Astragaloside-IV Inhibits Pancreatic Cancer Cell Proliferation in Vitro and in Vivo by Inducing Cell Cycle Arrest and Apoptosis

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Research Article

Keywords: Astragaloside IV, Pancreatic cancer, apoptosis, caspase, CDK

Posted Date: January 3rd, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1149253/v1

Abstract

Astragaloside IV (AS-IV) or 3-O-β-D-xylopyranosyl-6-O-β-D-glucopyranosylcyl-cloastragenol is a bioactive saponin extract from the root of Astragalus membranaceus. It has been proven to have an anti-tumor effect in a variety of tumors by inducing cell apoptosis and inhibiting cell proliferation. Its effects on pancreatic cancer have not been investigated. This study investigated the effects of AS-IV on proliferation, apoptosis and migration of pancreatic cancer cells in vitro and in vivo and explored its underlying mechanism. Pancreatic cancer cell lines SW1990 and Panc-1were treated with different doses of AS-IV. Plate clonality, CCK-8, EDU and flow cytometry were used to explore the effect of AS-IV on pancreatic cancer cell proliferation and cell cycle in vitro. Wound healing was used to investigate the effects of AS-IV on pancreatic cell migration. The protein expression levels of Bax/Bcl2, caspase3/7, cyclin D1, cyclin E and CDK4 were analyzed by western blotting. The results showed that AS-IV significantly inhibited tumor cell proliferation and cell cycle, induced apoptosis both in vitro and vivo on a dose-dependent basis and significantly inhibited the growth of pancreatic cell xenograft tumor in nude mice. Wound healing assays indicated that AS-IV also inhibited the migration of pancreatic cancer cells in a dose-dependent manner. This research confirmed that AS-IV inhibited pancreatic cancer cell proliferation by blocking the cell cycle and inducing apoptosis. It was hypothesized from this experiment that the potential mechanism of AS-IV inducing apoptosis of pancreatic cancer cells may be understood by activating the Bcl2/Bax/Caspase-3/Caspase-7 signaling pathway.

Intruduction

Pancreatic cancer is one of the most deadly malignancies and is projected to be the second leading cause of cancer death in the USA by 2030[1, 2]. Despite decades of research, the prognosis for pancreatic cancer patients continues to be poor as the five-year survival rate at the time of diagnosis is only about 10%, as approximately 80 to 85% of patients present with either unresectable or metastatic disease[3, 4]. Unfortunately, the current treatment options are limited and surgery is the only effective treatment. However, only about 10 to 20% of patients diagnosed with pancreatic cancer have the opportunity to undergo surgery and even among those patients who can undergo surgery, their five-year survival rate is also less than 20%[4, 5]. In addition, epidemiological studies have shown that both incidence and mortality rates increased by an average of 0.3% per year during the past decade so there is an urgent need to develop new diagnostic and therapeutic approaches.

Previous studies have shown that some Chinese herbal medicines have a therapeutic effect on tumors[6]. Astragaloside IV or AS-IV (3-O-β-D-xylopyranosyl-6-O-β-D-glucopyranosylcyl-cloastragenol) is a bioactive saponin extract from the root of Astragalus[7] that has shown a wide of pharmacological effects including strengthening the immune system and lowering blood pressure as well as anti-inflammatory and anti-tumor effects[8–12]. Previous studies have shown that AS-Ⅳ induces cell apoptosis and inhibits proliferation and autophagy through the TGF-β/Smad signaling pathway[13]. Other studies have shown that AS-IV can inhibit the ability of invasion and migration in human lung cancer and breast cancer[14,

15]. In addition to directly inhibiting the occurrence and development of tumors, AS-IV can also enhance the sensitivity of cisplatin, fluorouracil and gefitinib in the treatment of tumors[16–18].

The imbalance of cell proliferation and apoptosis plays an important part in the occurrence and development of tumors. Previous studies have shown that AS-IV may cause cell cycle arrest and apoptosis induction both in vivo and vitro [19, 20]. Although AS-IV has shown anti-tumor properties in other cancers, its role in pancreatic cancer is unclear.

This study investigated the therapeutic effect of AS-IV on pancreatic cancer and explored its underlying mechanism. The effects of AS-IV on cell cycle and apoptosis in pancreatic cancer cells were specifically examined.

Materials And Methods

Reagents

The AS-IV powder ($C_{41}H_{68}O_{14}$) with a molecular weight of 784.97 and purity exceeding 98% was purchased from ShanghaiyuanyeBio Co, batch number: S31401. (Shanghai, China). Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich (MA, USA). Antibodies against Bax (50599-2-Ig), Bcl-2 (26593-1-AP), caspase3 (19677-1-AP), Caspase7 (27155-1-AP), cyclin D1 (60186-1-Ig), cyclin E (11554-1-AP), CDK4 (11026-1-AP), Ki-67 (27309-1-AP) and β-actin (66009-1-Ig) were obtained from Proteintech (Proteintech, Rosemont, USA). The haematoxylin and eosin (H&E) staining kit (G1120), was purchased from Slarbio (Slarbio, Beijing, China).

Cell lines and cell culture

The Panc-1 and SW1990 cell lines used in the study were purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. The cells were cultured in Dulbecco's modified Eagle's media containing one mg/mL D-glucose with 10% fetal bovine serum (FBS) (Gibco, USA) and supplemented with penicillin/streptomycin (Sigma, USA) at 37°C in an atmosphere of 5% CO_2 and 95% air.

Cell treatment

The AS-IV was dissolved in DMSO for the treatment of cell lines. The final concentration of DMSO was less than 0.1% (v/v).

