Original Article
HSP70 inhibitor VER155008 suppresses pheochromocytoma cell and xenograft growth by inhibition of PI3K/AKT/mTOR and MEK/ERK pathways

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Abstract: According to the most recent World Health Organization classification, all pheochromocytomas have metastatic potential. Up until now there has been an absence of effective therapeutic methods to inhibit tumor growth and metastasis, especially in metastatic foci. Therefore, the discovery of new and effective drugs is urgently needed. Because overexpression of HSP70 frequently occurs in a variety of tumor tissues, VER155008, a new inhibitor targeting HSP70, has shown an anti-tumor effect through inhibition of PI3K/AKT/mTOR and MEK/ERK pathways, both of which are closely connected with pheochromocytoma proliferation, migration, and biologic behaviors. In our research, we reveal that VER155008 can reduce proliferation of the pheochromocytoma cell line PC12 and induce apoptosis at a relatively low dose. Most importantly, VER155008 can effectively suppress cell migration and invasion. Subsequently, drug-effect mechanisms of VER155008 were further detected by western blot, and we found that VER155008 exhibited an anti-tumor effect through down-regulating phosphorylation of the PI3K/AKT/mTOR and MEK/ERK signaling pathways. Finally, the above phenomena were further confirmed in a mouse model in vivo, and the results showed that the drug significantly inhibited xenograft tumor growth. In summary, VER155008 is a potential and promising effective drug for treating patients with pheochromocytoma, and furthermore, it could delay/inhibit tumor metastasis.

Keywords: Pheochromocytoma, HSP70, VER155008, signaling pathway, PC12

Introduction

According to 2017 World Health Organization classification of pheochromocytoma (PHEO), the term "malignant pheochromocytoma" is not used, and all PHEOs are considered to have metastatic potential, because of an absence of a histologic system-until now-for evaluating their biologic behavior. Such a system would avoid the confusion of attempting to distinguish between locally invasive and metastatic PHEOs [1]. PHEO is a tumor that originates from the intra-adrenal medulla, and an extra-adrenal PHEO is defined as a paraganglioma. Although PHEOs are exceptionally rare catecholamine-secreting tumors, with an incidence of 2-8 per million population, they are a relatively common cause of secondary hypertension, which causes severe cardiovascular complications [2, 3]. Among these PHEOs, it has been revealed that up to 36% of SDHB (succinate dehydrogenase subunit B) mutations eventually undergo metastasis [4]. It has been well noted that surgery offers the best treatment option. The five-year survival rate for metastatic and invasive PHEO (< 50%) is poorer than that for non-metastatic PHEO (84-89%) [5, 6]. Furthermore, nowadays, the applying of traditional chemotherapy is of little benefit to patients with metastatic PHEO [7]. Hence, discovery of new targeted agents is urgently needed.

The heat shock protein (HSP) family is known as a family of molecular chaperones that play crucial roles in cellular processes [8], regulating correct conformation and maintaining the activ-

ity, function, and stability of more than 200 known proteins [9, 10]. Recently, some researchers have focused on the 70 kDa heat shock protein, HSP70-one member of the HSP family-because overexpression of HSP70 frequently occurs in a variety of tumor tissues, such as lung and pancreatic cancer [11-14]. In addition, abnormal expression of HSP70 is closely correlated with PI3K/AKT/mTOR and MEK/ERK signaling pathways, which are two of the most important pathways involved in tumor progression [5, 15]. Under the inhibition of HSP90 in the PC12 cell line, HSP70 also shows upregulation, and silencing of HSP70 intensifies 6-hydroxydopamine (6-OHDA)-induced apoptosis and HSP90 upregulation in PC12 cells [16]. Thus, we can reasonably hypothesize that HSP70 is a promising therapeutic target for developing novel small molecular drugs.

It is possible that VER155008, a small molecule inhibitor, binds to HSP70 ATPase and inhibits its activity through mimicking the ATPase binding domain, thereby inhibiting HSP70-associated protein activities. VER-155008 binding occurs within the ATPase site of Hsc70/Bag-1, and the adenosine portion of VER-155008 adopts protein interactions that correspond to those occurring in the adenosine portion of ATP [17]. Until now, few studies have examined whether inhibition of HSP70 could affect PHEO progression. Here, we evaluated the effect of VER155008 on PHEO cell line PC12 in *in vitro* and *in vivo* mouse models, and explored the possible molecular mechanisms involved.

