Original Article
Apogossypolone (ApoG2) induces ROS-dependent apoptosis and reduces invasiveness of PC12 cells *in vitro* and *in vivo*

Dengqiang Lin[1*], Xiaoxia Li[2*], Lieyu Xu[1,3*], Jianpo Lian[1*], Yunze Xu[4], Li Meng[1], Xin Xie[1], Xiaojing Wang[1], Hongchao He[1], Danfeng Xu[1], Chenghe Wang[1], Yu Zhu[1]

[1]Department of Urology, Ruijin Hospital Affiliated to Medical School of Shanghai Jiaotong University, No.197, Ruijin'er Road, Shanghai, China; [2]Department of Radiology, Shanghai Ninth People's Hospital Affiliated to Medical School of Shanghai Jiaotong University, No.639, Zhizaoju Road, Shanghai, China; [3]Department of Urology, Jiangxi Provincial People's Hospital, No.92, Aiguo Road, Donghong District, Nanchang, Jiangxi, China; [4]Department of Urology, Renji Hospital Affiliated to Medical School of Shanghai Jiaotong University, No.1630, Dongfang Road, Shanghai, China. *Equal contributors and co-first authors.

Abstract: Malignant pheochromocytoma is accurately diagnosed only at occurrence of metastatic foci. However, at that time, patients are less likely to get many benefits from traditional chemotherapy. Over-expression of BCL-2 family proteins is tightly correlated with progression of pheochromocytoma. ApoG2, as the most potent gossypol derivative, has exhibited anti-tumor activities in various tumors. In the present study, we found that the staining degree of Bcl-2 being stronger than Bax was more frequently observed in pheochromocytoma than adrenocorticohyperplasia, which was possibly related to shorter overall survival. In addition, ApoG2 could induce apoptosis through up-regulation of Bax and down-regulation of Bcl-2, increasing reactive oxygen species (ROS) levels, inducing cytochrome C release and cleaving caspase proteins. Most importantly, those inhibition effects were blocked by caspase activation inhibitor Z-VAD-fmk and antioxidant *N*-acetyl-L-cysteine. The above results were further confirmed *in vivo*. Furthermore, ApoG2 could effectively inhibit tumor movement capabilities. Altogether, our results indicated that ApoG2 was a potential effective target drug for pheochromocytoma.

Keywords: Pheochromocytoma, apogossypolone, ApoG2, Bcl-2, reactive oxygen species

Introduction

Although pheochromocytoma (PHEO) is a rare catecholamine-secreting adrenal medullary tumor, it is a relatively common cause of secondary hypertension, potentially leading to severe or even fatal cardiovascular complications [1, 2]. The yearly incidence is approximately 2-8 per million population [3]. Among patients with PHEO, up to 36% show malignancy [4], even a higher-than-expected rate proved by extensively evidences. Early radical surgical resection was considered as the only curable means until recently. Compared with the 84-96% five-year survival rate of the benign form of PHEO, the five-year survival rate for the malignant form is poor at less than 50% [5-7] and recurrence rate post-operation is 65.4%

within five years [8]. Worse still, owing to the absence of clinically and pathologically reliable indices to allow the early differentiation of malignant PHEO from the benign form, the majority of patients are diagnosed with malignant PHEO solely by the presence of metastatic lesions. Hence, they are less likely to get many benefits from traditional chemotherapy. Therefore, discovering new and effective anti-tumor drugs to improve prognosis is quite important for benign or malignant PHEO.

Apoptosis or programmed cell death helps to retain homeostasis of the internal environment of an organism through orderly and effective clearing of damaged cells. Once the process becomes dysregulated, it is frequently related to excessive proliferation, finally leading to

Sketch map of c BCL-2 family members
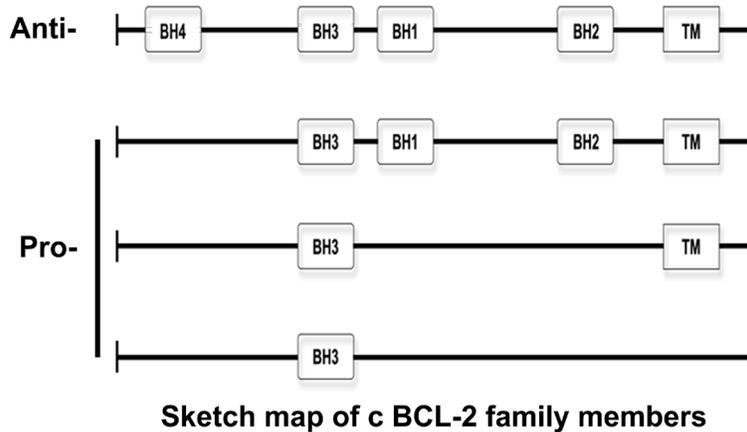
Figure 1. Sketch map of BCL-2 family members. TM, transmembrane domain.

tumor occurrence and tumor drug resistance [9, 10]. Accordingly, drugs targeted at apoptosis signaling pathways could potentially induce cell apoptosis and restore sensitivity for chemotherapy [11, 12]. B-cell lymphoma/leukemia-2 (BCL-2) family proteins are characterized by the inclusion of at least one of four BCL-2 homology (BH) domains, which are regarded as one of the most vital regulators for the apoptosis process. Based on structure and function, BCL-2 family proteins are divided into three groups: anti-apoptotic proteins (including BH1 to BH4 structures) such as Bcl-2, Bcl-xl, and Mcl-1; pro-apoptotic proteins (including BH1 to BH3 structures) such as Bax and Bak; and BH3-only proteins such as Bim, Bid, and Bad [13] (**Figure 1**). Remarkably, BH domains of anti-apoptotic proteins (Bcl-2 or Bcl-xl) can form a hydrophobic surface pocket which attaches to BH3 of pro-apoptotic proteins and then inhibits pro-apoptotic activity [14].

Research suggests that over-expression of Bcl-2 protein is a universal phenomenon in more than half of all tumor tissues [15] and is also tightly correlative to tolerance of different kinds of chemotherapeutics [16, 17]. Moreover, inhibition of autophagy is an alternative mechanism for Bcl-2 protein to promote tumor progression [18, 19]. In other words, the Bcl-2 family of proteins are closely associated with tumor formation and development. In PHEO, the expression rate of Bcl-2 varies from 39.5% to 86% [20-24]. Hence, we reasonably hypothesize that Bcl-2 protein may be a potential and effective target for PHEO.

Gossypol, used as a male contraceptive for 35 years in China [25], is a natural polyphenolic compound extracted from cotton seed. Recently, several studies have demonstrated that gossypol is a native BH3-mimic capable of blocking protein-protein interactions between pro-apoptotic and anti-apoptotic members, thus freeing the pro-apoptotic effect. Two aldehyde groups in gossypol are relevant to toxicity and non-specific reaction [26]. Therefore, a third-generation derivative of gossypol, apogossypolone (ApoG2), with the two aldehyde groups removed has shown greater performance and less toxicity in various cancers, including prostate cancer, nasopharyngeal carcinoma, lymphoma, pancreatic cancer, carcinoma and others [27-34]. However, the effect of ApoG2 on PHEO remains unclear. In the present study, we sought to investigate the effect of ApoG2 on PHEO *in vitro* and *in vivo*. The results indicated that ApoG2 is a potential effective target drug for PHEO, by not only promoting apoptosis but also inhibiting invasiveness.

Materials and methods

Cell line and chemical reagents

The mouse PHEO cell line (PC12) was originally donated by the Institute of Neurology in Ruijin Hospital affiliated to the Medical School of Shanghai Jiaotong University, China. Apogossypolone (ApoG2) was purchased from ApexBio (USA). On the day of experiment, ApoG2 powder was diluted to 20 mmol/L stock solution with pure dimethylsulfoxide (DMSO; Sigma, Germany), and then subsequently diluted to the appropriate working solution concentration with culture medium. Primary antibodies (Bcl-2, Bax, caspase, beta-actin) in this study were purchased from Cell Signaling Technology (USA), and the Hoechst 33258 Staining Kit was provided from Beyotime Company.

Cell culture

PC12 cells were cultured in 60 cm² dishes in an incubator under conditions of 37°C, 5% CO_2,

Table 1. Clinical features of patients in our study

Parameter	PHEO	AH
Total number	21	23
Gender		
Male	7	6
Female	14	17
Age	47.33±9.40	40.35±10.72
Tumor location		
Left	8	8
Right	13	14
Bilateral	0	1
Mean tumor size	5.34±2.41	0.91±0.36
Follow-up (month, x̄±s)	46.71±14.71	/

and 90% humidity. The medium for PC12 cells was composed of 5% fetal bovine serum (Invitrogen), 10% horse serum (Thermo Fisher Scientific), and 85% DMEM. PC12 cells were subcultured or the culture medium was replaced every three days.

Tissue samples

Following approval by institutional ethics review board, all tissue samples (demographic features are summarized in **Table 1**) were collected between December 2005 and December 2015 from those patients who received an adrenalectomy in the Department of Urology in Ruijin Hospital affiliated to the Medical School of Shanghai Jiaotong University in China. Methods were carried out in accordance with the relevant guidelines and regulations, and informed consent was obtained from all subjects.