CCK-8 assays

Cell viability was determined by CCK-8 assay (Dojindo Laboratories, Kumamoto, Japan) after exposure to different concentrations of 0, 20, 40 and 80 μM of AS-IV. The Panc-1 and SW1990 cells were placed on a 96-well culture plate at a density of 5 x10^3 cells/well and then exposed to increasing doses of AS-IV for 24, 48 and 72h.

At a specified time, 10 μL CCK8 solution were added to each well for four hours. The absorbance was determined with the wavelength of 450 nm using a MK3 ELISA reader (Thermo Fisher Scientific, USA).

EDU assays

Panc-1 and SW1990 cells were inoculated into 96-well plates and treated with different concentrations of AS-IV. Cell proliferation rates were determined according to the instructions using a 5-ethynn-20 deoxyuridine (EdU) kit (RiboBio, Guangzhou, China). 100 μL culture medium containing 50 M EdU was added to each well and incubated for 12 hours. The cells were then fixed with 4% paraformaldehyde for 30 minutes and followed by treatment of 0.5% Triton for 10 minutes and Apollo reaction cocktail (RiboBio, Guangzhou, China) for 30 minutes. The cells were then contaminated with DAPI for 30 minutes for DNA analysis and observed under a fluorescence microscope (Olympus CX41-72C02, Tokyo, Japan).

Wound healing assay

Wound healing assays were used to assess cell migration. Initially $5x10^5$ Panc-1 or SW1990 cells/Well were added to the six-well plate. The cells were cultured overnight to produce a fused monolayer. A 10μL pipette tip was used to make a direct scratch on the cell monolayer. The suspension cells were washed with phosphate buffered saline (PBS) three times. 1ml serum-free medium containing different concentrations of AS-IV was then added. The wound healing process was photographed at 0, 24 and 48 hours.

Plate clonality assays

Pancreatic cancer cells of Panc-1 and SW1990 were suspended in 2 ml complete medium and seeded into six-well plates at $1×10^3$ cells per well. After different treatments 40 and 80 μM AS-IV , they were cultured at 37℃ in air containing 5% CO_2 for two weeks. Cultures containing different concentrations of AS-IV were changed every two days. After 14 days, the colonies were fixed with 4% paraformaldehyde and then stained with 0.5% crystal violet for 20 minutes. The separate experiments involving Panc-1 and SW1990 were each conducted three times.

Cell cycle analysis

Panc-1 and SW1990 cells at the logarithmic growth phase were incubated. After being treated with the varying doses of AS-IV (0, 40 and 80 μM) for 48 h, cells were harvested and resuspended with cold 75% ethanol at -4˚C overnight. The ethanol was then removed and 150 μl propidium iodide (PI) was added and incubated at 4℃ for 30min in darkness (Mei Lun Bio, Dalian, China). Flow cytometry was used to measure cell cycle distribution ((BD, Franklin Lakes, NJ, United States).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

To explore the apoptotic in Panc-1 and SW1990 cells, apoptosis was examined using a one-step TUNEL apoptosis assay kit (Yeasen, Shanghai, China). Cell crawling were washed with phosphate buffered

saline (PBS) three times and fixed with 4% paraformaldehyde for 30 minutes. This was then washed with PBS three times and 100μL Proteinase K was added for 20 min at 37℃. Equilibration buffer was then added and the cells were incubated at room temperature for 20min and 50μl TDT enzyme incubation buffer containing 34μl ddH2O,10ul 5×Equilibration Buffer, 5μl FITC-12-dUTP Labling Mix and 1 μl Recombinant TdT Enzyme, was then added for 60min at 37℃. This was rinsed with PBS three times and cells were contaminated with DAPI for 10 minutes. The TUNEL stained cells were observed under a fluorescence microscope (Motic BA410T, China).

JC-1 prob assay

The mitochondrial depolarization of Panc-1 and SW1990 cells with different treatments were determined by JC-1 probe (Beyotime, Jiangsu, China). Cells exposed to different concentrations of AS-IV in a six-well plate were incubated with an equal volume of JC-15 μg/ml staining solution at 37℃ for 20 minutes and then washed twice with JC-1 staining buffer. After treatment, cells were resuspended with JC-1 staining buffer and the ratio of green to red fluorescence intensity was measured with an FACS Calibur flow cytometer (BD, Franklin Lakes, NJ, United States).

Flow cytometry

Cell apoptosis was assessed by flow cytometry after Annexin-V-fluorescence isothiocyanate (FITC)/ PI staining. The Panc-1 and SW1990 cells were exposed to differing doses of AS-IV for 48 hours respectively and then stained with 5 μL of FITC Annexin V and 5 μL PI for 10 minutes at room temperature in the dark and analyzed by flow cytometry according to the manufacturer's instructions (KeyGEN BioTECH, Jiangsu, China). Three independent experiments were conducted.

Western blotting assays

Western blot assays were performed similarly to the procedure reported previously (Transient receptor potential vanilloid-type 2 targeting on stemness in liver cancer[21]. Total proteins were extracted with RIPA cleavage buffer, separated by 10% sodium dodecyl sulfate SDS-PAGE, and transferred to polyvinylidene fluoride (PVDF) membranes purchased from Millipore (MA, *USA*). The membrane was sealed with 5% skim milk, incubated with a primary antibody and then incubated with a secondary antibody. β-Actin was used as an internal reference.

