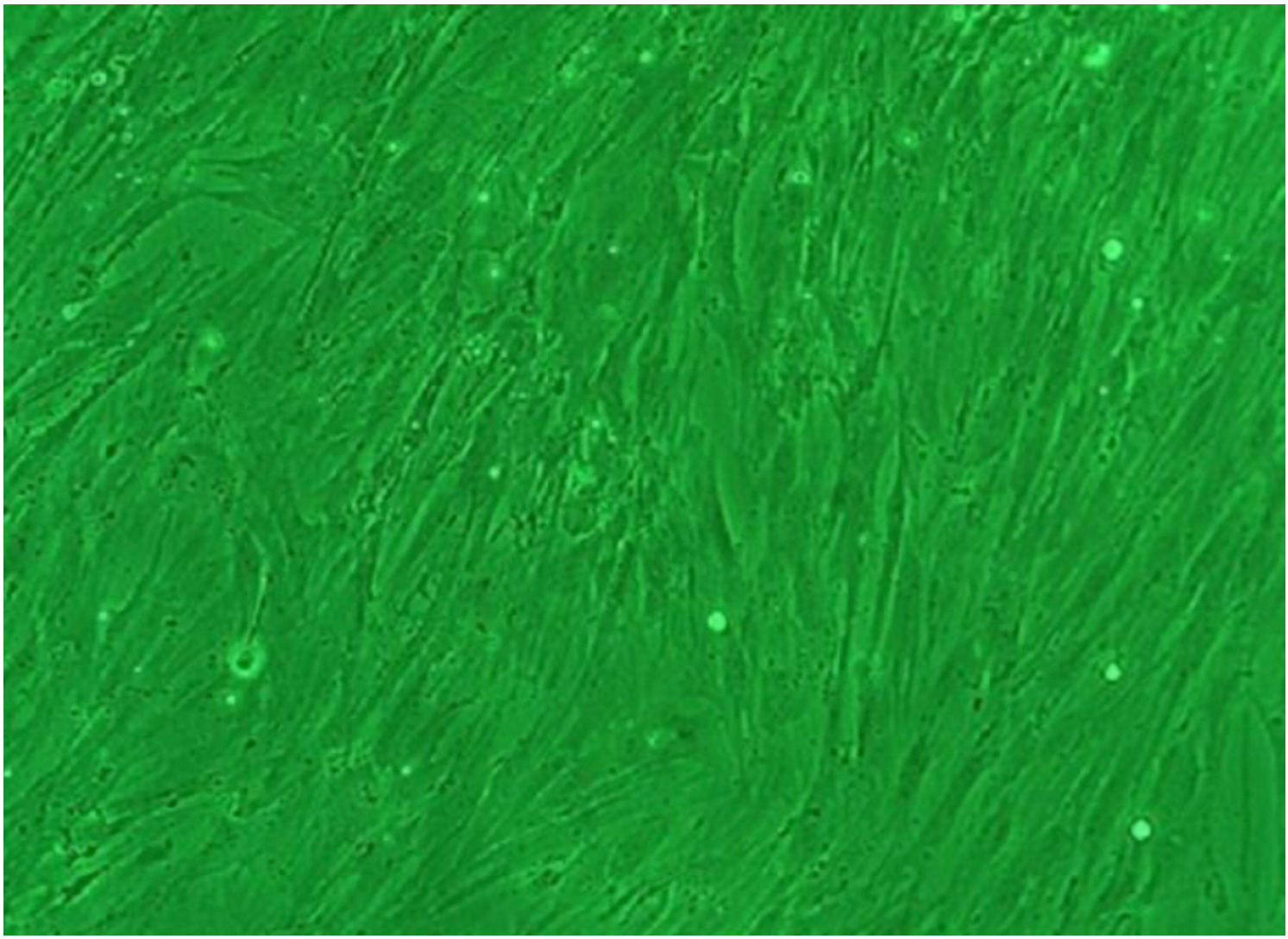


Figure 2

After 4 weeks of culture, hemangioma cells scattered and crawled out (× 100)