Immunohistochemistry

After fixing in 10% neutral formalin and dehydrating in an alcohol concentration gradient, samples were embedded in paraffin, then cut into 3-μm serial sections, which were dewaxed twice in xylene, rehydrated in alcohol, and then rinsed with phosphate buffered saline (PBS) followed by treatment with 3% H_2O_2 for 10 min to inactivate endogenous peroxidase. After antigen retrieval, the slides were incubated with 10% goat serum for 10 min under constant temperature to block non-specific reactions. Subsequently, sections were treated with polyclonal rabbit anti-human Bcl-2 and Bax antibody for 12 h at 4°C. After washing with PBS

three times, the slides were incubated with secondary antibody at 37°C for 30 min. Slides were then washed again with PBS, developed in diaminobenzidine (DAB) substrate, counterstained in hematoxylin, and dehydrated with ethanol and xylene before being mounted. For negative controls, the slides were incubated with PBS instead of primary antibodies.

The staining intensity of Bcl-2 and Bax were assessed by two pathologists blinded to each other and clinical data. Yellowish-brown granules located in the cytoplasm or nucleus for Bcl-2 and in the cytoplasm for Bax were considered as positive staining, otherwise the lack of any evident yellowish-brown granules was classed as negative.

Staining intensity was assessed as follows: score 0 (no staining), score 1 (weak), score 2 (moderate), and score 3 (strong). Three random high-power fields (×400 magnification) were score 0 (0-5%), score 1 (6-25%), score 2 (26-50%), score 3 (51-75%), and score 4 (76-100%). The final score was determined according to the combination of these two variables and divided into: score 0 negative (-), otherwise positive including score 1-4 weakly positive (+), score 5-8 moderately positive (++), score 9-12 strongly positive (+++).

Cell proliferation assay

Cells were seeded at a density of $3×10^3$/well in 200 μL culture medium in a 96-well plate. After incubation for 24 h, cells were treated with different concentrations of ApoG2. At the end of each time point, the cell counting kit CCK8 reagent (YeaSen, China) was added to each well and incubated at 37°C for 4 h according to the manufacturer's instructions. The absorbance of each well was then measured at 450 nm by gen5 software (BioTek, USA), and cell inhibition rates at every time point were calculated compared to the matched control group.

Apoptosis assay

ApoG2-induced-apoptosis was observed by using the Hoechst 33258 nuclear staining kit. Briefly, after treatment with ApoG2 at various concentrations for 48 h in 6-well plates, cells were stained with Hoechst 33258 for 15 min. Cells were then washed twice with PBS buffer, and slides were imaged with a fluorescence microscope (Olympus, Japan).

ApoG2-induced-apoptosis was also objectively quantified by flow cytometry through double staining with Annexin V and propidium iodide (PI) (BD, USA). Briefly, at the end of every time point, cells were harvested and centrifuged at 1000 rpm for 5 min. The cells were then re-suspended with binding buffer and stained with Annexin V-FITC for 10 min and PI for 5 min. Finally, ApoG2-induced-apoptosis were detected by flow cytometry (Bioscience, USA).

Protein extraction and western blot

Cells were harvested, washed twice with PBS and then lysed in ice-cold lysis buffer containing 1% phenylmethylsulfonyl fluoride (PMSF) and 10% cocktail, for 30 min at 4°C. Cell lysates were centrifuged to remove precipitate at 12000 rpm for 30 min, added with loading buffer and stored at -20°C until use. Proteins were separated by SDS-PAGE (10% or 12% gels) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA). 5% BSA was used to block non-specific binding sites for 1 h at normal atmospheric temperature and subsequently membranes were incubated overnight at 4°C with primary antibodies (1:1000) and β-actin (1:2000). Membranes were then washed three times with PBS-T (PBS with 1‰ Tween 20) and incubated with corresponding secondary antibodies at room temperature for 40 min. After washing three times with PBS-T, protein bands were detected using the enhanced chemiluminescence method in Image Lab software (Bio-Rad) through auto-or manu-exposure.

Co-immunoprecipitation

Co-immunoprecipitation was performed to detect whether ApoG2 potently binds to Bcl-2 affecting Bcl-2/Bax complex. Briefly, PC12 cells were lysed with cell lysis buffer for Western and IP (Beyotime, China) and were centrifuged at 12000 rpm for 30 min. The supernatants were then incubated with corresponding antibodies (1:50) overnight at 4°C. Protein G agarose beads pre-washed with lysis buffer were used to capture antibodies at room temperature for 90 min. Proteins bound to the beads were heated to denature compounds before separation by western blot analysis.

Colony formation assay

Cells at a density of 1×10^3/well were seeded into sterile 6-well plates with complete medium

for 24 h. Varying concentrations of ApoG2 or 1% DMSO as a matched group were added to the 6-well plate for 10 days and the medium was replaced every 3 days to maintain cell growth. The colonies were then fixed with methanol for 15 min and stained with crystal violet (Sigma, USA) for 15 min at room temperature. After washing three times with PBS and imaging by digital camera, the colony formation rate was calculated as: Colony formation rate = number of colonies/number of seeded cells×100%.

Cell cycle analysis

Cells were re-suspended with 300 µL PBS and fixed with 700 µL absolute ethanol. Cellular DNA was stained with PI (0.05 mg/mL) and analyzed by flow cytometry.

Mouse xenograft model

Equivalent amounts of PC12 cells (20×10^6, 200 µL) were subcutaneously injected into the left hind flank in female athymic nude mice (Shanghai Institute of Material Medical, China) at 4 weeks of age. After being left for a week to form macroscopic tumors, mice were randomized to two groups: control and treatment. The treatment group received ApoG2 continuously by intraperitoneal injection at 100 mg/kg every 3 days for a total of 18 days. Tumor length and width were measured every 3 days. Tumor volume was calculated as: Volume (mm^3) = length×width^2×0.5236.

Mice were humanely killed and tumors were excised, weighed, and subsequently imaged by digital photography.

Reactive oxygen species (ROS) assay

Intracellular reactive oxygen species (ROS) level was measured by a DCFH-DA fluorescence probe (Sigma). PC12 cells were treated with or without ApoG2. Before harvesting, cells were incubated with 10 µmol/L DCFH-DA for 30 min in an incubator-37C, 5% CO_2. Cells were then washed twice with PBS and analyzed with a Microplate Reader (BioTek, USA) and fluorescence microscope (Olympus, Japan).

Cell migration and invasive assay

Tumor cell migration was assayed in transwell chambers (Corning, USA) and membrane used for the invasive assay was purchased from BD. Briefly, PC12 cells at a density of 1×10^5/well

A

B
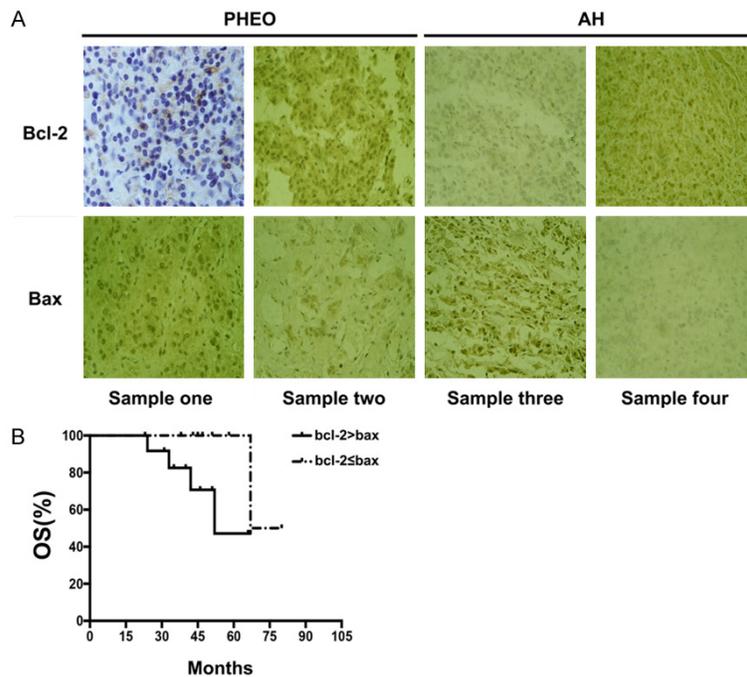
Figure 2. Kaplan-Meier curves showed overall survival distribution in patients with different BCL-2 family proteins abundance. A: Expression of Bcl-2 (upper row) and Bax (lower row) in the same samples from PHEOs (four pictures on left) and AHs (four pictures on right). Sample one: Bcl-2≤Bax; Sample two: Bcl-2<Bax; Sample three: Bcl-2≤Bax; Sample four: Bcl-2>Bax. All images are at ×400 magnification. B: Overall survival distribution in patients with PHEO.

were seeded into upper transwell chambers with or without ApoG2 diluted by 200 uL DMEM, and 10% FBS mediums were added to lower transwell chambers. After incubation for 6 h in a 37°C incubator, cells were fixed with methanol for 15 min and then stained with crystal violet for 15 min. Cells on the upper surface of the filter were then carefully wiped away with a cotton swab and the cells on the lower surface of the filter were counted using an inverted microscope (Olympus, Japan).