Materials and methods

Cell line and reagents

We purchased rat PCC PC12 cells from the American Type Culture Collection (Manassas, VA, USA) and cultured these in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% horse serum, 5% fetal bovine serum (FBS; Sigma-Aldrich, St Louis, MO, USA) and antibiotic/antimycotic at 37°C with 5% CO_2. The HSP70 inhibitor VER155008 was purchased from Selleck Chemicals (Houston, TX, USA) and solubilized in dimethyl sulfoxide (DMSO) to the concentration of 1 mM, as the operating fluid. We purchased a Cell Counting Kit-8 (CCK-8) from Dojindo (Tokyo, Japan). All primary antibodies (anti-GADPH, anti-cyclin D1, anti-Bax, anti-PARP, anti-cleaved PARP, anti-heat-shock

protein 70 [HSP70], anti-PI3K, anti-phospho-PI3K, anti-phospho-AKT [S473], anti-AKT, anti-phospho-ERK1/2 [T202/Y204], anti-ERK1/2, anti-phospho-MEK, anti-MEK, anti-mTOR and anti-phospho-mTOR) were bought from Cell Signaling Technology (Boston, MA, USA).

Cell viability assay

We used a CCK8-assay to test the effect of HSP70 inhibitor VER155008 on the cell viability of the PC12 cell line. We cultured tumor cells in 96-well plates at a density of 3×10^3/well in 200 µL of the complete culture medium for 24 h. We added various concentrations of VER155008 to each well and incubated the system for 24, 48 or 72 h. At each of these time points, we added 100 µL of the culture medium, containing 10% CCK8, to each well and incubated it for 1 h. Finally, we measured the absorbance of the medium at 450 nm.

Colony formation assay

After trypsinization, the cells were collected and reseeded at a density of 3×10^3/well into six-well plates with complete medium for 24 h. Varying concentrations of VER155008 were used, in addition to the control group. We replaced the medium every 3 days to maintain cell growth until day 10. The colonies were fixed with methanol for 30 min and then stained with crystal violet for 15 min at room temperature. After washing the stained plates with PBS (phosphate-buffered saline), we used a digital camera to record images of the colonies.

Cell migration assay and wound healing assay

We used transwell chamber assay (Corning, USA) to evaluate the ability of the PC12 cells to migrate, following the manufacturer's protocol. We seeded 2×10^5 PC12 cells onto one plate per chamber. To trigger cell migration, we used a culture medium with 10% serum to attract cell migration from a serum-free culture medium containing 50 µM, or a medium that had been exposed to 100 µM VER155008 for 8 h, in addition to the control group (CTR).

Cell migration capacity was determined by a wound healing assay. PC12 cells were seeded into six-well plates at a density of 2×10^6 cells per well, to grow into a monolayer. The monolayers were wounded by scratching lines with a

plastic tip. The wells were then washed twice with PBS to remove any debris, and photographed under a microscope. Thereafter, the plates were incubated at 37°C under 5% CO_2 for 0 h, 8 h, 24 h and 48 h, with DMEM supplemented with 1% FBS in control, 50 µM, and 100 µM group respectively, after which the cells were observed and photographed. The area in which there was no migration of cells was recorded using ImageJ software. The relative non-migration area was calculated as non-migration area in pictures between the cells of two sides.

Protein extraction and western blot analysis

Following treatment with the indicated concentrations of VER155008, the PC12 cells were trypsinized and collected. The cells were pelleted, washed once with PBS, and then incubated in ice-cold lysis buffer (50 mM tris-HCl pH 8.0, 150 mM NaCl, 1% Triton X-100, 100 ug/mL phenylmethylsulfonyl fluoride and 1 mM DL-dithiothreitol) for 30 min. The cell lysates were centrifuged at 12,000 rpm for 30 min at 4°C, and the protein concentrations were determined using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Each sample, corresponding to 15 µg protein in the final assay volume, was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 10% polyacrylamide gels (10% gels) and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with 5% BSA (bovine serum albumin), the membranes were incubated overnight at 4°C with primary antibodies (anti-PARP, anti-cleaved PARP, anti-BAX, anti-Cyclin D1, anti-PI3K, anti-phospho-PI3K anti-AKT, anti-phospho-AKT, anti-MEK, anti-phospho-MEK, anti-ERK, anti-phospho-ERK, anti-mTOR, anti-phospho-mTOR anti-HSP70 and anti-GAPDH). Next, the membranes were washed three times-for 10 min-with PBS-T, containing 0.05% Tween 20 and tris-buffered saline, and then incubated with horseradish peroxidase-conjugated respective secondary antibodies (Abcam, Cambridge, MA, USA) at room temperature for 1 h. Membranes were again washed three times with PBS-T for 10 min. Western chemiluminescent HRP substrate (Millipore, Darmstadt, Germany) was mixed in the ratio of 1:1 to highlight the presence of protein. The target bands were visualized using Pierce enhanced chemiluminescence (Thermo Scientific) to observe the relative expression of protein. The values of the protein bands were normalized by dividing them by their corresponding GADPH values.