Animal work and experimental protocols

Four-week-old male nude mice were provided by Hunan SJA Laboratory Animal Technology, Inc. (Hunan, China). Each nude mouse was given a single subcutaneous injection on the right flank of 1×10^6 Panc-1 cells, suspended in a matrix glue (BD Biosciences, CA). After seven days, the tumor size was measured twice weekly using a digital caliper and was calculated as $(D^2 \times d) / 2$, where D is the large diameter and d is the small diameter of the tumor. Mice were randomly divided into two groups when the tumors were up to 100 mm^3 as a control group treated only with DMSO and an AS-IV treatment group receiving

0.1mg/10g/day AS-IV dissolved in DMSO. Each mouse was gavaged daily for 21 days. After anesthesia, tumor-bearing mice were sacrificed and the tumors were removed for further study. Animal experiments were conducted in accordance with the guidelines for animal care and use issued by the National Laboratory of the United States, and the experimental program was approved by the Animal Care and Use Committee of South China University (Hengyang, China).

Immunohistochemistry (IHC)

Immunohistochemistry was performed similarly to the procedure reported previously (Camellia oil (Camellia oleifera Abel.) Attenuates CCl4-induced liver fibrosis via suppressing hepatocyte apoptosis in mice)[22]. Immunohistochemical studies were performed on paraffin sections using anti- ki67 antibody developed using a biotinylated alkaline phosphatase-conjugated secondary antibody and diaminobenzidine (DAB) substrate kit according to standard methods in routine pathology. The positive cells were evaluated using ImageJ software and all measurements were made in three microscope fields randomly selected from each section.

Statistical analysis

Data is shown as mean ± standard deviation (SD). Statistical analysis was conducted using the SPSS (Chicago, IL, USA) and GraphPad Prism 8 (San Diego, CA) software. The significance of the variance between two or more groups was evaluated using student's t-test or ANOVA. $P<0.05$ had statistical significance.

Results

AS-IV inhibited cell proliferation and induced cell cycle arrest in pancreatic cells

To uncover the functional roles of AS-IV in pancreatic cells, Panc-1 and SW1990 cells were treated with a series of AS-IV concentrations for 24, 48 and 72h. The CCK-8 data showed that AS-IV inhibited the proliferation of Panc-1 and SW1990 cells in a dose-dependent manner (Fig. 1a). Colony formation assays and the EDU experiment also showed that the clonogenic ability of Panc-1and SW1990 cell lines were significantly decreased after being treated with differing concentrations of AS-VI (Fig. 1b and c).

The regulation of the cancer cell cycle plays a significant role in cancer cell proliferation. To explore the impact of AS-IV on pancreatic cancer cell cycle regulation, flow cytometry assays of Panc-1 and SW1990 cell lines were conducted. As shown in Fig. 2a, the data indicated that AS-IV induced an accumulation of cells in G1 phase, accompanied by a decrease of cells in the S phase and the effect was dose-dependent.

Previous research had shown that mammalian cells encode three D cyclins that coordinately function as allosteric regulators of cyclin-dependent kinase 4 (CDK4) and cyclin-dependent kinase 6 (CDK6) to regulate cell cycle transition from G1 to S phase[23]. These experimental results showed that the expression levels of cyclin D1, cyclin E and CDK4 decreased significantly after treatment with differing

concentrations of AS-IV. This data confirmed that AS-IV blocked the transition from G1 to S phase by decreasing the expression of cyclinD1, cyclin E and CDK4 (Fig. 4).

AS-IV suppressed the migration of Panc-1 and SW1990 cells

To evaluate the effects of AS-IV on pancreatic cells of Panc-1 and SW1990 migration, wound healing assays were performed. The results showed that AS-IV significantly suppressed the wound healing in the experiments with both Panc-1 and SW1990 cells line compared with the control group (Fig. 2b).

AS-IV promoted apoptosis and mitochondrial events in Panc-1 and SW1990 cells

Flow cytometry analysis, JC-1, TUNEL assay, caspase-3 and caspase-7 were used to explore the potential effect of AS-IV on the apoptosis of Pan-1 and SW1990 cells. As shown in Fig. 3, those results showed that after treatment with differing doses of AS-IV for 48h, the apoptosis rate of Panc-1 and SW1990 cells increased significantly in a dose-dependent manner.

The ratio of Bax/Bcl-2 in Panc-1 and SW1990 cells was increased by AS-IV (Fig. 4). Since mitochondria play a crucial role in the transduction of apoptotic signaling, the expression of mitochondria related apoptogenic proteins was further examined. The results showed that AS-IV increased the release of caspase 3 and caspase 7 in a dose-dependent manner and promoted the occurrence of apoptosis.

Previous research suggested that Bcl-2 family proteins could play a significant role in regulating cell growth and death. In this study western blot analysis proved that the expression of Bax protein was activated but the protein level of Bcl-2 were suppressed. Caspase-3, caspase-7, Bcl-2 and Bax are closely correlated with mitochondrial pathway mediated apoptosis. In this study, the expression of Bax/Bcl2, caspase-3 and caspase-7 were activated after treatment with differing concentrations of AS-IV (Fig. 4).

AS-IV suppressed pancreatic cancer tumor formation and growth in vivo

To confirm whether the AS-IV can affect tumorigenesis in vivo, Panc-1 cells were subcutaneously injected into four-week-old male nude mice separately. After 17 days post-inoculation treatment of AS-IV significantly suppressed the growth of pancreatic xenografts as shown in Fig. 5a and b. In addition, the expression levels of Bax, Bcl2, caspase3/7, cyclin D1, cyclin E and CDK4 in vivo were detected by western blot. The results showed that the expressions of Bcl2, caspase7, cyclin E and CDK4 were significantly reduced after AS-IV treatment in mice (Fig. 5c and d). Immunohistochemical staining indicated a decreased proliferative index Ki67 expression in treatment of AS-IV group (Fig. 5e).