Statistical analysis

All data are presented as means ± standard deviation. Statistical analyses were performed with SPSS 21.0 software (IBM) and corresponding bar graph or line charts were drawn by GraphPad Prism 7 software. Differences of measurement data and enumeration data were compared respectively with Student's *t*-test, chi-square test and analysis of variance. A Kaplan-Meier survival plot was generated to compare overall survival with the log-rank statistic. *P* values less than 0.05 were considered to be statistically significant.

Results

Expression of Bcl-2 and Bax in PHEO and adrenocorticohyperplasia (AH)

As shown in **Figure 2A**, the frequency of Bcl-2-positive expression in PHEOs and AHs was respectively observed in 16/21 (76.19%) and 6/13 (37.5%) ($P≥0.05$), while Bax-positive expression was respectively 14/21 (66.67%) and 10/13 (76.92%) ($P≥0.05$). Of note, the number of staining intensity of Bcl-2 stronger than Bax (Bcl-2>Bax) from the same samples in PHEOs and AHs was 13 and 3 ($P<0.05$), respectively.

We subsequently analyzed overall survival (OS) in PHEOs alone, including Bcl-2>Bax group and Bcl-2≤Bax group (staining intensity of Bcl-2 not stronger than Bax). Although there was no significant difference ($P = 0.1694$), Kaplan-Meier survival analysis showed that the median OS in the Bcl-2>Bax group was 53.82 months (95% confidence interval (CI), 44.02-63.62 months) compared with 73.50 months (95% CI, 64.49-82.51 months) in the Bcl-2≤Bax group (**Figure 2B**).

ApoG2 could inhibit viability of PC12 cell line

To attest the anti-tumor effect of ApoG2, a CCK8 assay was firstly applied to examine the viability of PC12 cells treated with various concentrations of ApoG2. The results (**Figure 3A**) showed that ApoG2 could effectively inhibit cell viability by a dose- and time-dependent manner, with an IC50 value of 64.39, 34.89 and 31.60 µmol/L at 24, 48, and 72 h, respectively. Under treatment with 50 µmol/L ApoG2, the percentages of dead cells were 45.1±1.07, 80.4±0.60 and 89.4±0.43% at 24, 48 and 72 h, respectively.

ApoG2 affected cell viability by inducing apoptosis but not inhibiting cell proliferation

We observed morphological characteristics of apoptosis in treatment groups compared to the
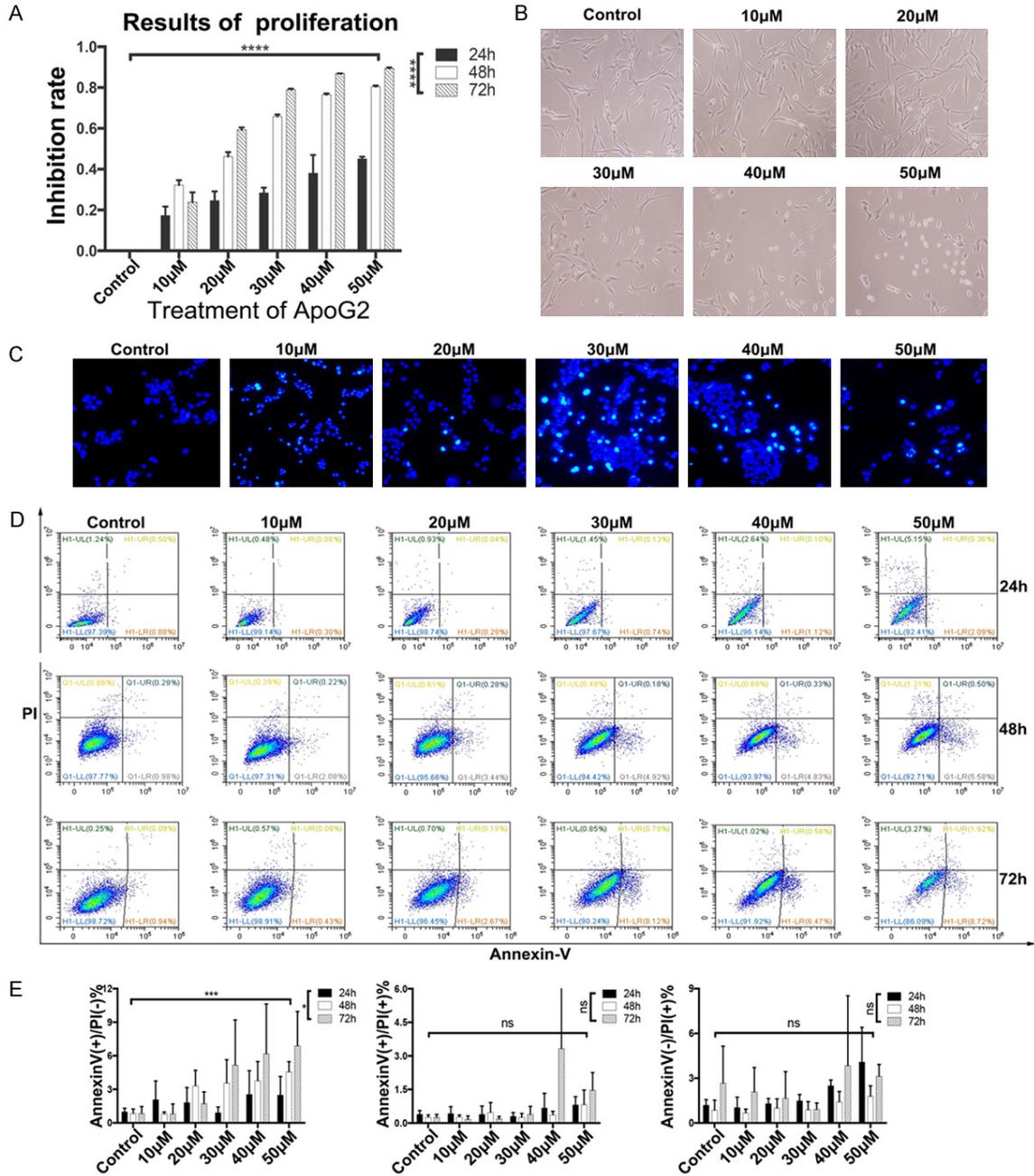
Figure 3. ApoG2 could induce cell apoptosis. A: Growth inhibition rate, as measured by CCK8 assay, of cells treated with varying concentrations of ApoG2. B: Morphological characteristics of apoptosis such as becoming shrunken, rounded and decrease of synaptic structures were observed by microscopy in cells treated with ApoG2. C: Apoptosis cell nucleus presented bright blue in Hoechst 33258 assay. D: Apoptosis was determined with Annexin-V/PI labeling by flow cytometry. E: Statistical analysis of the number of apoptotic cells following treatment with varying concentrations of ApoG2, including early apoptosis (Annexin V+/PI-), late apoptosis (Annexin V+/PI+) and necrosis (Annexin V-/PI+). ns, $P>0.05$; *$P<0.05$; **$P<0.01$; ***$P<0.001$; ****$P<0.0001$.

matched group (**Figure 3B**), including cells becoming shrunken, rounded and decreased of synaptic structure. Therefore, Hoechst 33258 nuclear staining and flow cytometry were performed to objectively evaluate whether apoptosis occurred in the cells. As shown in **Figure 3C**

and **3D**, ApoG2 primarily induced early apoptosis in cells as time and concentration increased. The early apoptosis percentages of the group treated with 50 μmol/L ApoG2 were 2.49±1.66% vs. 0.99±0.32% for the control group, 4.57±0.90% vs. 0.84±0.39% and 6.89±
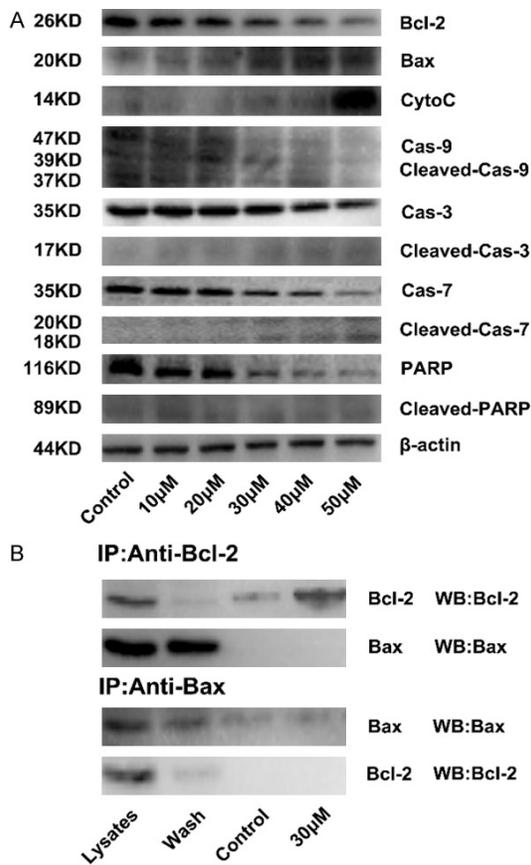
Figure 4. Effects of ApoG2 on apoptotic pathway in PC12 cells. A: Cells were treated with or without indicated concentrations of ApoG2 for 24 h. Proteins were then extracted from those cells and subjected to western blot analysis. B: Cell lysates buffer (lane 1) served as a positive control; supernatant buffer (lane 2) served as a negative control. Control group (lane 3) and ApoG2-treated group (lane 4) were immunoprecipitated by primary anti-Bax or anti-Bcl-2 antibodies. Western blots were used to identify whether ApoG2 affected Bcl-2/Bax compound.