Flow cytometry

Following treatment for 24 h with VER155008, the cells were harvested and washed. The PC12 cells were stained with propidium iodide (PI) to assess cell cycle progression. After exposure to VER155008 for 48 h, the percentage of cells undergoing apoptosis was measured using an Annexin V-FITC Apoptosis Detection Kit (Becton Dickinson, NJ, USA) to double-stain the apoptosis-associated proteins, following the manufacturer's instructions.

PCC mouse xenograft model

The entire animal studies were conducted consistent with the principles and procedures outlined in the National Institutes of Health's Guide for the Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee in Teaching and Research of Shanghai Jiaotong University, complying with ARRIVE guidelines, in accordance with the U.K. Animals (Scientific Procedures) Act.

Mouse xenograft models were developed using four-week-old female athymic nude mice (Shanghai Institute of Material Medical, China). We used 10 nude mice to perform the tumor model study. They were all maintained under specific pathogen-free conditions, and all our procedures were conducted under aseptic conditions. Following the trypsinization and collection, the PC12 cells (1×10^6 cells/100 µL) were subcutaneously injected into the left hind flank of the female athymic nude mice. After about a week, the injected tumor cells formed visibly evident tumors. Tumor volume was measured using Vernier calipers, and the mice received intraperitoneal injections of VER155008 (40 mg/kg) once the tumor volume was found to be measurable (80-150 mm^3, ~5-8 days post injection). The mice were administered VER-155008 daily for two weeks. We measured the tumor volume every day, using the following formula: V (mm^3) = length × width^2 × 0.5236. After the administration of the HSP70 inhibitor, the mice were sacrificed and the tumors were excised and weighed.
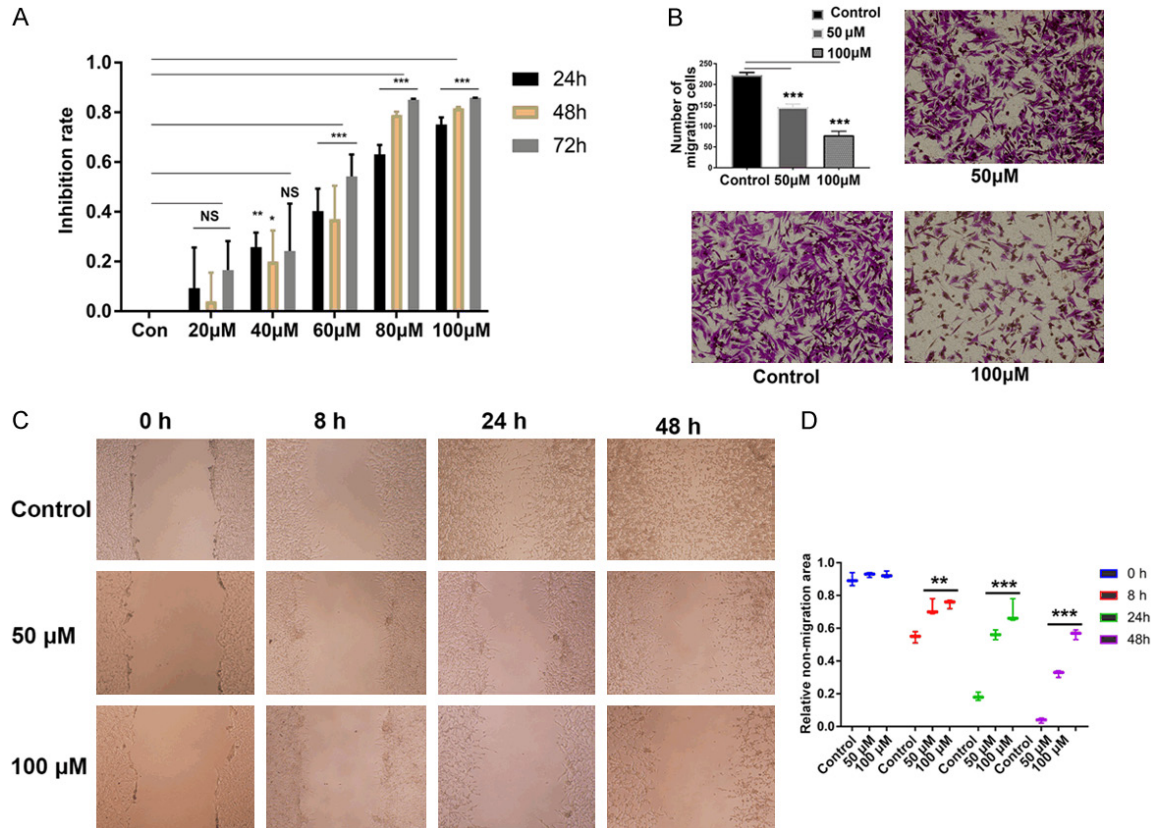
Figure 1. VER155008 inhibited PC12 growth and migration *in vitro*. A. CCK8 assays were used to evaluate the proliferation of PC12 cells under exposure to a variety of concentrations of VER155008 for 24 h, 48 h, and 72 h. B. Transwell assays were used to mimic and measure cell migration in PC12 cells in different groups with treatment of 50 μM or 100 μM VER155008 or control. C. Wound healing assays were used to test the inhibition of migration after VER155008 exposure in 50 μM or 100 μM or control for 0 h, 8 h, 24 h, and 48 h. D. Statistical quantification of **Figure 3C** with the non-migrating area indicating the area remaining between the two side cells. All data are representative of three independent experiments and expressed as the mean ± SD. ns; *P < 0.05; **P < 0.01; ***P < 0.001.

