# AKT inhibitor SC66 inhibits proliferation and induces apoptosis in human glioblastoma through down-regulating AKT/β-catenin pathway

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**Primary research**

**Keywords:** SC66, GBM, proliferation, apoptosis, migration, invasion, AKT/β-catenin pathway

**Posted Date:** March 16th, 2020

**DOI:** https://doi.org/10.21203/rs.3.rs-17363/v1



**Version of Record:** A version of this preprint was published at Frontiers in Pharmacology on July 31st, 2020. See the published version at https://doi.org/10.3389/fphar.2020.01102.

# Abstract

Background  Glioblastoma multiforme (GBM) is the most common and deadliest type of primary malignant tumor in the adult central nervous system. Temozolomide (TMZ) has limited effectiveness on glioblastoma, so it is urgent to develop new drugs to improve the prognosis of patients. SC66, a novel AKT inhibitor, was reported to exert antiproliferative activity in many types of cancer cells. However, it remains unclear whether SC66 has antitumor effects in GBM.

Methods : Cell count kits-8(CCK8) assay, EdU-DNA incorporation assay and colony formation assay were used to evaluate cell proliferation of U87 and U251 cells.Wound -healing assay and transwell assay were used to detect migration and invasion. The cell cycle and apoptosis were detected by flow cytometry. Finally, xenograft mouse model was established to demonstrate the antitumor effect of SC66 in vitro.

Results :SC66 obviously suppressed U87 and U251 cells proliferation in a dose-dependent manner. Additionally, SC66 treatment was found to significantly inhibit the invasion and migration of GBM cells as detected by Transwell invasion and wound healing assays. Moreover, treatment of SC66 induced GBM cells apoptosis through up-regulating BAX, Cleaved -caspase3 and down-regulating Bcl-2. SC66 also could arrested cell cycle in G0/G1 phase by decreasing cyclin D1.Furthermore, the results of western blot showed that SC66 significantly reduced level of phosphorylation of AKT, p-GSK-3β and β-catenin, while no change was observed in level of AKT and GSK3β.Then a GSK3-inhibitor,IM-12 was used and IM-12 could significantly restored proliferation, migration and invasion of glioma cells treated with SC66.Meanwhile, SC66 showed significantly suppressed the tumorigenicity compared to the control group in the xenograft mouse model.

Conclusion : AKT inhibitor SC66 exerted powerful antitumor activity via down-regulating AKT/β-catenin pathways in vitro and in vivo. It might be as a potential chemotherapy drug to improve the therapeutic efficacy of GBM treatments.


# Background

Glioblastoma multiforme (GBM) is the most prevailing, malignant and lethal primary tumor in the adult central nervous system, accounting for approximately 50% of all gliomas [1]. The standard strategy of GBM includes surgical resection, radiotherapy and adjuvant Temozolomide (TMZ) chemotherapy. TMZ was considered to be the most effective drug for treating glioblastoma. In 2005, TMZ treatment in phase III clinical trials was shown to increase the median survival from 12.1 to 14.6 months and the two-year survival rate from 10 to 26.5%, as compared with postoperative radiotherapy alone in GBM patients [ 2 3]. Unfortunately, at least 50% of GBM patients do not respond to TMZ, and prone to acquire resistance after treatment with TMZ, which limit TMZ's effectiveness [4–5]. Therefore, it is urgent to develop new drugs and explore new therapeutic strategies to improve the prognosis of GBM patients.

The phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway is one of the most frequently activated in the majority of human cancers. AKT is considered to be the hub protein of PI3K/AKT signaling

pathway, regulating the activity of over 100 downstream substrates [6–8]. AKT is a serine/threonine protein kinase, plays an important role in cell malignant transformation after activating by phosphorylation. AKT activation is considered to be a hallmark of a variety of human cancers, which contributes to cancer development by inhibiting apoptosis, increasing cell proliferation, and accelerating oncogenic mutation rates [9–10]. Successful inhibition of AKT may have a significant antineoplastic effect, therefore, targeting AKT is an important field of oncological therapy.

SC66 is a novel allosteric AKT inhibitor, which promotes AKT ubiquitination and deactivation by directly interfering with the pleckstrin homology (PH) domain binding to PIP3[11]. SC66 has been demonstrated to promote cell death in cervical cancer through disruption of mTOR signaling and glucose uptake [12]. SC66 combined with cisplatin showed a more effective inhibition of tumor growth in the xenograft model of ovarian cancer mice [9]. In addition, SC66 suppressed colon cancer cell growth and induced apoptosis [13]. These studies indicated that SC66 might be a potential antitumor drug. However, whether SC66 can exert antitumor activity in GBM cells is indistinct.

In this study, we investigated the antitumor activity of SC66 in GBM cells in vitro and in vivo, and demonstrated that SC66 effectively suppressed the cell proliferation, invasion and migration. It also arrested the cell cycle in G0/G1 phase and induced cell apoptosis. Furthermore, SC66 displayed potential tumor growth reduction in nude mouse model of GBM. In summary, SC66 might be a new drug for suppressing GBM progression.