3.07% vs. 0.83±0.62%, respectively, at 24, 48, and 72 h ($P<0.05$, **Figure 3E**).

As previously described, ApoG2 is a BH3 mimic, and BCL-2 family proteins regulate homeostasis between survival and death. Hence, western blot analysis was employed to examine the expression levels of Bcl-2 and Bax proteins to further probe into molecular mechanisms of ApoG2-induced apoptosis activity. It was dependent on up-regulation of Bax protein and down-regulation of Bcl-2 protein (**Figure 4A**). To our knowledge, regardless of extracellular and intracellular apoptosis signals, caspase family members are quite crucial in the process of apoptosis transduction. Additionally, caspase family proteins initiate a cascade reaction followed by release of cytochrome C from mitochondria. Therefore, we next detected the expression levels of cytochrome C, initiator caspase-9, executor caspase-7, and caspase-3 and its target effector PARP. ApoG2 could effectively promote release of cytochrome C and subsequently cleave caspases-9, 7, and 3, finally leading to inactivation of PARP to trigger apoptosis (**Figure 4A**).

As ApoG2 is a BH3 mimic, we speculated that interaction between Bcl-2 and Bax was possibly blocked by ApoG2. However, our co-immunoprecipitation results suggested that Bcl-2/Bax compound did not participant in the ApoG2-induced-apoptosis process (**Figure 4B**).

Cell cycle arrest is another aspect affecting cell viability so a colony formation assay and cell cycle analysis by flow cytometry were performed to detect changes in the cell cycle. There were significantly fewer colonies formed in ApoG2-treated groups compared to the control group (**Figure 5A** and **5B**). Although the G2 phase of the cell cycle was slightly blocked as ApoG2 dose increased in the low dose interval (control group versus 30 μmol/L group, 15.59± 4.44% vs. 23.18±6.38%) (**Figure 5C** and **5D**), cell cycle arrests were not significantly statistically different. Similarly, expression of protein CDK4 related to G2 phase arrest did not significantly decrease with increased concentrations of ApoG2 (**Figure 5E**).

In vivo, we found tumor volumes in experimental groups treated with ApoG2 were significantly smaller than matched groups (**Figure 5F** and **5G**, 82.38±54.78 mm^3 vs. 301.68±48.77 mm^3, $P<0.0001$).

ApoG2-reduced-cell-viability capacity was reliant on caspase activation and ROS increase

From the above results, we questioned whether caspase activation in the PC12 cell line was a required process for ApoG2-induced apoptosis. To investigate this, we firstly determined the expression level of apoptosis inducing factor (AIF) involved in the caspase-independent apoptosis pathway. However, the AIF expression level was constant with or without ApoG2 (**Figure 6A**). Next, cells were treated without or
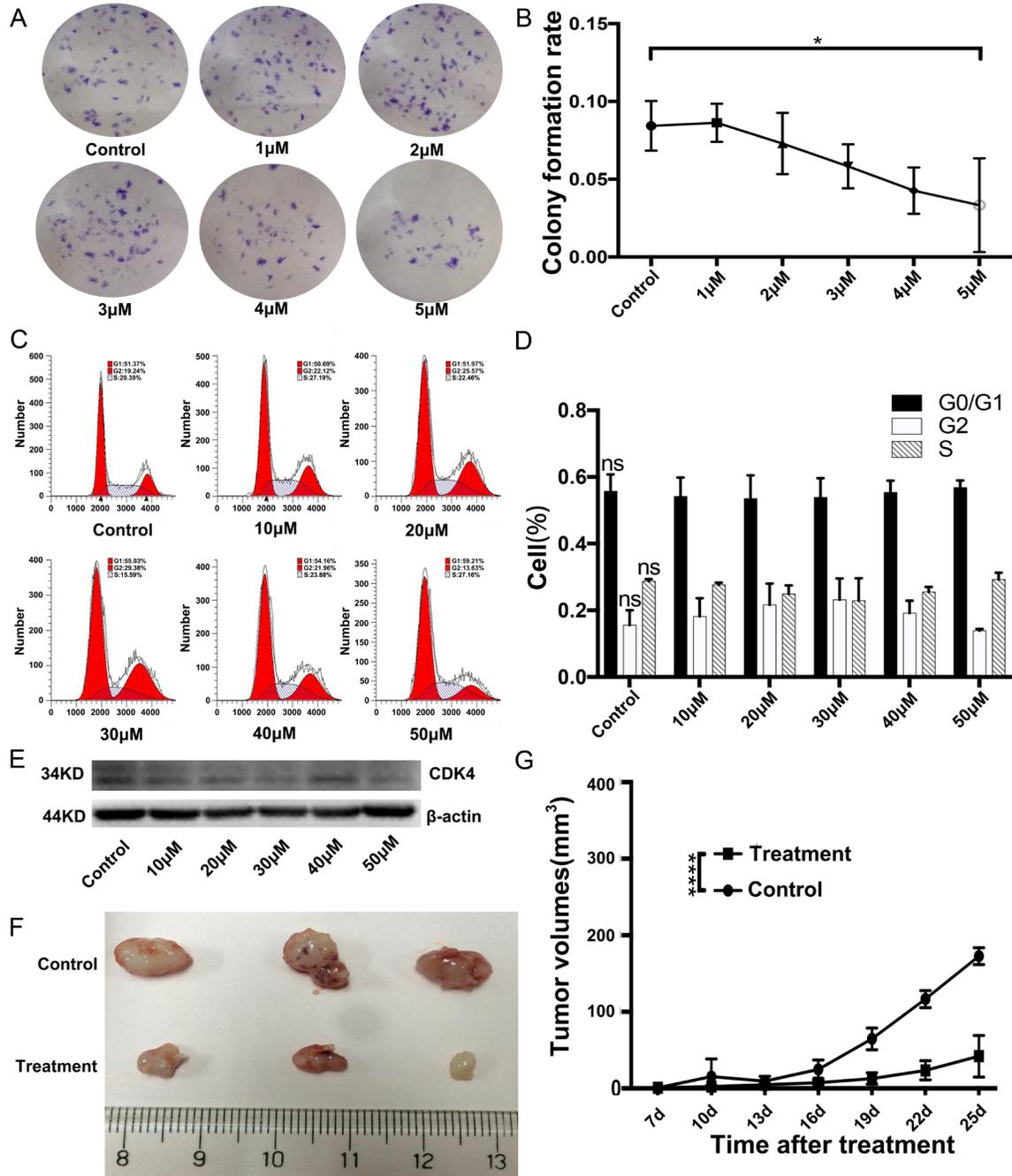
Figure 5. ApoG2 couldn't induce cell cycle arrest, but could retard growth of xenograft tumors. A: Cells were treated with or without indicated concentrations of ApoG2 for 10 days, and a colony formation assay was then performed. B: Statistical analysis of the colony formation rate. C: Results of cell cycle analysis by flow cytometry. D: Statistical analysis of cell cycle. ns, Not significant. E: CDK4 expression level assayed by western blot. F, G: Xenograft tumors extracted from euthanized nude mice. Tumor length and width were measured every three days for 21 days, and tumor volume was calculated.