Statistical analysis

All of the relevant characteristic data are presented as mean ± standard deviation (SD) in three independent experiments. Differences in measurement and enumeration data were compared using Student's t-test. We conducted all the statistical analyses and graphical presentations using GraphPad Prism7 software (TNC, La Jolla, CA, USA). In all the statistical data related to the experiment, a *P* value of < 0.05 was significant.

Results

VER155008 suppresses cell viability in a dose-dependent manner

A CCK8 proliferation assay was conducted to examine the effect of VER155008 on PC12

cells. We found that VER155008 inhibited the PC12 cell viability as the time and dose increased. The 50% inhibitory concentrations (IC-50) at 24 h, 48 h and 72 h were 64.3 μM, 61.8 μM and 50.5 μM, respectively (**Figure 1A**).

VER155008 inhibits PC12 cell migration

Generally, metastasis is closely associated with poor prognosis in patients with PHEOs. Thus, it is inevitable to evaluate the effect of VER155008 on PC12 cell migration. The results showed that the number of penetrated cells were 222 ± 6.6, 145 ± 8 and 78 ± 10 in the control group, 50 μM (P < 0.001) group and 100 μM (P < 0.001) group, respectively. In the transwell assay, our results indicated that after an 8-h exposure to VER155008, when the migrated PC12 cells are not inclined to proliferate fast and affect the migrated cell number,

cell migration capacity was significantly suppressed (**Figure 1B**).

In the wound healing assay, we revealed that VER155008 showed significant migration-suppressing ability in a time- and dose-dependent manner. The relative non-migration areas between the two side cells were 0.547 ± 0.035, 0.723 ± 0.049 (P < 0.01), 0.750 ± 0.026 (P < 0.01) after 8-h treatment in the control, 50 μM and 100 μM group, respectively; 0.183 ± 0.025, 0.560 ± 0.030 (P < 0.001) and 0.697 ± 0.072 (P < 0.001) in the 24-h group; and 0.037 ± 0.015, 0.323 ± 0.021 (P < 0.001) and 0.563 ± 0.031 (P < 0.001) in the 48-h group (**Figure 1C**, **1D**).

VER155008 inhibits colony formation and induces cell-cycle arrest and apoptosis in PC12

As shown in the results of the colony formation assay (**Figure 2A**), we discovered that VER155008 inhibits the ability of PC12 cells to form colonies in a dose-dependent manner.

To further identify which biologic functions were affected by the underlying decreased cell viability of VER155008, cell cycle and apoptotic functions were monitored, to support this hypothesis. Cell cycles were analyzed through corresponding assays. Compared with the control group, the cell cycle in the treatment group was arrested in the G0/G1 phase, accounting for 44.8 ± 1.6%, 52.4 ± 0.7% (P < 0.01) and 56.4 ± 0.7% (P < 0.01) in the control, 50 μM and 100 μM group, respectively, contributed to a decreased percentage in the G2 phase, accounting for 30.0 ± 0.3%, 30.7 ± 1.2 (P > 0.05) and 22.4 ± 1.0 (P < 0.01) separately, and S phase, accounting for 25.2 ± 1.3%, 16.8 ± 1.7% (P < 0.01) and 21.1 ± 0.5% (P < 0.01), respectively (**Figure 2B**, **2C**). These phenomena were further confirmed through western blot analysis, where down-regualtion of cyclin-B1, a protein associated with cell cycle progression, was also observed in the VER155008 group (**Figure 2D**).