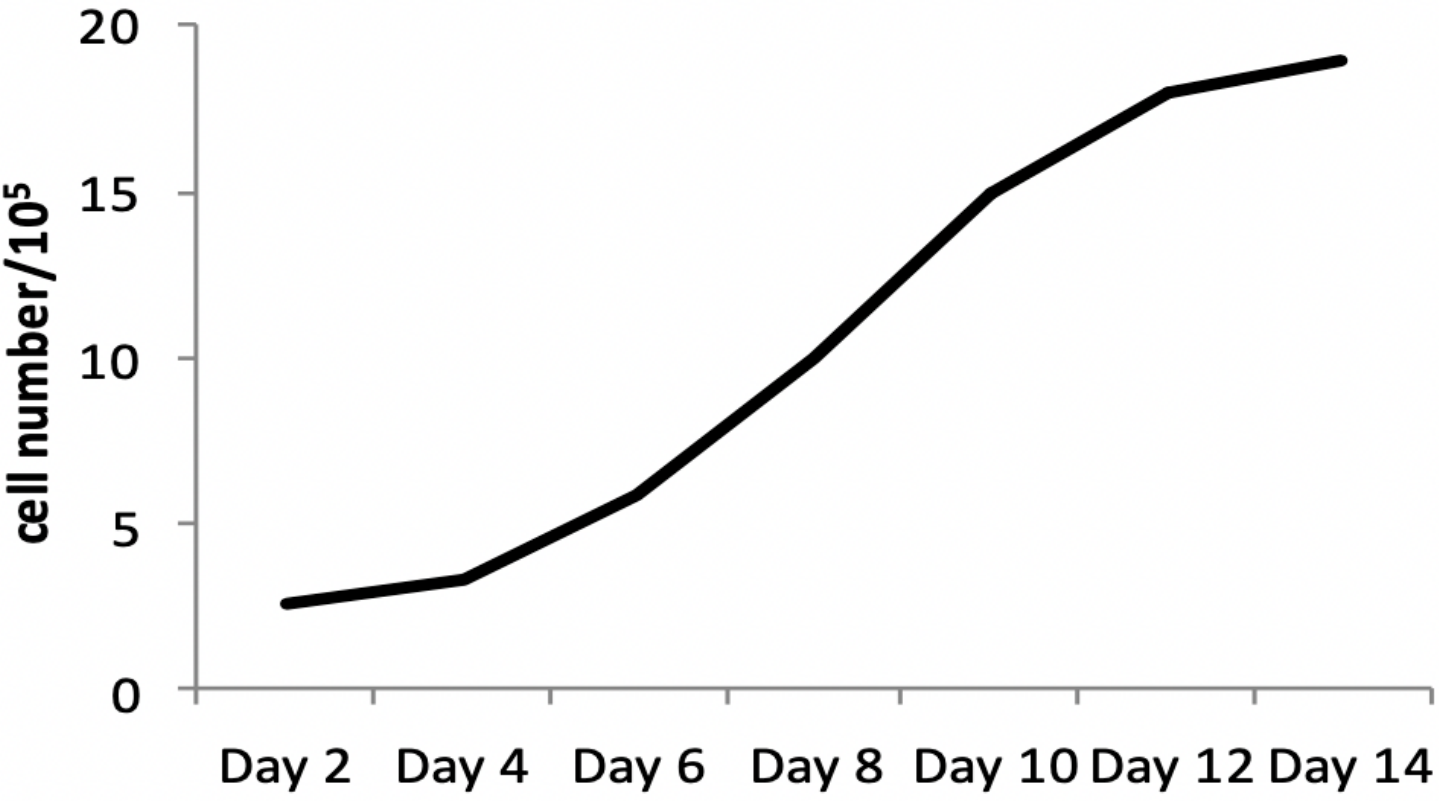