Discussion

Pancreatic ductal adenocarcinoma is the most common type of pancreatic cancer[24]. Over the past few decades, with the advancement of surgical technology and the improvement of neoadjuvant therapy, remarkable progress has been made in treating pancreatic cancer. However, pancreatic carcinoma has high malignancy affecting the digestive system with insidious onset, invasive fast-growth, high recurrence rate and fatality. The treatments of pancreatic cancer are often refractory, but diagnosis is

often made at advanced stages of the disease, making few patients eligible for surgical intervention [4]. The development of new antineoplastic drugs will offer opportunities for the treatment of pancreatic cancer by new ‘traditional Chinese medicine’.

Several studies have shown that AS-IV could play an important role in controlling immunodeficiency and reducing the side effects of anti-tumor drugs [25]. Other studies have indicated that AS-IV showed antiproliferation and anti-migration activities in vitro and vivo and provides the experimental basis for preparing a new antitumor medicine[19, 26–28]. However, its pharmacological effect on pancreatic cancer remains unclear. This study demonstrated that AS-IV inhibited the proliferation, apoptosis and migration of pancreatic cells in vitro and inhibited the growth of pancreatic cancer xenograft tumor in vivo. Further studies indicated that cell apoptosis induction and cell cycle arrest were affected by AS-IV as part of its antitumor activity in pancreatic cancer. In this study, it was demonstrated that AS-IV significantly impaired the cell cycle in Panc-1 and SW1990 cells by inducing a G1 phase arrest and therefore a reduction in the percentage of cells progressing to S phase.

Invasion and metastasis are the main causes of tumor relapse and are regarded as important characteristics of pancreatic cancer. Previous studies have shown that AS-IV had a significant effect on tumor invasion and metastasis. Xu et al. found that AS-IV inhibited lung cancer metastasis by influencing the AMPK signaling pathway[29]. Studies have also shown that AS-IV can inhibit epithelial-mesenchymal transition (EMT) through the Akt/GSK-3β/β-catenin pathway, inhibiting the metastasis and invasion of pancreatic cancer cells[27]. To detect the effect of AS-IV on pancreatic cell metastasis, this study performed a wound healing assay that confirmed that AS-IV inhibited the metastasis of Panc-1 and SW1990 cells in a concentration-dependent manner in vitro.

Accumulating evidence suggests that the imbalance between apoptosis and proliferation plays a significant role in the occurrence and development of cancer[30]. This study proved that pancreatic cancer cells multiplication capacities were affected after treatment with varying concentrations AS-IV. Previous studies have shown that D-type cyclins D1, D2 and D3 associate with CDK4 or CDK6 to form heterodimeric complexes, which control the progression of G1 phase (G1-to-S phase) and initiate DNA replication[31–33]. The oncogenic capacity of cyclin D1 has been established in assorted studies. Cyclin D1 is essential for G1 progression and inhibiting the expression of cyclin D1 can block the cellular entry into S phase[34, 35]. In addition, cyclin D1 and cyclin E limit the rate progress of G1 in early and late G1 phase, respectively[34, 36]. In this study, flow cytometric analysis was used to explore the change of cell cycle after treatment with differing concentrations of AS-IV. The results showed that the cell cycle was arrested in the G1 phase in a dose-dependent manner and the western blot research further confirmed that the expression of cyclin D1, cyclin E and CDK4 significant decreased after treatment with differing doses of AS-IV. These results proved that AS-IV blocked the pancreatic cancer cell cycle from G1 phase to S phase and inhibited the expression of cyclin D1, cyclin E and CDK4.

The blockage of cell apoptosis is one of the important mechanisms needed to control abnormal cell proliferation in carcinogenesis. In this study, TUNEL, JC-1 and flow cytometric analysis were used to

detect Panc-1 and SW1990 cells apoptosis rate after different doses of AS-IV in vitro. Those experiments also found that with increases in the dose of AS-IV, the rate of apoptosis also increases.

Some past studies have found that Bax, Bcl2 and caspases3/7are associated with the mitochondrial apoptosis pathway[37]. Of these, Bcl2 is an antiapoptotic molecule that inhibits cell apoptosis by reducing reactive oxygen species[38] and it has been also observed that the down-regulation of Bcl2 or over-expression of Bax can promote tumor cell apoptosis[39, 40]. Bax promotes cell apoptosis by enhancing the permeability of mitochondria and mediating the synthesis of apoptotic complexes[41–43]. This study found that AS-IV treatment increased the ratio of Bax/Bcl-2, which further demonstrated the role of the mitochondria in AS-IV induced apoptosis. Bcl2 and Bax regulates the release of caspases by changing mitochondrial membrane potential and permeability. Several studies strongly suggested that caspase-3 and caspase-7 have critical roles in apoptosis cell death and in normal development[44, 45]. They can trigger the cascade of cell apoptosis and therefore play pivotal roles in regulating apoptosis[46–48]. In this study, the results of western blot experiments showed that AS-IV elicited the expression of Bax, caspase-3 and caspase-7 and down-regulated Bcl-2 synthesis in a dose dependent manner.

Conclusion

It is hypothesized from this study that the potential mechanism of AS-IV inducing apoptosis of pancreatic cancer cells may be the activation of the Bcl2/Bax/Caspase-3/Caspase-7 signaling pathway. This study confirmed that AS-IV inhibits the pancreatic cell proliferation in vivo and vitro by blocking the cell cycle and inducing apoptosis. However, there are still limitations that make it impossible to be absolutely certain and the underlying regulatory mechanisms of AS-IV in pancreatic cancer need to be further investigated.

Declarations

- **Author Declarations**

Ethics approval and consent to participate

Animal experiments were conducted in accordance with the guidelines for animal care and use issued by the National Laboratory of the United States, and the experimental program was approved by the Animal Care and Use Committee of South China University (2019111008012).

Consent for publication

All authors have read and consented to publish.

Availability of data and materials

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

Competing interests

The authors declare no conflicts of interest.