with increasing concentrations of ApoG2 combined with 30 µmol/L caspase inhibitor Z-VAD-fmk (fmk). The results suggested that Z-VAD-fmk could significantly block the ApoG2-induced apoptosis effect (**Figure 6D**). The inhi-

bition rates of 30 µmol/L ApoG2 without and with Z-VAD-fmk were 28.41±2.54% vs. 9.26± 21.61%, 65.80±1.02% vs. 54.02±5.23% and 78.96±0.54% vs. 69.70±11.23% at 24, 48 and 72 h, respectively.
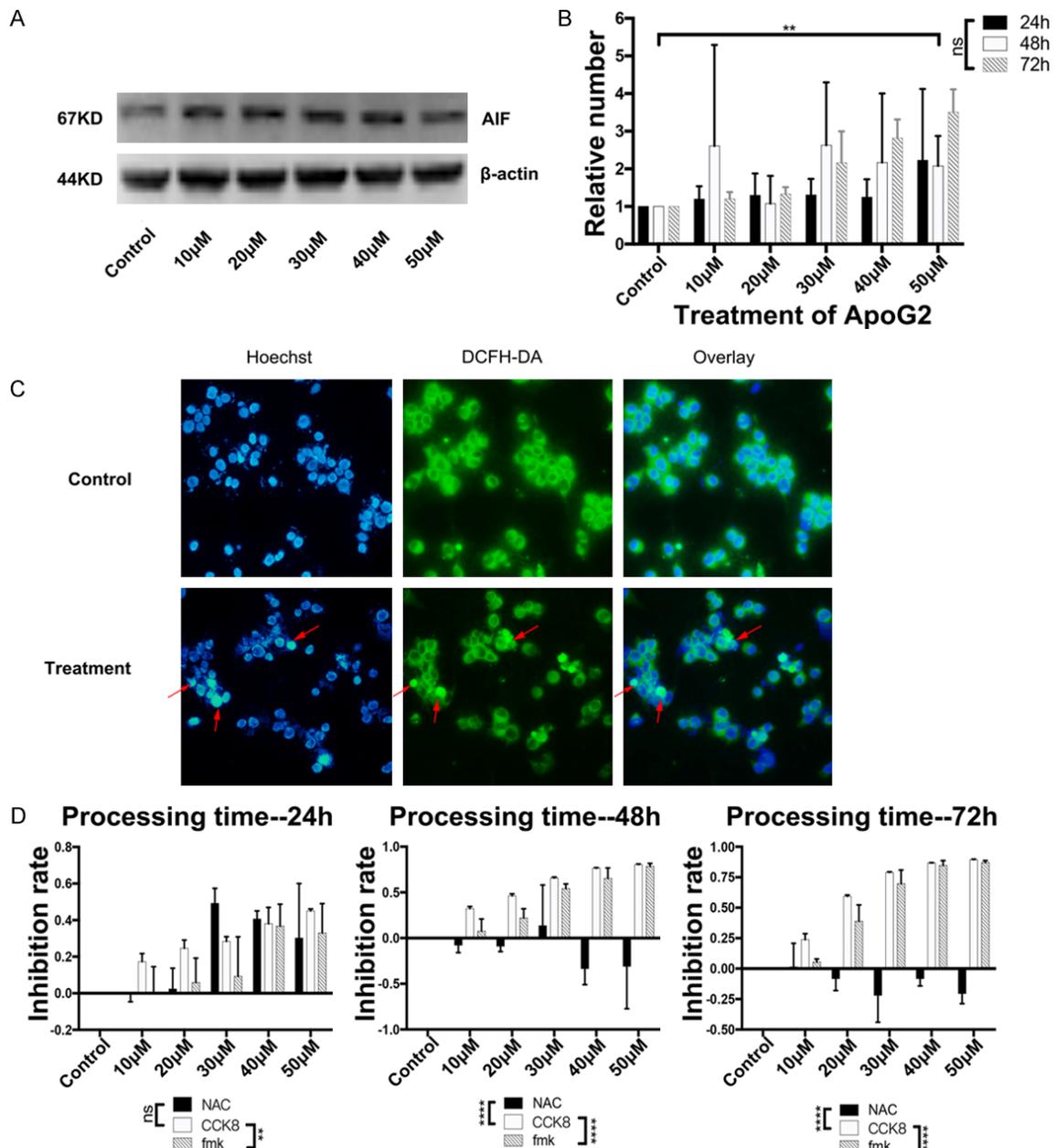
Figure 6. ApoG2 anti-tumor mechanism analysis. A: Expression level of AIF protein involved in caspase-independent pathway. B: ROS level measured by Microplate Reader. C: ROS level increased in apoptosis cells (red arrows) as seen through fluorescence microscope (×200 magnification). D: Cells were treated without or with indicated concentration ApoG2 combined with caspase inhibitor fmk or NAC. Cell viability was then measured with the CCK8 assay through Microplate Reader.

Reactive oxygen species (ROS) not only induce apoptosis, but are also closely related to cell necrosis. Some studies have previously demonstrated that ApoG2 could significantly increase ROS production [35, 36]. Consequently, ROS levels were detected using a DCFH-DA fluorescence probe in our study. ROS levels in the experimental groups markedly increased compared with the control group as dose, but not time, increased (**Figure 6B**). Increased ROS generation in the cytoplasm of apoptotic cells was detected by fluorescence microscopy (indicated by red arrows in **Figure 6C**. Next, cells were treated with antioxidant *N*-acetyl-L-cysteine (NAC) combined with or without ApoG2 to prove whether the ApoG2-mediated cell death

	Migration			Invasion
ApoG2	+	+	+	+
Z-VAD-fmk	-	+	-	-
NAC	-	-	+	-
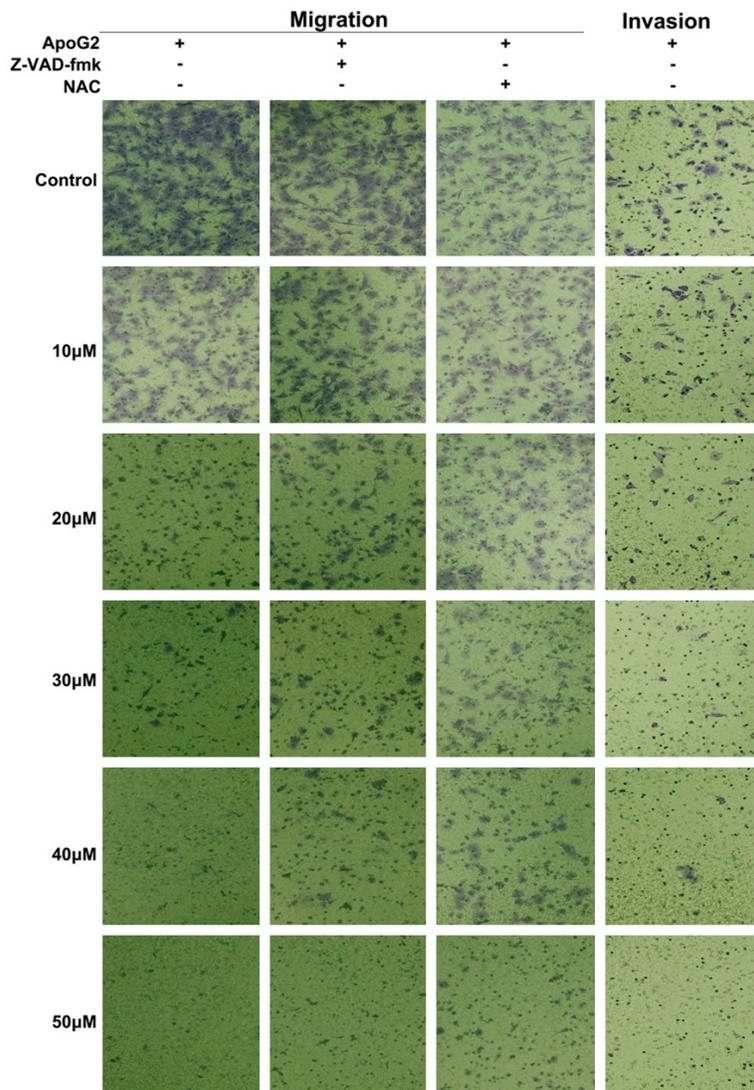
Figure 7. ApoG2 could inhibit migration and invasion in PC12 cells.

6 h, migration and invasion characters of PC12 cells were significantly inhibited (**Figure 7**). To avoid the effects of apoptosis factors, cells were tentatively treated by fmk or NAC combined with or without ApoG2. As expected, fmk or NAC did not reverse the above phenomena. This indicates that ApoG2 could actually decrease the movement capacity of PC12 cells.

Discussion

More than half of all tumor tissues over-express anti-apoptotic protein Bcl-2 [15]. Consequently, in the field of oncology, researchers have paid more attention to role of anti-apoptotic proteins in treatment of various tumors, and also in the aspects of resistance to chemotherapy [16, 17]. As a BH3 mimic, ApoG2 has been confirmed to have greater anti-tumor effects and less side effects in multiple cancers.

Currently, there is an absence of clinically reliable indexes to allow early diagnosis of malignant PHEO, with exception of the gold standard that chromaffin tissue presents at the side where chromaffin tissue is normally absent. However once metastasis occurs, tho-se patients have regrettably missed the optimal treatment opportunity and only benefit partially from conventional chemotherapy. The anti-PHEO effect of ApoG2 is still poorly understood until now. In this research, we chiefly proved that ApoG2 could induce apoptosis and inhibit progression of PHEO.

effect depends on ROS production. As expected, NAC significantly protected PC12 cells from damage by ApoG2. The inhibition rates in cells treated with 50 μmol/L ApoG2 without and with NAC were 45.06±1.07% vs. 30.19±29.86%, 80.44±0.60% vs. -30.84±46.48% and 89.41± 0.43% vs. -20.48±8.25% at 24, 48 and 72 h, respectively (**Figure 6D**).