The effect of VER155008 on the apoptosis of PC12 cells was examined by Annexin V/PI double staining. From **Figure 2E** and **2F**, we observed that VER155008 could markedly induce cell apoptosis after treatment for 48 h. The apoptotic rates were 2.2 ± 0.1%, 4.0 ± 0.1% (P < 0.05), 7.2 ± 0.6% (P < 0.001), 12.6 ± 0.4% (P < 0.001), 14.7 ± 0.4% (P < 0.001) and 19.6 ± 1.6% (P < 0.001) in the control, 20 μM, 40 μM, 60 μM, 80 μM and 100 μM group, respectively. Similarly, those phenomena were also attested by western blot analysis. Bax is a protein overexpressed in the case of apoptosis triggering, and PARP plays a role in DNA repair and cell apoptosis. Up-regulation of protein Bax and cleaved-PARP, and down-regulation of PARP, were detected in the corresponding groups (**Figure 2G**).

VER155008 exhibits anti-tumor effects by inhibiting phosphorylation of the PI3K/AKT/ mTOR and MEK/ERK signaling pathways and up-regulating the HSP70 protein level

It has been well-documented that PI3K/AKT/ mTOR and MEK/ERK signaling pathways are closely associated with tumorigenesis and progression [18, 19]. Therefore, we performed western blot analysis to explore possible molecular mechanisms for the anti-tumor effect of VER155008. As illustrated in **Figure 3A-E**, we revealed that VER155008 could significantly down-regulate the phosphorylation levels of PI3K/AKT/mTOR and MEK/ERK, while barely affecting the expression levels of total PI3K/ AKT/mTOR and MEK/ERK proteins. Unexpectedly, the expression level of HSP70 protein was up-regulated after treatment with various concentrations of VER155008 (**Figure 3B**).

VER155008 impedes tumor growth in vivo

Based on the above revelations-that VER-155008 impeded cell growth, and inhibited cell migration *in vitro*-we naturally believed that VER155008 might exert an important effect on tumor growth *in vivo*. To this end, we established a xenograft model of PC12 cells, to examine the effect of VER155008 *in vivo*. After two weeks of VER155008 treatment, we observed that the group exposed to VER155008 showed a significant reduction in tumor size compared with the matched group. The volume of tumors was 2584.7 ± 525.6 mm^3 in the control group and 1253.9 ± 157.8 mm^3 (P < 0.001) in the treatment group (**Figure 4A-C**). Moreover, it was noteworthy that compared with the mice in the control group, VER155008 did not significantly affect body weights (**Figure 4D**), which were found to be 20.4 ± 0.7 g in the control group and 19.5 ± 1.5 g (P > 0.05) in the treatment group (assessed separately), partial-