Funding

This work was supported by the Health Commission of Hunan Province Research Project (Grant number 20200284), Health Commission of Hunan Province Research Project (Grant number 20200430). Key Project of Science and Technology Plan of Hunan Provincial Health Commission (Grant number 20201919). Natural Science Foundation of Hunan Province (Grant number 2565). Hunan Province Clinical Medical Technology Innovation Guidance Project (Grant number 2020SK51817).

Author Contributions

Xiaohua Lei and Guodong Cheng conceived and designed the work; Nanxiang Zhang and Chengming Ding coordinated technical support and funding; Chuanfu Li and Pengyu Zhou wrote the manuscript; Xiaohua Lei, Weiping Tang and Fan Wu performed the experiments and collected the samples; Weiping Tang and Nanxiang Zhang acquired, analyzed, and interpreted the data; Shuo Qi and Guodong Chen participated in data collection and analysis. All authors read and approved the final manuscript. All authors declare that this manuscript has not been submitted for possible publication to another journal.

Acknowledgments

We thank International Science Editing (http://www.internationalscienceediting.com) for editing this manuscript.

• Compliance with Ethical Standards

Disclosure of potential conflicts of interest

The authors declare no conflicts of interest.

Research involving Human Participants and/or Animals

Animal experiments were conducted in accordance with the guidelines for animal care and use issued by the National Laboratory of the United States, and the experimental program was approved by the Animal Care and Use Committee of South China University (2019111008012). The authors declare that this article does not contain any studies involving the use of human participants.

Informed consent

Not applicable.

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Figures

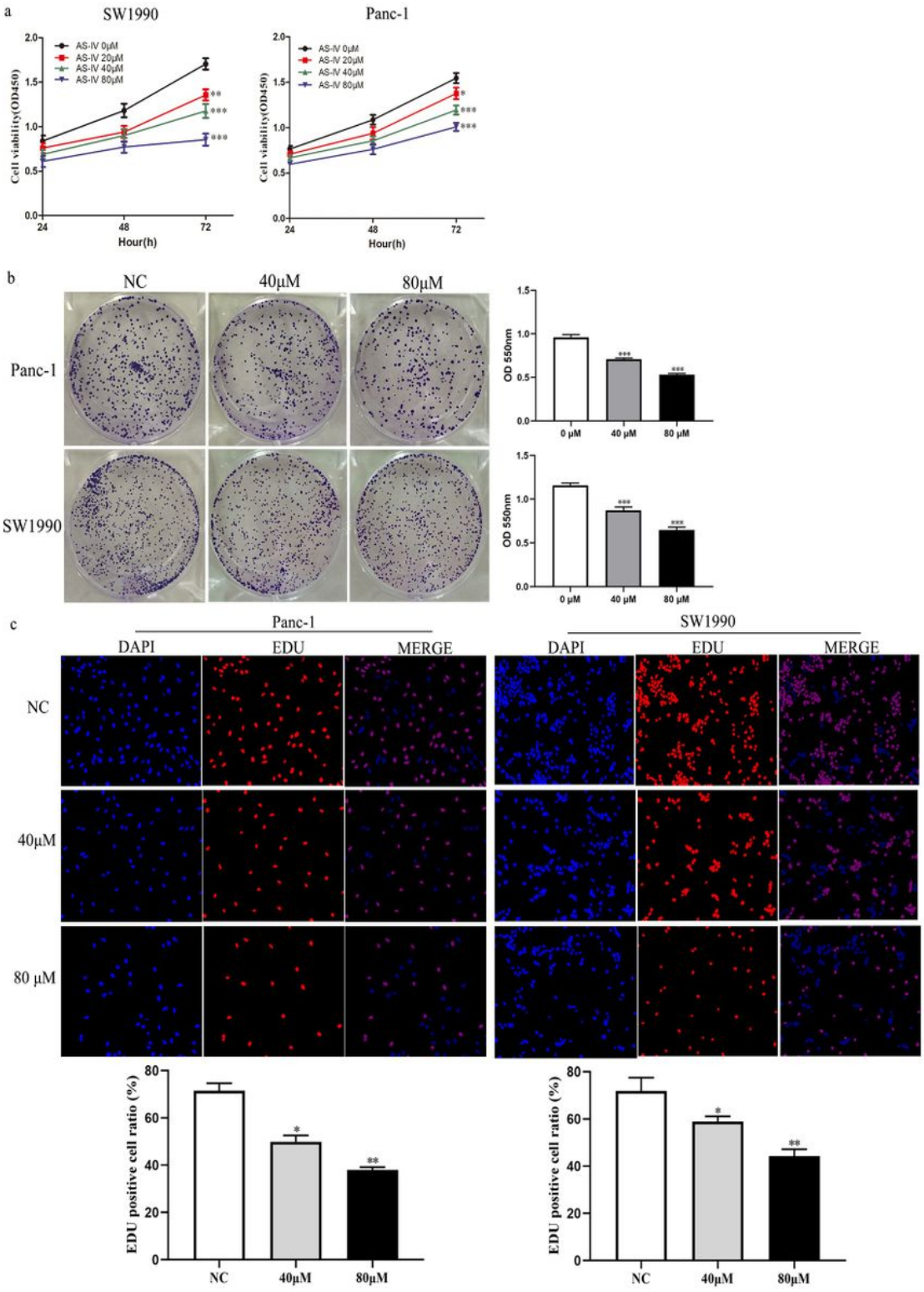

Figure 1

AS-IV inhibits cell proliferation in Panc-1 and SW1990 cells. (A) Panc-1 and SW1990 cells proliferation assayed by CCK-8 Kit after treatment with different doses of AS-IV. (B) The colony formation capacity of Panc-1 and SW1990 cells measured by clonogenic assay after AS-IV treatment. (C) EDU experiment measured the proliferation ability of Panc-1 and SW1990 cells after AS-IV treatment. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