ApoG2 could inhibit PC12 cell-line migration and invasion

Metastasis is relevant to the prognosis of tumors. Therefore, it would be very useful if ApoG2 could inhibit the aggressive properties of tumors. To investigate this, we performed migration and invasion detection using transwell chambers. After treatment with ApoG2 for

In the present study, we initially corroborated that the expression intensity of Bcl-2 was stronger than Bax in PHEOs, suggesting critical roles of Bcl-2 in PHEO pathogenesis. Additionally, when the staining intensity of Bcl-2 was stronger than Bax in the same patient, the median overall survival may be shorter, though this was

not observed to be a statistically significant difference. Subsequently, we showed that ApoG2 exactly inhibited viability of PC12 cell lines through inducing apoptosis rather than cell cycle arrest. These results were further verified by western blotting, which demonstrated up-regulation of pro-apoptotic proteins like Bax, cleaved caspase proteins and cleaved PARP, and down-regulation of anti-apoptotic proteins like Bcl-2, precursor caspase proteins and PARP. ApoG2 could impede colony formation while cell cycle arrest and expression level of CDK4 did not show any significant difference. We hypothesized that the significant colony formation results were owing to apoptosis-induced effects caused by prolonged exposure to ApoG2 at low concentration. Finally, the mouse xenograft model experiment indicated that ApoG2 inhibited growth of tumors *in vivo*. Therefore, based on the above study, we confirmed that ApoG2 possessed anti-PEHO effects and show for the first time that this is primarily through pro-apoptotic effects but not blocking of proliferation.

Unfortunately, our co-immunoprecipitation results suggested that ApoG2 did not affect Bcl-2/Bax complex to induce apoptosis, which was different to results from other studies [27]. This could be explained in that ApoG2 did not affect Bcl-2/Bax compounds, but could affect other compounds such as Bcl-xl/Bak or Bcl-2/Beclin-1, etc. A limitation of our study was that we only concentrated on Bcl-2/Bax compound, but ignored other related compounds. The other limitation was that we did not clearly elucidate the cause of ROS production. The production of ROS might be because ApoG2 reacted with Bcl-2 to damage mitochondria and further increase its permeability, subsequently resulting in an increase in ROS generation. On the other hand, two para-quinone moieties in ApoG2 are strong electrophilic groups easily attaching to nucleophiles so that the cells could not scavenge ROS and experienced oxidative stress at a high level of ROS.

However notwithstanding the limitations, this study clearly indicated that ApoG2 could contribute to an increase in ROS levels just like other studies [37], and most importantly, the antioxidant NAC could almost totally take a turn for ApoG2-apoptosis-induced effects. In addition, a broad spectrum caspase activation inhibitor z-VAD-fmk was the same as NAC, capable of partially reversing ApoG2-induced-apoptosis. Therefore, we believe that ApoG2-induced-apoptosis is involved in, but not totally dependent on, the caspase cascade reaction. Unfortunately, we do not know the role of other molecules such as autophagy-related proteins in the process of ApoG2-induced effects.

Another failure of therapy of tumors is attributed to the occurrence of metastasis. Accordingly, it would be best if the target drugs inhibited the movement character of tumors. Encouragingly, our results indicated that ApoG2 could impede the migration and aggressive nature of PC12 cells compared with the control group. It is noteworthy that fmk or NAC did not intervene in ApoG2-decreased movement of PC12 cells, suggesting that ApoG2 exactly inhibited movement of PC12 cells independent of ROS- or caspase-mediated cell death. Regrettably, our study did not refer to further mechanisms such as expression level of genes or proteins related to the epithelial-mesenchymal transition (EMT) process.

In conclusion, our study showed that Bcl-2>Bax was more frequently found in PHEO and possibly related to poor prognosis, although the latter needs to be further verified in a large number samples. In addition, ApoG2 is a potential target drug with significant anti-tumor activity through up-regulation of Bax and down-regulation of Bcl-2 in PHEO. ApoG2 could induce apoptosis dependent on the activation of caspase proteins and ROS increase. Furthermore, it could effectively inhibit migration and invasion of PC12 cells. More attention should therefore be paid to ApoG2 in the treatment of PHEO.

Additional files: All original western images were showed in Supplementary Figure 1.

Disclosure of conflict of interest

None.

Authors' contribution

Dengqiang Lin and Lieyu Xu conceived the experiments; Dengqiang Lin and Xiaoxia Li conducted the experiments; Xiaoxia Li and Jianpo

Address correspondence to: Yu Zhu and Chenghe Wang, Department of Urology, Ruijin Hospital Affiliated to Medical School of Shanghai Jiaotong University, No.197, Ruijin'er Road, Shanghai, China. E-mail: zhuyuruijin@163.com (YZ), E-mail: wangch8603@163.com (CHW)

References

[1] Zelinka T, Petrak O, Turkova H, Holaj R, Strauch B, Krsek M, Vrankova AB, Musil Z, Duskova J, Kubinyi J, Michalsky D, Novak K and Widimsky J. High incidence of cardiovascular complications in pheochromocytoma. Horm Metab Res 2012; 44: 379-384.

[2] Prejbisz A, Lenders JW, Eisenhofer G and Januszewicz A. Mortality associated with phaeochromocytoma. Horm Metab Res 2013; 45: 154-158.

[3] Walther MM, Herring J, Enquist E, Keiser HR and Linehan WM. von Recklinghausen's disease and pheochromocytomas. J Urol 1999; 162: 1582-1586.

[4] Pacak K, Eisenhofer G, Ahlman H, Bornstein SR, Gimenez-Roqueplo AP, Grossman AB, Kimura N, Mannelli M, McNicol AM and Tischler AS. Pheochromocytoma: recommendations for clinical practice from the first international symposium. October 2005. Nat Clin Pract Endocrinol Metab 2007; 3: 92-102.

[5] Edstrom Elder E, Hjelm Skog AL, Hoog A and Hamberger B. The management of benign and malignant pheochromocytoma and abdominal paraganglioma. Eur J Surg Oncol 2003; 29: 278-283.

[6] Schurmeyer T, Dralle H, Schuppert F and von zur Muhlen A. [Preoperative diagnosis of suspected pheochromocytoma--retrospective assessment of diagnostic criteria]. Acta Med Austriaca 1988; 15: 106-108.

[7] Walther MM, Keiser HR and Linehan WM. Pheochromocytoma: evaluation, diagnosis, and treatment. World J Urol 1999; 17: 35-39.

[8] Kopf D, Goretzki PE and Lehnert H. Clinical management of malignant adrenal tumors. J Cancer Res Clin Oncol 2001; 127: 143-155.

[9] Plati J, Bucur O and Khosravi-Far R. Dysregulation of apoptotic signaling in cancer: molecular mechanisms and therapeutic opportunities. J Cell Biochem 2008; 104: 1124-1149.

[10] Fulda S. Tumor resistance to apoptosis. Int J Cancer 2009; 124: 511-515.

[11] Gimenez-Bonafe P, Tortosa A and Perez-Tomas R. Overcoming drug resistance by enhancing apoptosis of tumor cells. Curr Cancer Drug Targets 2009; 9: 320-340.

[12] Fulda S. Targeting apoptosis for anticancer therapy. Semin Cancer Biol 2015; 31: 84-88.

[13] Theofilas P, Bedner P, Huttmann K, Theis M, Steinhauser C and Frank S. The proapoptotic BCL-2 homology domain 3-only protein Bim is not critical for acute excitotoxic cell death. J Neuropathol Exp Neurol 2009; 68: 102-110.

[14] Reed JC, Zha H, Aime-Sempe C, Takayama S and Wang HG. Structure-function analysis of Bcl-2 family proteins. Regulators of programmed cell death. Adv Exp Med Biol 1996; 406: 99-112.

[15] Alsabeh R, Wilson CS, Ahn CW, Vasef MA and Battifora H. Expression of bcl-2 by breast cancer: a possible diagnostic application. Mod Pathol 1996; 9: 439-444.

[16] Poelman SM, Adeyanju MO, Robertson MA, Recant WM, Karrison T, Fleming GF, Olopade OI and Conzen SD. Human breast cancer susceptibility to paclitaxel therapy is independent of Bcl-2 expression. Clin Cancer Res 2000; 6: 4043-4048.

[17] Jaroszewski JW, Kaplan O and Cohen JS. Action of gossypol and rhodamine 123 on wild type and multidrug-resistant MCF-7 human breast cancer cells: 31P nuclear magnetic resonance and toxicity studies. Cancer Res 1990; 50: 6936-6943.

[18] Pattingre S and Levine B. Bcl-2 inhibition of autophagy: a new route to cancer? Cancer Res 2006; 66: 2885-2888.

[19] Kanematsu S, Uehara N, Miki H, Yoshizawa K, Kawanaka A, Yuri T and Tsubura A. Autophagy inhibition enhances sulforaphane-induced apoptosis in human breast cancer cells. Anticancer Res 2010; 30: 3381-3390.

[20] Zhang PJ, Genega EM, Tomaszewski JE, Pasha TL and LiVolsi VA. The role of calretinin, inhibin, melan-A, BCL-2, and C-kit in differentiating adrenal cortical and medullary tumors: an immunohistochemical study. Mod Pathol 2003; 16: 591-597.

[21] Wang DG, Johnston CF, Marley JJ, Phenix KV, Atkinson AB, Russell CF and Buchanan KD. Expression of the apoptosis-suppressing gene BCL-2 in pheochromocytoma is associated with the expression of C-MYC. J Clin Endocrinol Metab 1997; 82: 1949-1952.