# Methods

### Drugs and antibodies

AKT inhibitor SC66 was purchased form MedChem Express ((HY-19832, Shanghai, China) and dissolved in dimethyl sulfoxide (DMSO), which was purchased form Servicebio (G5051, Wuhan, China). The GS3K-β inhibitor IM-12was obtained from Selleck (S7566, USA). The antibodies included the following: anti-Phospho-AKT (#4060, Cell Signaling Technology, USA), anti-AKT (#4691, Cell Signaling Technology), anti-Phospho-GSK-3β (#9323,Cell Signaling Technology),anti-GSK-3β (#12456,Cell Signaling Technology)，anti-Phospho-β-catenin (#9561,Cell Signaling Technology), anti-cleaved-caspase3(ab32042, Abcam, UK), anti-β-catenin (ab32572, Abcam，anti-GAPDH (60004-1-Ig,Proteintech,Wuhan, China),anti-Snai1（13099-1-AP,Proteintech），anti -BAX (50599-2-Ig,Proteintech),anti-Bcl-2 ( 12789-1-AP, Proteintech),anti-Cyclin D1(60186-1-Ig, Proteintech),anti-caspase3 (19677-1-AP,Proteintech),anti-MMP2(10373-2-AP,Proteintech),anti-Vimentin ( sc-6260 Santa Cruz Biotechnology, USA)

### Cell culture

Humans glioblastoma cell lines (U87 and U251) were purchased from the Cell Bank of the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in high-glucose DMEM (Genom, Hangzhou, China) supplemented with 10% foetal bovine serum

(FBS) (Gibco, Thermo Fisher Scientific, USA) and 1% penicillin/streptomycin (Genom, Hangzhou, China). These cells were incubated at 37°C in a humidified atmosphere containing 5% carbon dioxide.

### Cell viability assay

The ability of SC66 exerts antiproliferative activity was measured with Cell Counting Kit-8 (CCK-8) (Dojindo, Shanghai, China) according to the manufacturer's instructions. U87 and U251 cells were planted into 96-well plate (5000 cells/well) and treated with 0,5,10,15,20,25,30umol/L of SC66 for 24 hours. Then 10u per well of CCK8 was added and then incubated at 37°C for 1 hour. The absorbance value (OD) was measured with a spectrophotometric plate reader at 450 nm. Three independent assays were carried out.

### Colony formation assay

An approximate number of 500 cells per well were planted into 6-well plate and cultured with 2ml DMEM containing 10%FBS.Then, the cells were treated with 0,6,10 and 15umol/L of SC66 for two to three weeks until there was significant single-cell colony formation. The cells were fixed in 4% paraformaldehyde for 30 minutes and then stained with 0.5% crystal violet for 15 minutes. The counts of colonies were counted using Image J software (National Institutes of Health, Bethesda, MD, USA).

### 5-Ethynyl-2'-deoxyuridine (EdU) incorporation assay.

Approximately $5x10^3$cells were seeded into 96-well plates containing 100ul DMEM and treated with 0,6,10,15umol/L SC66 for 24 hours the next day. The cell growth was measured using the Cell -Light Edu imaging detecting kit (RiboBio, Guangzhou, China) according to the manufacturer's protocol. Cells were cultured in medium supplement with 50uM EdU for 2 hours, and fixed in 4% paraformaldehyde for 30 minutes. Subsequently, 100ul of 1X Apollo® reaction cocktail was added to each well and incubated at room temperature for 30 minutes, and then the DNA was stained with 1X Hoechst 33342 in dark for 30 minutes. Fluorescence images of the Hoechst 33342 and EdU were visualized using a fluorescence microscope (Olympus BX51; Olympus, Tokyo, Japan). The number of DAPI and EdU-positive cells was quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

### Wound -healing assay

Cells were seeded into 6-well plates and growth until 70% confluency. A wound was created by scratching the monolayer cell gently and slowly with a yellow 200-ul pipette tip in each well. Whereafter, the cells were gently washed twice with PBS and cultured with DMEM supplement with serum-free medium. Meanwhile ,0 or 10 uM of SC66 was added into DMEM at 0 and 24 hours respectively. The images were captured under a microscope (Olympus BX51; Olympus, Tokyo, Japan).

### Invasion assay

Invasion assay was performed using a Transwell chamber with an 8.0-μm pore polycarbonate

membrane. The polycarbonate Transwell filters coated with Matrigel, a number of $8x10^3$cells treated with 0,6,10 or 15uM of SC66 for 24 hours were seeded into the top chambers. Simultaneously, 200 ul serum-free DMEM was added into the top chambers, and 600 ul DMEM supplement with 10%FBS was added into the bottom as a chemoattractant. The cells was incubated at 37℃ for 36 hours and fixed in 4% paraformaldehyde for 30 minutes. The non-invasive cells in the top chambers were moved with cotton swabs, and the cells on the lower side of membrane surface were stained with 0.5% crystal violet for 15 minutes. Air dried and the results were obtained under a microscope (Olympus BX51; Olympus, Tokyo, Japan).