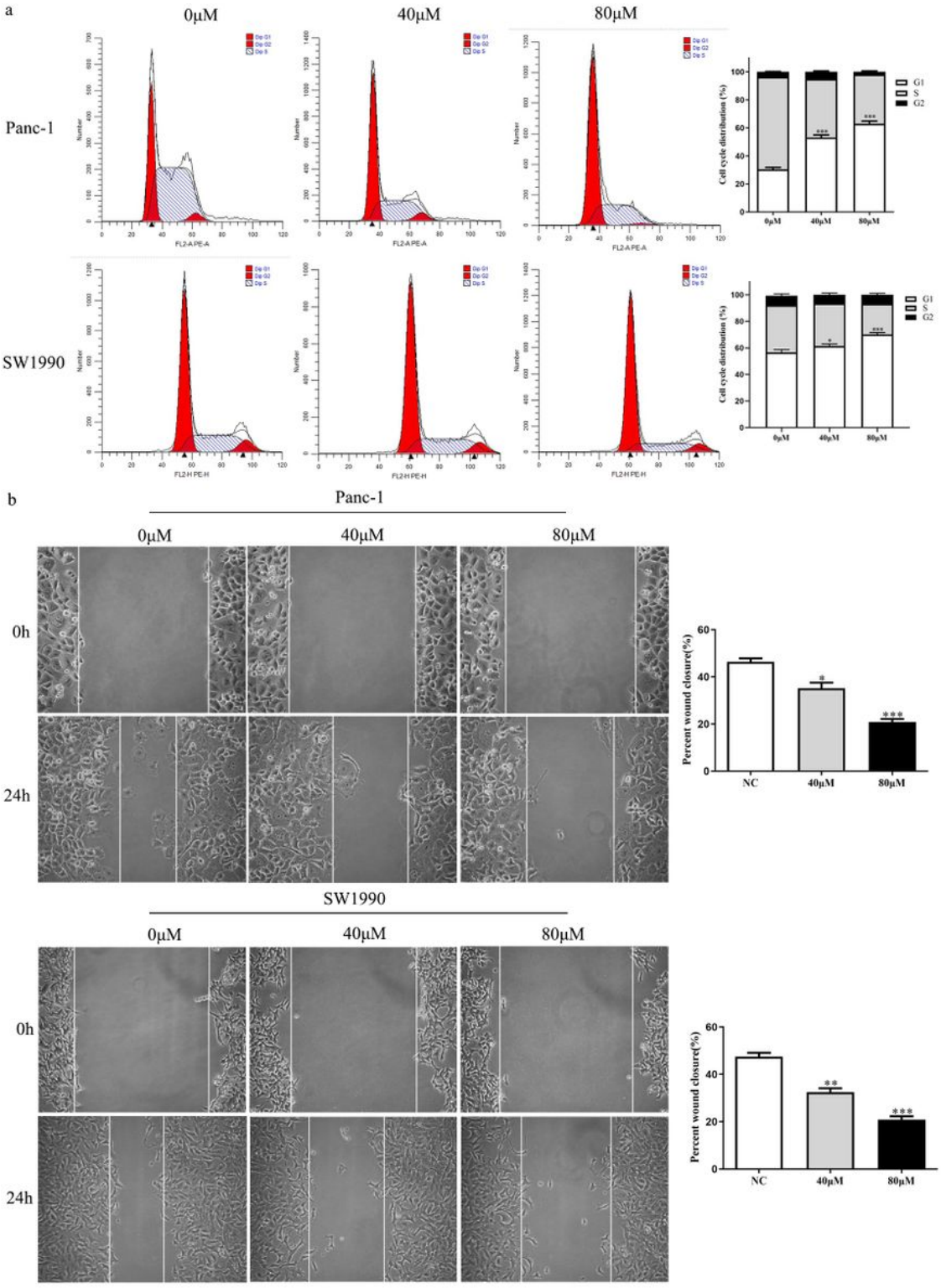

Figure 2

AS-IV induced cell cycle arrest and decreased cell migration potential in Panc-1 and SW1990 cells. (a) The populations of cells were stained with PI to determine the percentages of cells in each phase by flow cytometry. (b) The migration of Panc-1 and SW1990 cells after treated with AS-IV examined by wound healing assay. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