[22] de Krijger RR, van der Harst E, van der Ham F, Stijnen T, Dinjens WN, Koper JW, Bruining HA, Lamberts SW and Bosman FT. Prognostic value of p53, bcl-2, and c-erbB-2 protein expression in phaeochromocytomas. J Pathol 1999; 188: 51-55.

[23] Cadden IS, Atkinson AB, Johnston BT, Pogue K, Connolly R, McCance D, Ardill JE, Russell CF and McGinty A. Cyclooxygenase-2 expression correlates with phaeochromocytoma malignancy: evidence for a Bcl-2-dependent mechanism. Histopathology 2007; 51: 743-751.

[24] Carlsen E, Abdullah Z, Kazmi SM and Kousparos G. Pheochromocytomas, PASS, and immunohistochemistry. Horm Metab Res 2009; 41: 715-719.

[25] Wu D. An overview of the clinical pharmacology and therapeutic potential of gossypol as a male contraceptive agent and in gynaecological disease. Drugs 1989; 38: 333-341.

[26] Shelley MD, Hartley L, Groundwater PW and Fish RG. Structure-activity studies on gossypol in tumor cell lines. Anticancer Drugs 2000; 11: 209-216.

[27] Hu ZY, Zhu XF, Zhong ZD, Sun J, Wang J, Yang D and Zeng YX. ApoG2, a novel inhibitor of anti-apoptotic Bcl-2 family proteins, induces apoptosis and suppresses tumor growth in nasopharyngeal carcinoma xenografts. Int J Cancer 2008; 123: 2418-2429.

[28] Hu ZY, Sun J, Zhu XF, Yang D and Zeng YX. ApoG2 induces cell cycle arrest of nasopharyngeal carcinoma cells by suppressing the c-Myc signaling pathway. J Transl Med 2009; 7: 74.

[29] Zhang XQ, Huang XF, Hu XB, Zhan YH, An QX, Yang SM, Xia AJ, Yi J, Chen R, Mu SJ and Wu DC. Apogossypolone, a novel inhibitor of anti-apoptotic Bcl-2 family proteins, induces autophagy of PC-3 and LNCaP prostate cancer cells in vitro. Asian J Androl 2010; 12: 697-708.

[30] Sun Y, Wu J, Aboukameel A, Banerjee S, Arnold AA, Chen J, Nikolovska-Coleska Z, Lin Y, Ling X, Yang D, Wang S, Al-Katib A and Mohammad RM. Apogossypolone, a nonpeptidic small molecule inhibitor targeting Bcl-2 family proteins, effectively inhibits growth of diffuse large cell lymphoma cells in vitro and in vivo. Cancer Biol Ther 2008; 7: 1418-1426.

[31] Sun J, Li ZM, Hu ZY, Zeng ZL, Yang DJ and Jiang WQ. Apogossypolone inhibits cell growth by inducing cell cycle arrest in U937 cells. Oncol Rep 2009; 22: 193-198.

[32] Arnold AA, Aboukameel A, Chen J, Yang D, Wang S, Al-Katib A and Mohammad RM. Preclinical studies of Apogossypolone: a new non-peptidic pan small-molecule inhibitor of Bcl-2, Bcl-XL and Mcl-1 proteins in follicular small cleaved cell lymphoma model. Mol Cancer 2008; 7: 20.

[33] Banerjee S, Choi M, Aboukameel A, Wang Z, Mohammad M, Chen J, Yang D, Sarkar FH and Mohammad RM. Preclinical studies of apogossypolone, a novel pan inhibitor of bcl-2 and mcl-1, synergistically potentiates cytotoxic effect of gemcitabine in pancreatic cancer cells. Pancreas 2010; 39: 323-331.

[34] Mi JX, Wang GF, Wang HB, Sun XQ, Ni XY, Zhang XW, Tang JM and Yang DJ. Synergistic antitumoral activity and induction of apoptosis by novel pan Bcl-2 proteins inhibitor apogossypolone with adriamycin in human hepatocellular carcinoma. Acta Pharmacol Sin 2008; 29: 1467-1477.

[35] Hu ZY, Wang J, Cheng G, Zhu XF, Huang P, Yang D and Zeng YX. Apogossypolone targets mitochondria and light enhances its anticancer activity by stimulating generation of singlet oxygen and reactive oxygen species. Chin J Cancer 2011; 30: 41-53.

[36] Zubair H, Khan HY, Ullah MF, Ahmad A, Wu D and Hadi SM. Apogossypolone, derivative of gossypol, mobilizes endogenous copper in human peripheral lymphocytes leading to oxidative DNA breakage. Eur J Pharm Sci 2012; 47: 280-286.

[37] Xin J, Zhan Y, Liu M, Hu H, Xia L, Nie Y, Wu K, Liang J and Tian J. ApoG2 induces ER stress-dependent apoptosis in gastric cancer cells in vitro and its real-time evaluation by bioluminescence imaging in vivo. Cancer Lett 2013; 336: 260-269.

Apogossypolone induces apoptosis and reduces invasiveness of PC12 cells
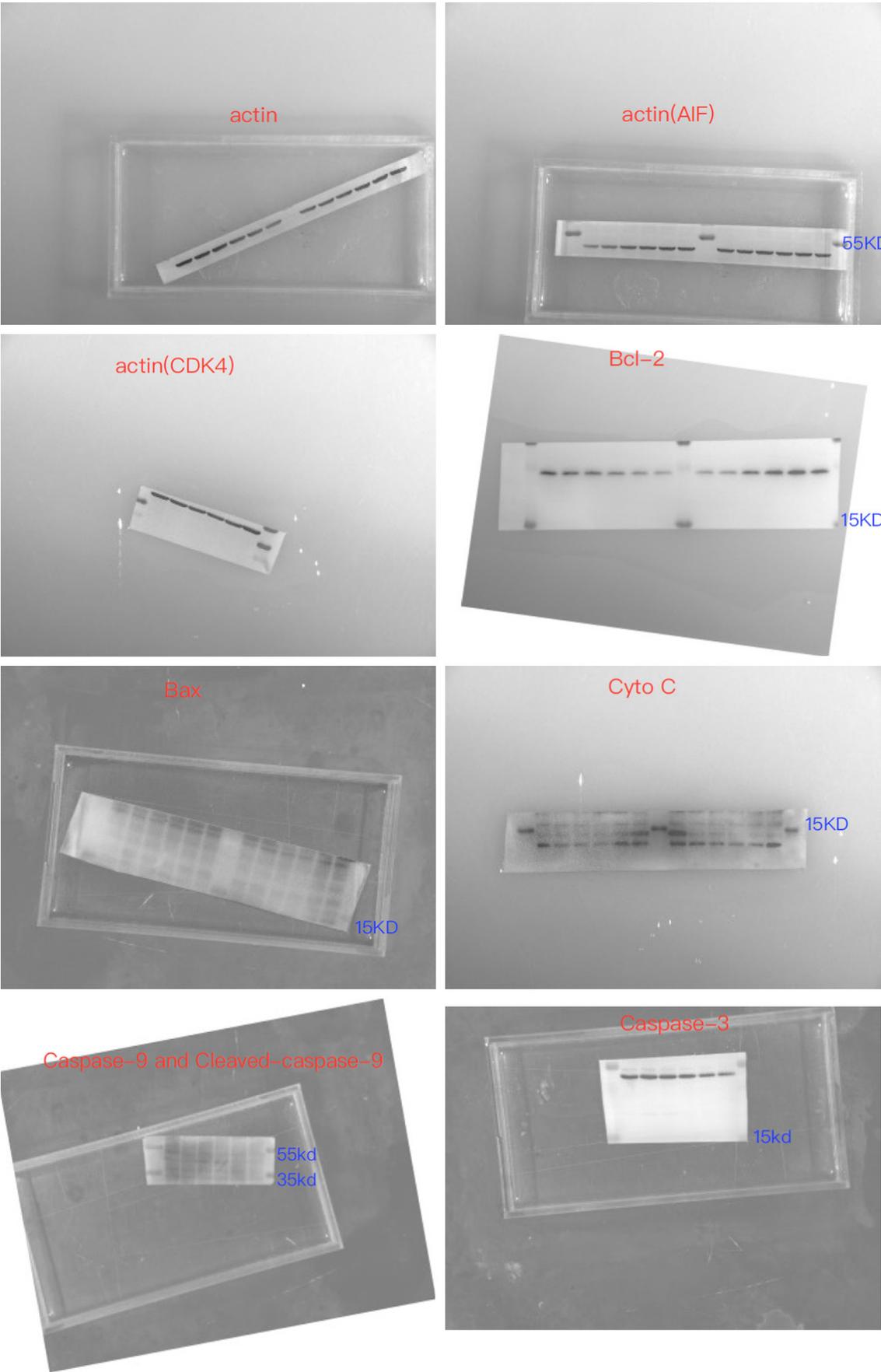
Cleaved–Caspase–3