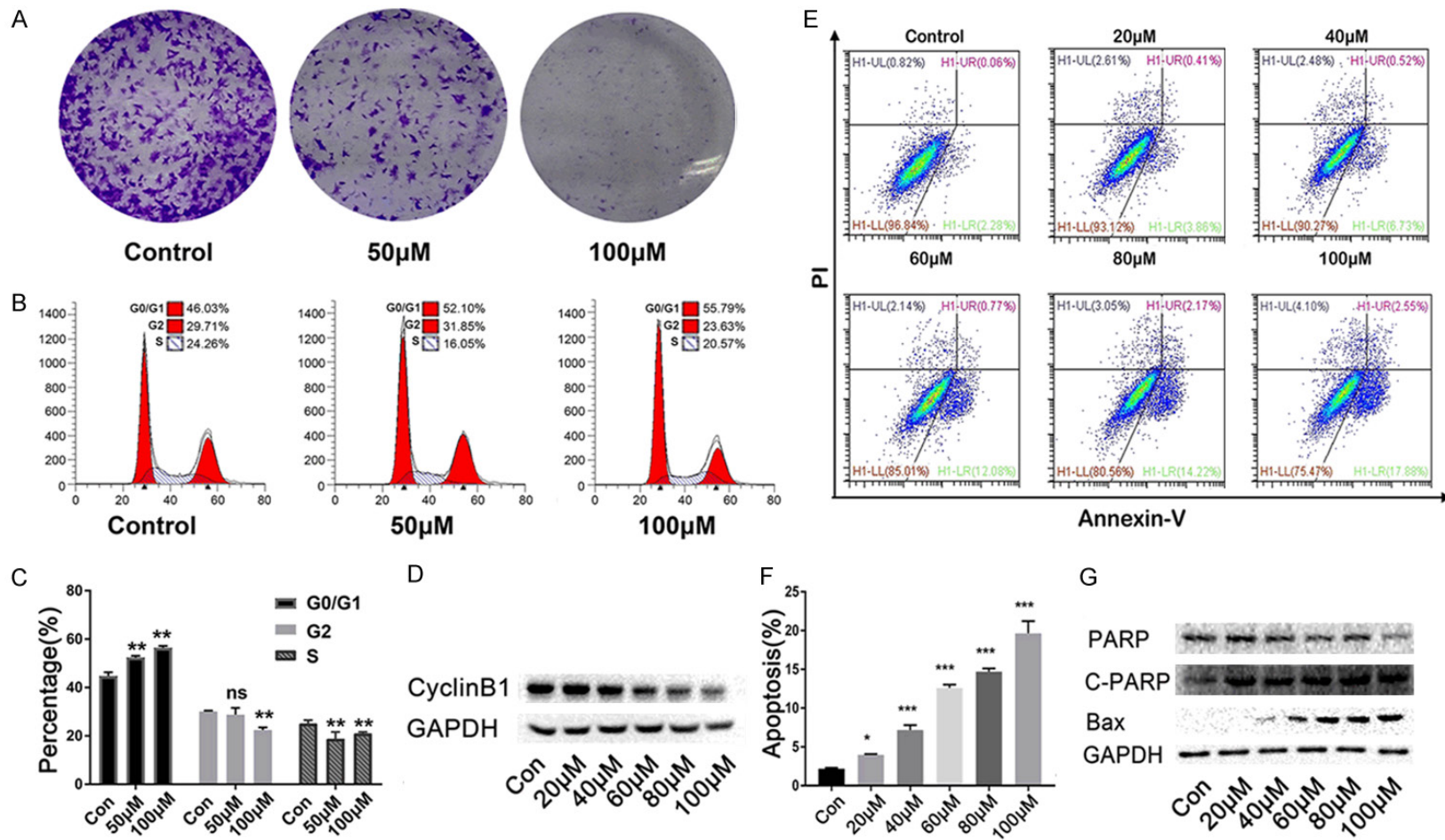# Effect of VER155008 in PC12 cell line

Figure 2. VER155008 suppressed PC12 colony formation, impeded its cell cycle progression and induced tumor cell apoptosis. (A) Colony formation assays were used to explore the effect of VER155008 on the ability of tumor cells to form colonies. (B) Cell cycle arrest induced by VER155008. VER155008 induced increased percentages of the G0/G1 phases of the cell cycle and decreased the percentages of the S phase and G2 phases. (C) Percentage of G0/G1, G2, and S phase under exposure to different VER155008 concentrations. (D) Cell cycle progression associated with protein-cyclin D1 was shown by western blot analysis. (E) Annexin-V and PI staining in PC12 after exposure to VER155008 with varying concentrations for 48 h. (F) Percentage of early-stage apoptotic cells in groups corresponding to (E, G) Protein levels of PARP, C-PARP and Bax in PC12 were affected by different concentrations of VER155008.
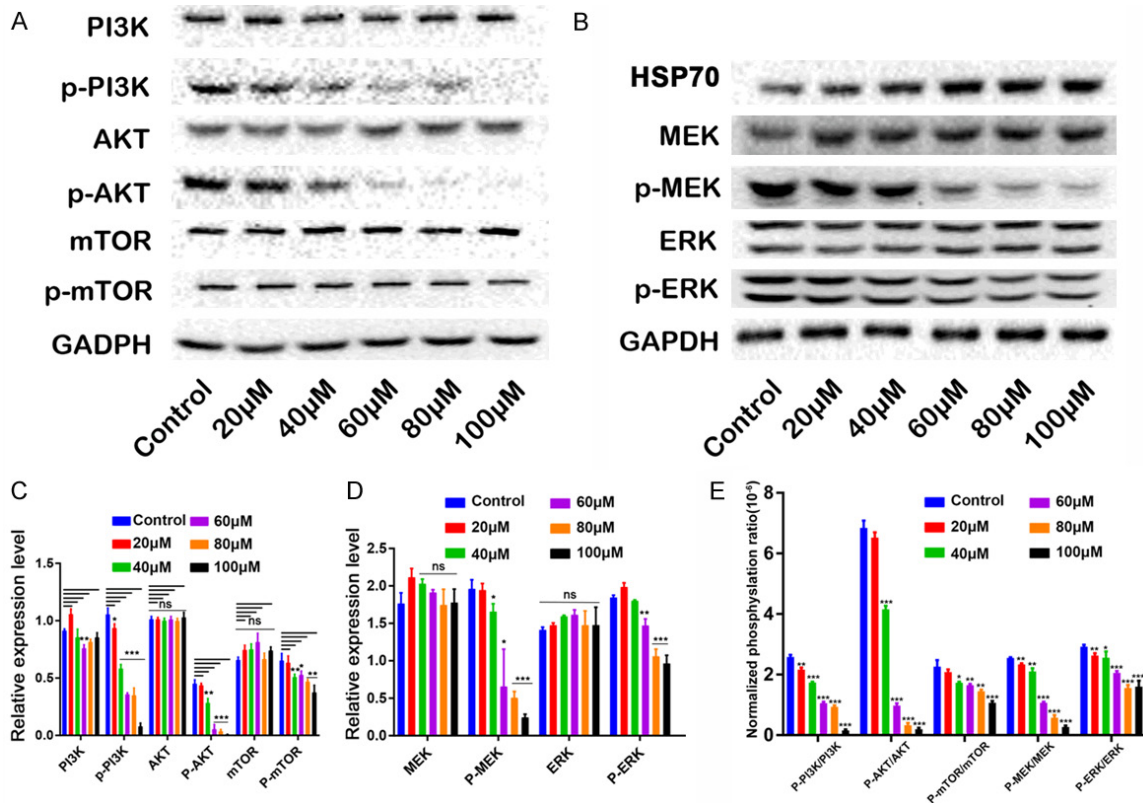
Figure 3. VER155008 down-regulated phosphorylation of the PI3K/AKT/mTOR, and MEK/ERK signaling pathways and stimulated an increase in HSP70 protein level after PC12 was exposed to it for 24 h. A. PI3K/AKT/mTOR total protein level and its phosphorylation level were affected by VER155008 after 24-h exposure, exhibited by western blot analysis. B. Total protein expression and phosphorylation levels of the MEK/ERK signaling pathway and HSP70 protein level of PC12 under exposure to VER155008 for 24 h, as revealed by western blot. C. Relative expression of phosphorylation and total protein levels of the PI3K/AKT/mTOR signaling pathways. D. Relative expression levels of phosphorylation and total protein of the MEK/ERK signaling pathways. E. Phosphorylation ratio of PI3K/AKT and MEK/ERK signal protein normalized by GAPDH.