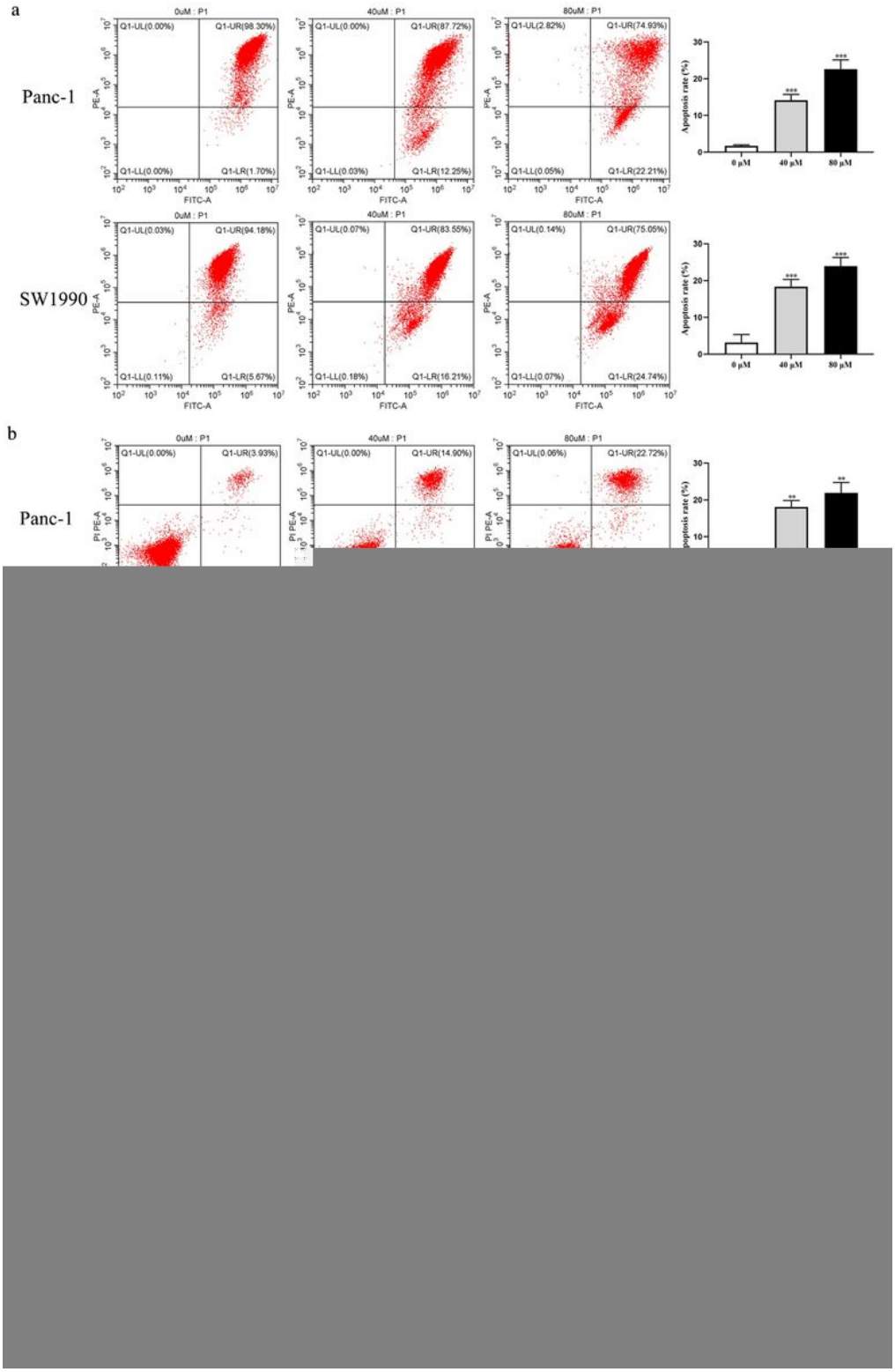

Figure 3

AS-IV promoted apoptosis in Panc-1 and SW1990 cells. (a) Panc-1 and SW1990 cells apoptosis rate measured by JC-1. (b) Panc-1 and SW1990 cells apoptosis rate measured by flow cytometry. (c) The apoptosis of Panc-1 and SW1990 cells evaluated by TUNEL staining assay after AS-IV treatment. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