15kd

Caspase–7

35kd

15kd

Cleaved–Caspase–7

35kd
25kd
15kd

PARP

250kd
130kd
110kd

Cleaved–PARP

250kd
130kd
110kd

IPbcl–2WBbcl–2

35kd

15kd

IPbcl–2WBbax

35kd
25kd

15kd
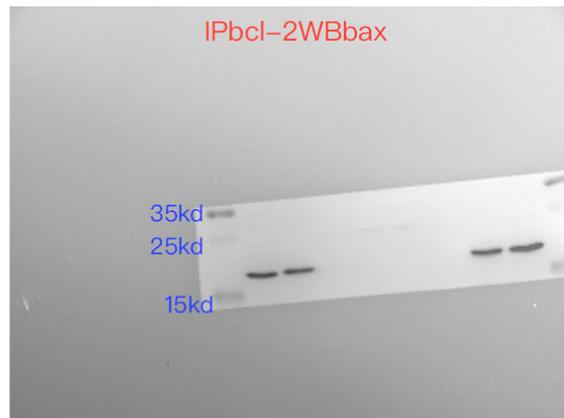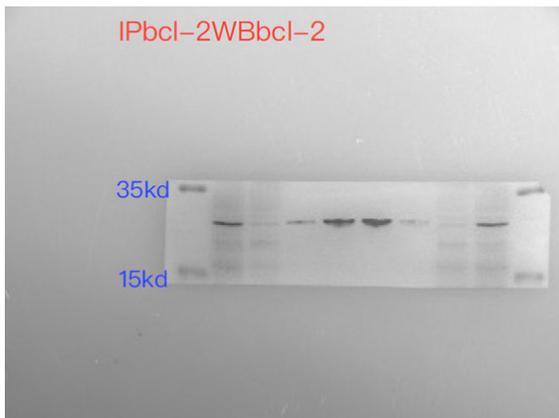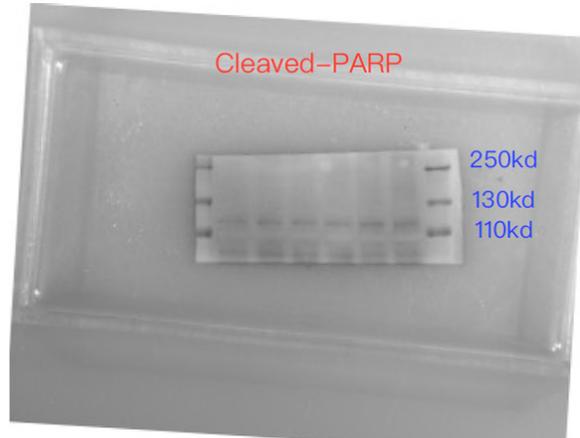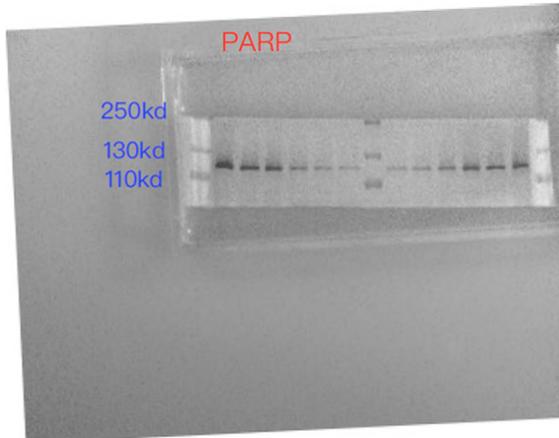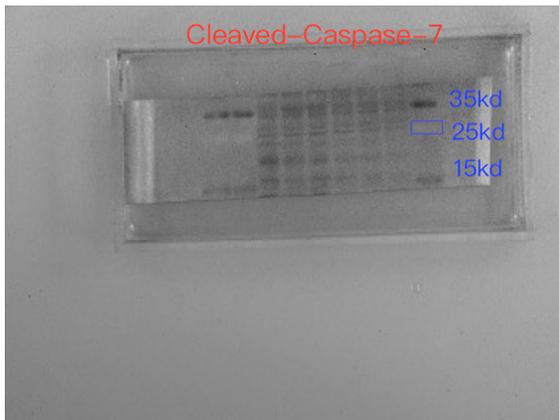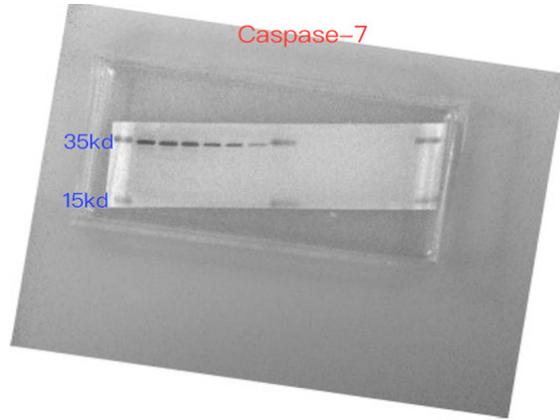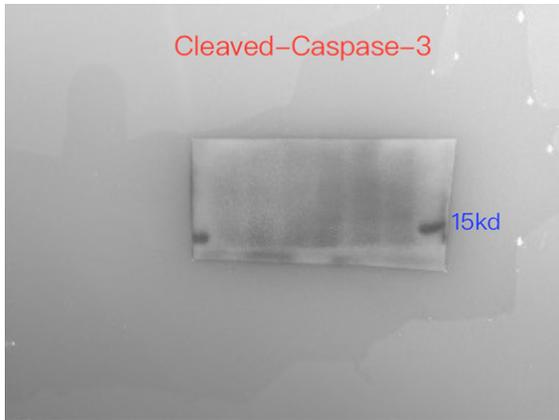
Apogossypolone induces apoptosis and reduces invasiveness of PC12 cells
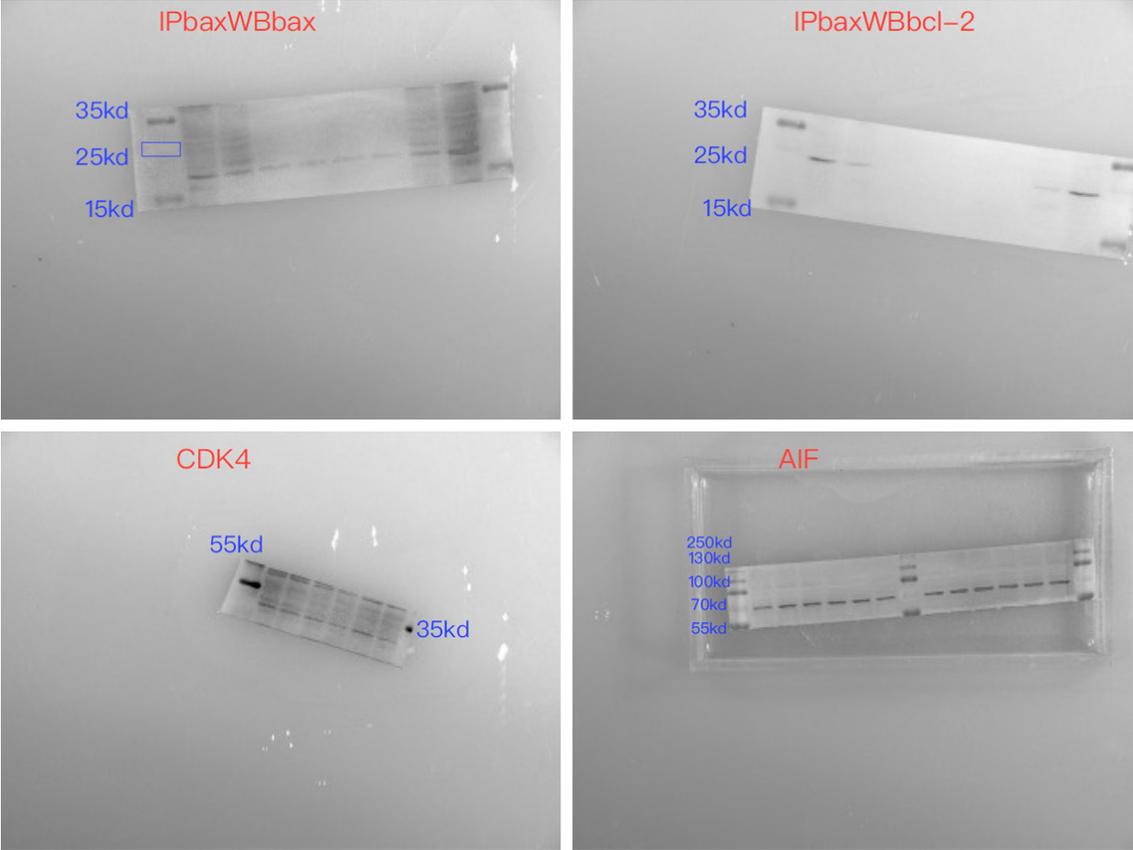
Supplementary Figure 1. Generally, the bands distribute symmetrically, which are blank, 10 µM, 20 µM, 30 µM, 40 µM and 50 µM group from the outside to the inside; Content marked by Blue Pen stands for Marker size.