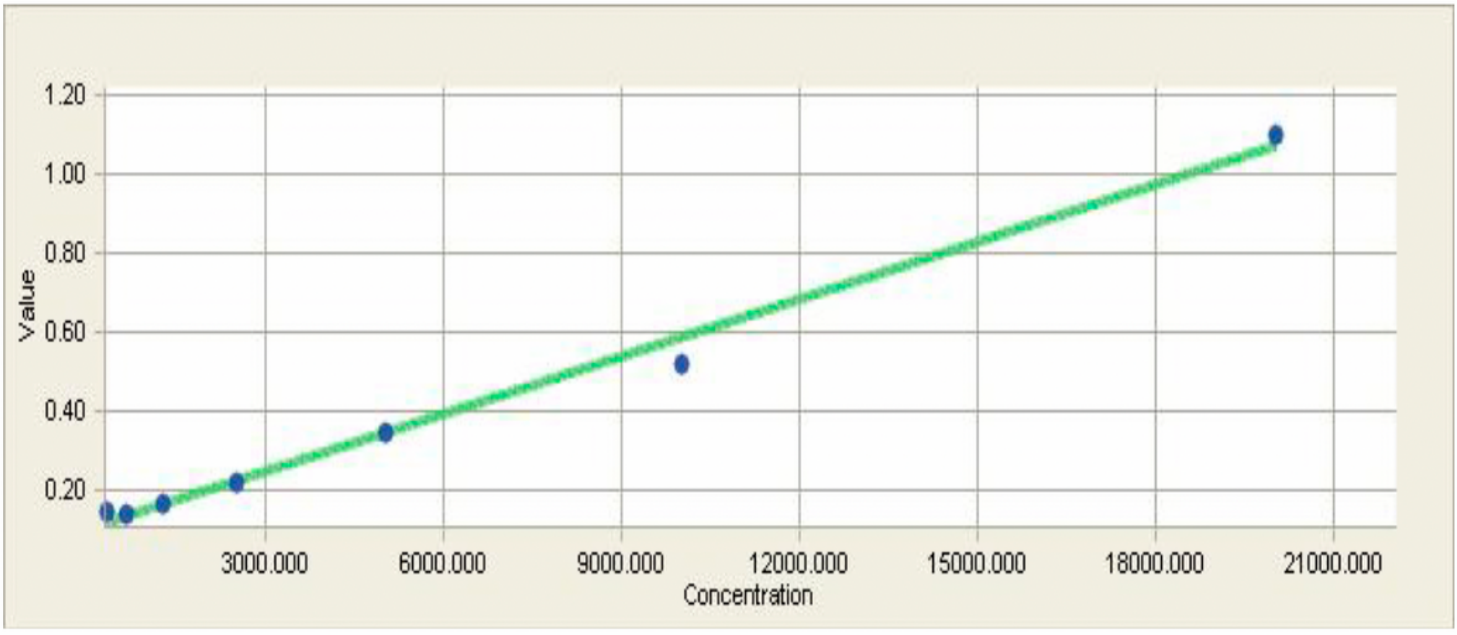