ly suggesting that VER155008 caused no toxic side effects.

Discussion

Until now, the therapeutic options for metastatic PHEO have remained limited because of the low incidence of PHEO and the fact that the mechanism underlying its pathogenesis is still not fully understood. In addition, there are no reliable indices for early diagnosis of metastatic PHEO, creating an even more serious problem. Metastatic PHEO shows a relatively high mortality rate, with a five-year survival rate of 44% [20]. Therefore, the discovery of novel and effective drugs is urgently needed to improve the current situation.

In recent years, HSP90 has emerged as an attractive therapy target for a broad spectrum of human cancers; nevertheless, few studies have considered HSP70 as an optional anti-tumor therapeutic strategy [21]. The HSP70 family of HSPs consists of molecular chaperones that are approximately 70 kDa in size and play important roles in cell proliferation and death homeostasis. These HSP70 ATPase activities in unfolded, misfolded or denatured proteins promote nascent protein translation, facilitating protein transportation and impeding abnormal protein aggregation [22]. Reportedly, high expression of HSP70 in various human cancers is a relatively common phenomenon, which promotes tumor progression by the action of extrinsic and intrinsic stimuli [13]. In Shabbir et al.'s research [23], 50 µmol/L VER155008 was used to inhibit HSP70 activity to suppress the neuroprotective role of HSP70 in an injury model of the rat spinal cord (*in vitro*). In the research of Cao et al. [24], 100 µmol/L VER155008 was used to treat the clone 9 cells. Our experiments limit the maxi-
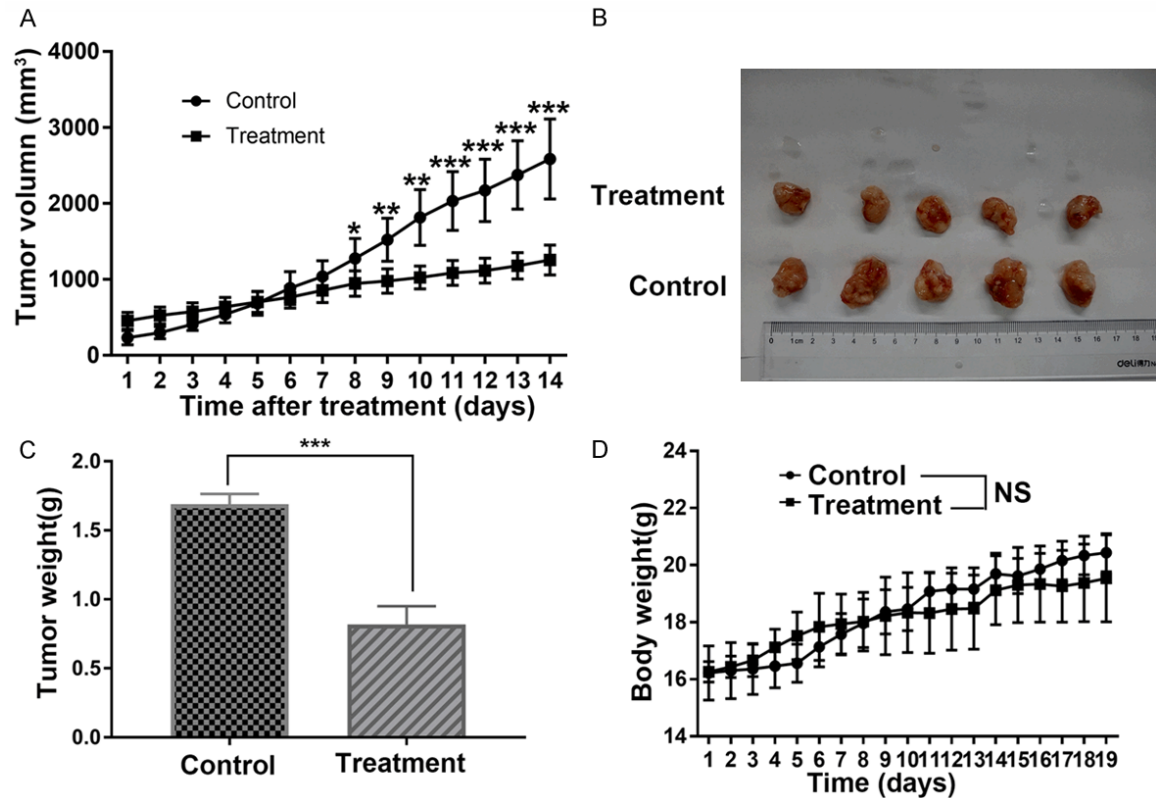
Figure 4. VER155008 inhibited PC12 tumor xenograft growth significantly. A. Tumor volumes of the control and treatment group were recorded daily. B. Images of the tumors in the control group and the VER155008 treatment group. C. Tumor weights were analyzed after excision of the tumors from the sacrificed nude mice. D. Nude athymic mice body weights of the control (20.4 ± 0.67 g) and treatment (19.5 ± 1.5 g) groups. Results are mean ± SD from three independent experiments. ns; *P < 0.05; **P < 0.01; ***P < 0.001.