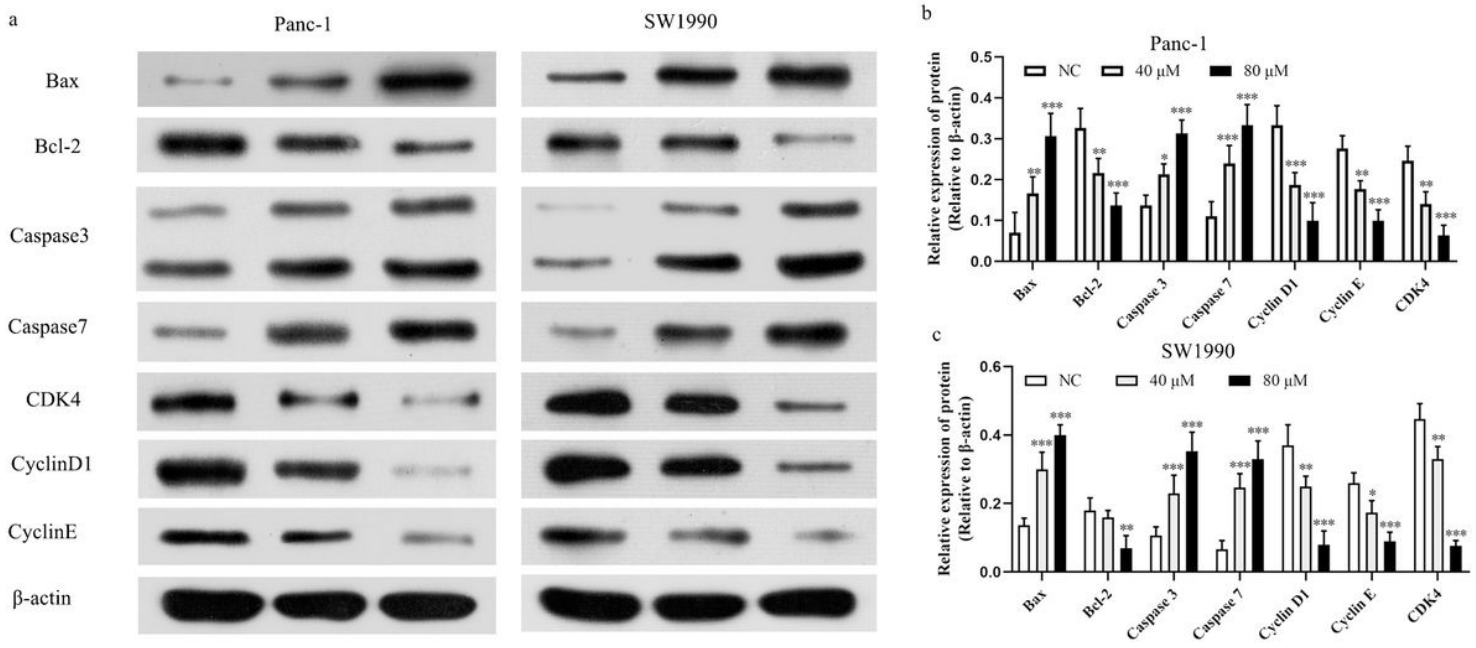

Figure 4

AS-IV promoted apoptosis and blocked cell cycle in Panc-1 and SW1990 cells. (a) The expressions of Bax, Bcl2, caspase3/7, cyclin D1, cyclinE and CDK4 detected by western blot. (b) Quantitation of the expressions of Bax, Bcl2, caspase3/7, cyclin D1, cyclinE and CDK4 in Panc-1 cells. (c) Quantitation of the expressions of Bax, Bcl2, caspase3/7, cyclin D1, cyclinE and CDK4 in Panc-1 cells. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

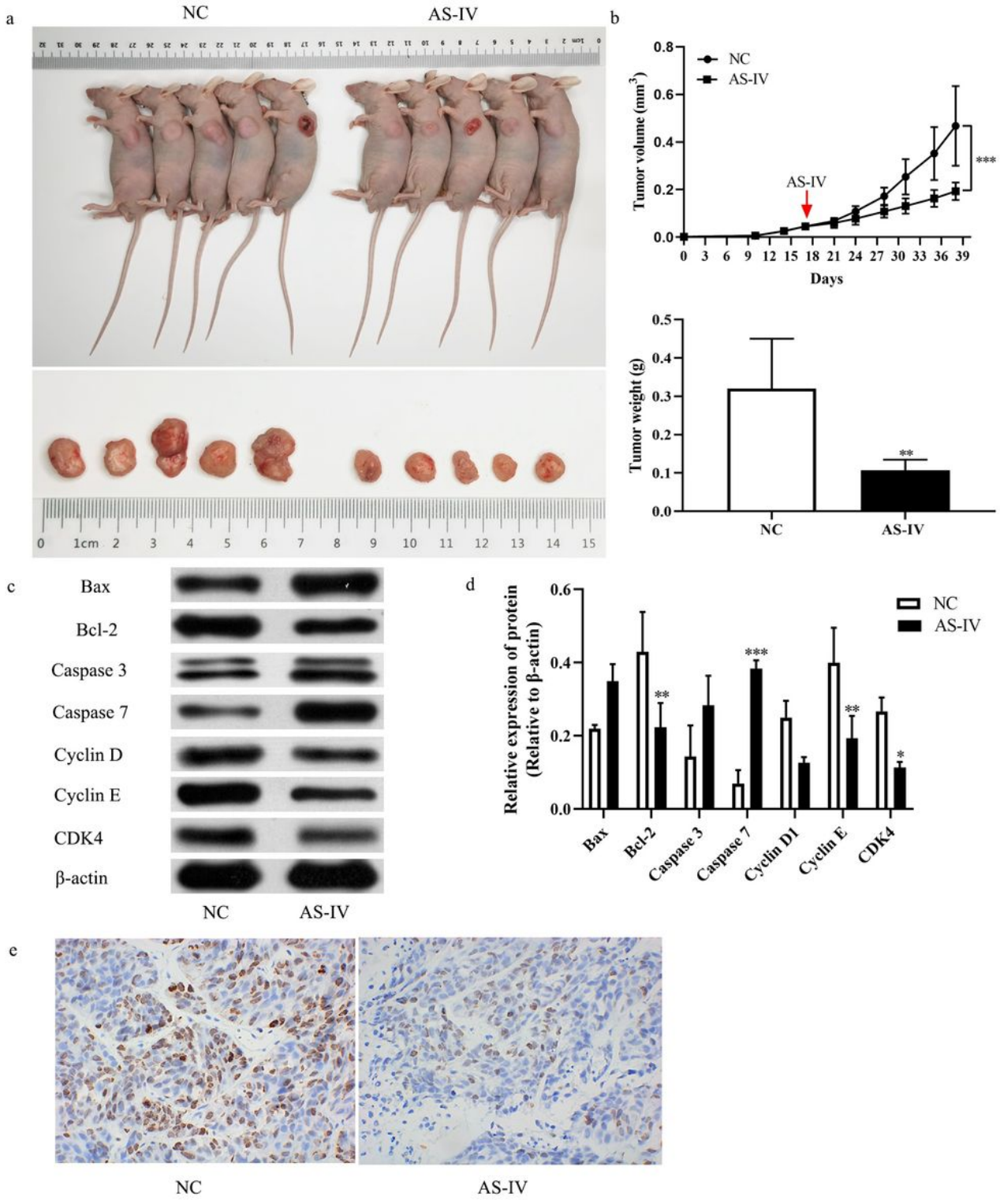

Figure 5

AS-IV induced apoptosis and blocked cell cycle to inhibit tumor growth in vivo. Panc-1 cells were subcutaneously injected into nude mice separately, followed by intragastrical administered with AS-IV (0.1 mg/10g/day) for consecutive 21 days. (a) Tumor formation in the NC and AS-IV groups of nude mice (N=5). (b) The tumor volumes were measured at different time points. The weights of isolated tumor tissues were measured at 38 days post Panc-1 cell injection. (c) The protein level of Bax, Bcl2,

caspase3/7, cyclin D1, cyclinE and CDK4 in xenograft tissues detected by western blot. (d) Quantitation of Fig 6C. (e) The expression level of Ki67 was detected by immunohistochemical staining. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$