Figure 3

Endothelial cell growth curve of hemangioma



$Y=4.873x+0.10E-1, R^2=0.99$

Figure 4

CCK-8 detection standard curve. Y=4.873x+0.10E-1, R2=0.99.

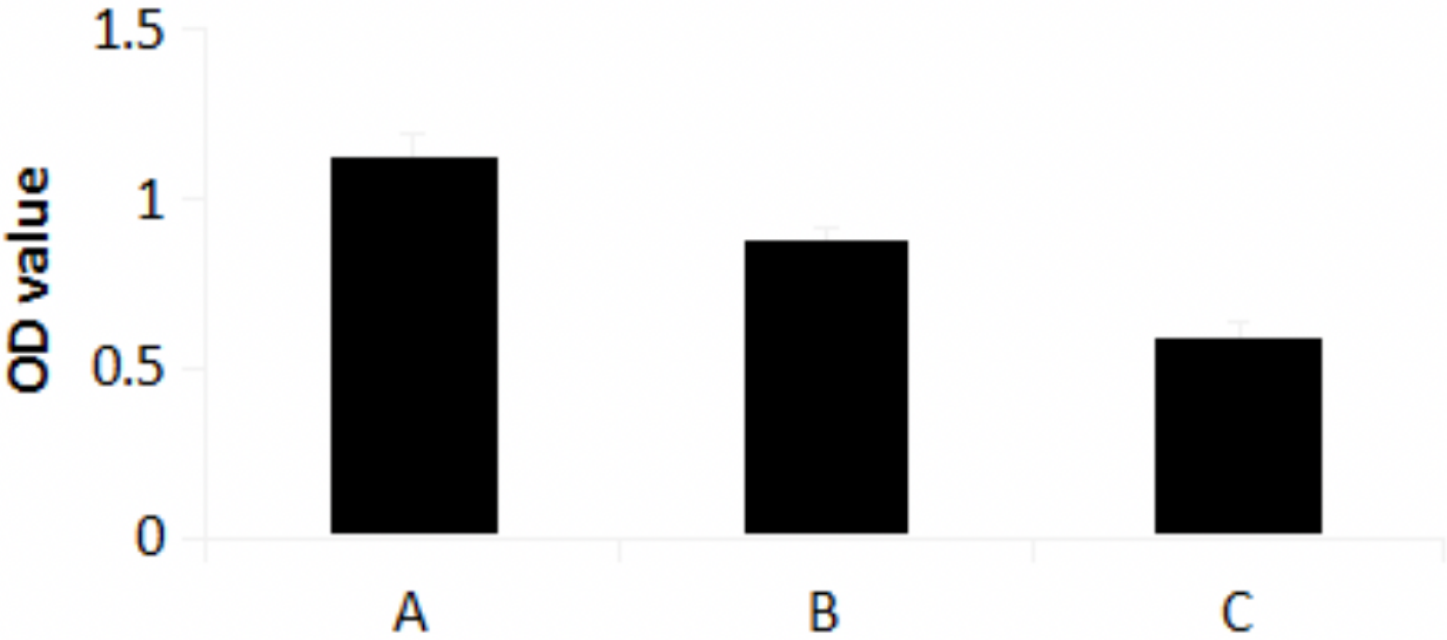


Figure 5

OD value changes inNVP-BEZ235 after intervention. A: control group B: 0.50μM group C: 1.00μM group.

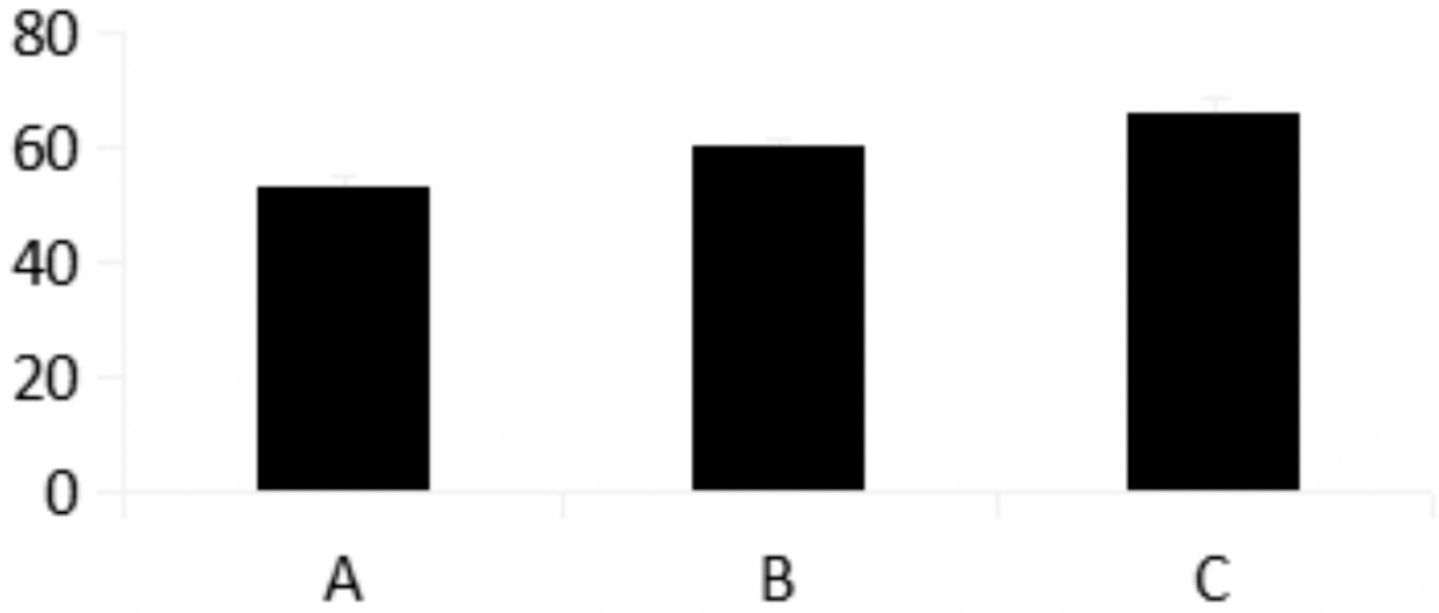


Figure 6

Changes of endothelial cell cycle in hemangioma after intervention with NVP-BEZ235, A: control group B: 0.50μM group C: 1.00μM group