mum concentration of VER155008 to 100 µmol/L in PC12 cells, which is consistent with these studies. To this end, we aimed to determine whether inhibition of HSP70 could retard PHEO progression, and thus improve the prognosis of patients with metastatic PHEO.

In the present research, we found that VER-155008 could not only effectively inhibit the proliferation and migration of PC12 cells, but also promote apoptosis and induce cell cycle arrest, in a time-and dose-dependent manner. The findings of our western blot analysis indicated that VER155008 could suppress tumor progression via down-regulated phosphorylation of PI3K/AKT/mTOR and MEK/ERK signaling pathways. Finally, we further verified-by comparing xenograft models with the control group-that sizes of tumor in the treatment group with VER155008 were significantly suppressed. In other words, VER155008 could effectively inhibit tumor growth *in vivo*.

Interestingly, our results showed that the expression level of HSP70 increased as the dose of VER155008 increased. Li et al. [25] revealed that VER155008 down-regulated HSP70 expression level in porcine granulosa cells (GCs). However, Wolf et al. [26] showed that the basal HSP70 expression was not affected by VER155008. Although the HSP70 expression level increased, we found that VER155008 significantly down-regulated the phosphorylation of the PI3K/AKT/mTOR and MEK/ERK signaling pathways, suggesting that VER155008 effectively works *in vitro*. In addition, cell viability within the treatment group was greatly inhibited compared with that in the matched group. This could have been because VER155008 alone, as a HSP70 ATPase inhibitor, inhibited HSP70 activity. The increased level of HSP70 may have occurred because competitive inhibition of the HSP70 binding site by VER155008 stimulates expression of HSP70 encoding genes expression (HSP70 family genes: HSPA1A/

HSPA1B), suggesting that competitive inhibition of the HSP70 binding site by VER155008 should also result in an increased expression of these genes as a compensation mechanism after treatment inducing cellular stress, which may consequently up-regulate the HSP70 protein level [17, 27]. However, further research is needed to elucidate the mechanisms involved.

In conclusion, we note that in the newest WHO-updated PHEO classification, all PHEOs are regarded as having metastatic potential. The results of our research demonstrated that VER155008 not only effectively inhibits PC12 cell proliferation and induces cell apoptosis *in vitro* and *in vivo*, but also may inhibit or delay PHEO metastasis. Thus, we regard VER155008 as a promising small molecular inhibitor for PHEO.

Acknowledgements

Disclosure of conflict of interest

None.

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