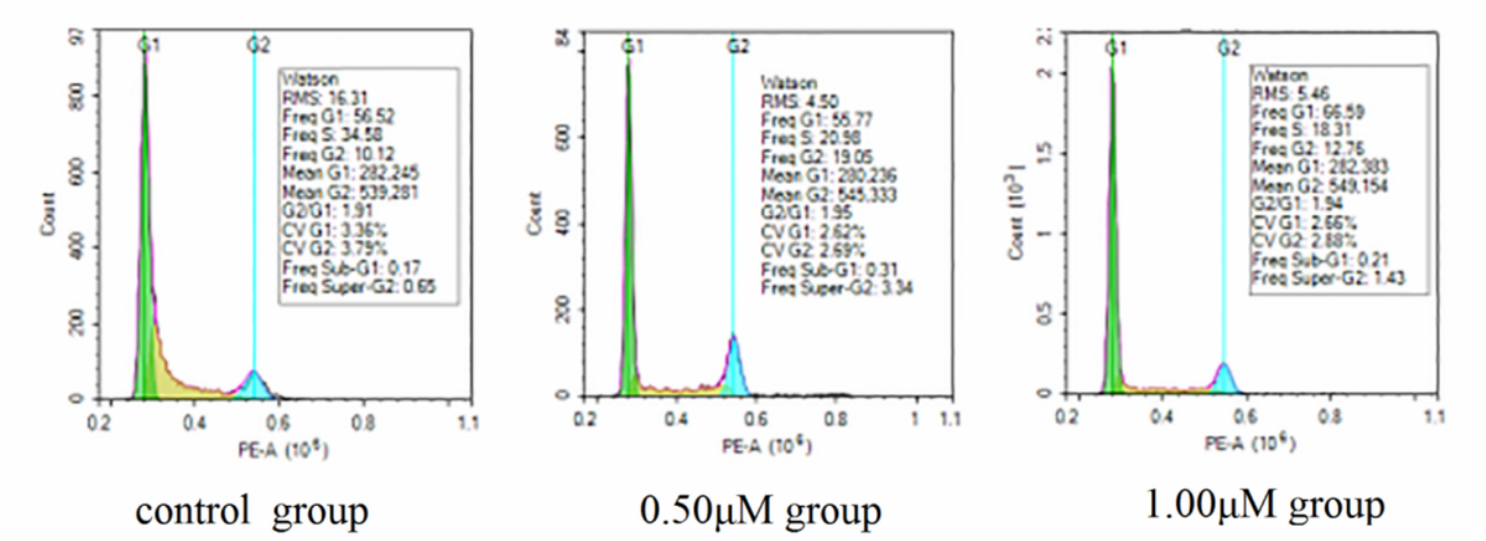


Figure 7

Apoptosis distribution of cells after intervention by NVP-BEZ235

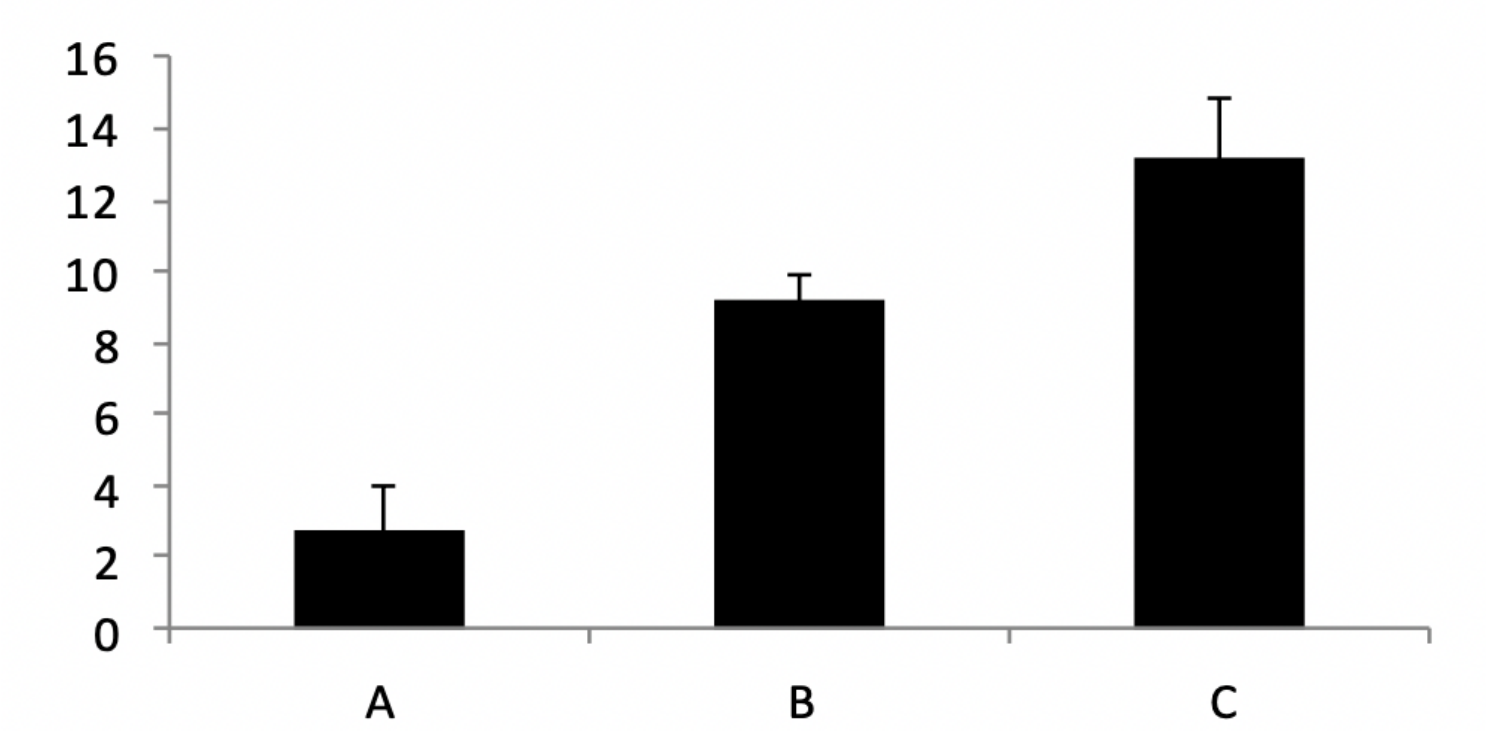


Figure 8

Changes of endothelial cell apoptosis in hemangioma after intervention with NVP-BEZ235. A: control group B: 0.50μM group C: 1.00μM group

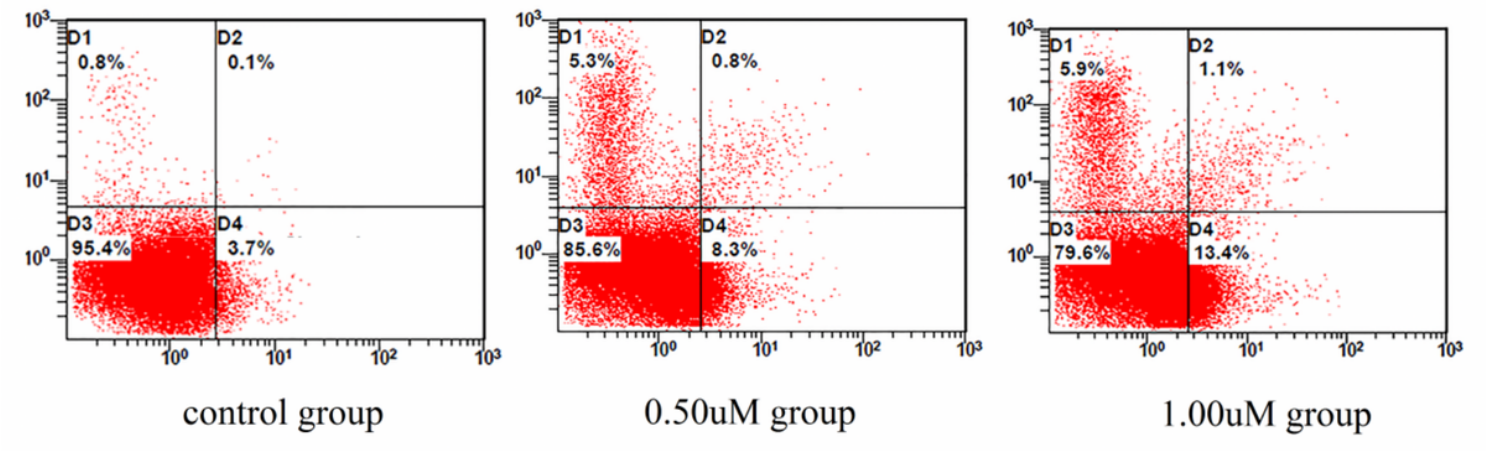


Figure 9

Scatter plot of apoptosis after intervention by NVP-BEZ235

Figure 10

Protein expression of p-Akt,p70s6k,PI3KandmTOR, A: control group B: 0.50μM group C: 1.00μM group