### Flow cytometry analysis of the cell cycle distribution

Cells were harvested with 0.25 trypsin after treated with 0.6,10 or 15 of SC66 for 24 hours. Next, cells were fixed in 70% cold ethanol overnight at −20 °C. Then the cells were washed twice with PBS and incubated with PBS containing RNAase for 30 minutes. Eventually, the cells stained with

propidium iodide (PI) and incubated in dark for 15 minutes. The cells were analysed by BD FACSAria flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and the data were quantified using ModFit LT 5.0 software (http://www.vsh.com/products/mflt/mfFeatures.asp; Verity Software House, Topsham, ME, USA).

### Flow cytometric analysis of apoptosis

Cells in each group was plated in 6-well plates, and harvested the cells after treated with 0,6,10 or 15 uM of SC66 for 24 hours. The cells were suspended in 1ml 1X binding buffer, and stained with 5 μl of PE Annexin V and 5 μl of 7-amino-actinomycin (7-ADD) for 15 minutes at room temperature away from light. For each experiment ,$2x10^5$ cells were analyzed using BD FACSAria flow cytomer (BD Biosciences, Franklin Lakes, NJ, USA).

### Western blot analysis

Cells treated with 0,6,10 or 15 uM of SC66 for 24 hours, and then lysed in RIPA buffer (Beyotime, Shanghai, China) supplemented with protease inhibitors (Roche, Indianapolis, IN) and phosphatase inhibitors (Applygen, Beijing, China) at 4 °C for 30 min. The cell lysate was centrifuged at $1.2x10^5$ rpm for 15 minutes at 4℃ and protein concentrations were measured by BCA method (Beyotime, shanghai, China).The protein was loaded onto a 10% or 12% SDS-PAGE and transferred to a PVDF membrane (Millipore, Germeny). After block with 5% skim milk powder for 1 hour, membranes were immunoblotted with primary antibodies to Phospho-AKT,Phospho-GSK-3β、Phospho-β-catenin、AKT、GSK-3β、β-catenin、GAPDH、BAX、Bcl-2,cleaved-caspase3,casapse3,snai1,MMP2,vimtenin and Cyclin D1 with an appropriate dilution concentration overnight at 4℃. Subsequently, the membranes were incubated with Alex Fluor 680/790-labelled secondary antibodies (LI-COR Bioscience, USA) for 1hour. The bands were captured using a LI-COR Odyssey Infrared Imaging System (LI-COR Biosciences).

### Immunofluorescence staining of cells

The sterilized slides were placed in a 6-well plate, and approximately $3x10^4$ cells were planted. After 12 hours of sc66 treatment, they were fixed in 4% paraformaldehyde for half an hour. Permeabilized with 0.5% Triton X-100 (Amresco, USA) for 20 min, and blocked with 5% bull serum albumin (Amresco, USA) for 30 min at room temperature. Cells were incubated with primary antibodies overnight at 4℃ according to the manufacturer's recommended concentration. The next day, cells were washed and then incubated with FITC-labeled goat anti-rabbit IgG (1:50 dilution; Servicebio, China) in dark for 1 hour at room temperature. Subsequently, cells nuclei were counterstained with diamidino-phenyl-indole (DAPI) (ANT046, Antgene,China) in dark for 5minutes.The images were visualized under a fully automatic Microscope (Olympus BX63; Olympus, Tokyo, Japan).

### Immunohistochemistry

The tissues were fixed in 4% paraformaldehyde and embedded in paraffin. The sections were deparaffinized, hydrated and antigen repaired with 10mM sodium citrate (pH, 6.0). After removing endogenous peroxidase by using 3% $H_2O_2$. Then, the samples were blocked with 5% bovine serum albumin (Amresco, USA) for 30 minutes. The samples were incubated with primary antibody overnight at 4 °C according to the manufacturer's recommended concentration. The reactions were visualized using a 3,3'-diaminobenzidine visualization kit (Servicebio,China) and counterstained with hematoxylin to visualize nuclei for 1minute.The images were visualized under a microscope (Olympus BX51; Olympus, Tokyo, Japan)

### T-cell factor/lymphoid enhancer factor (TCF/LEF) luciferase reporter assay

Cells were seeded in 6-well plates and treated with 0 or 6uM of SC66 for 24hours. The cells were harvested after co-transfection with TCF/LEF1 luciferase reporter plasmid and pGMLR-TK luciferase reporter plasmid (Yeasen Biotech Co, Ltd. Shanghai, China) for 48 hours. The Renilla and firefly luciferase activities was measured by the Dual Luciferase Reporter Gene Assay Kit (Yeasen Biotech Co., Ltd.Shanghai, China) according to manufacturer’s protocol.

### Animal experiments

All animal experiments were approved by the Institutional Animal Care and Use Committee at Renmin Hospital of Wuhan University and complied with the internationally recognized Animal Research: Reporting of In vivo Experiments guideline. U87 cells were resuspended in PBS at a concentration of $2x10^7$/ml and subcutaneously injected a 100 μL cell suspension into the left armpit of 5-week-old male Balb/c nude mice. After 15 days, when the tumor volume reached about 100 mm3, the mice were randomly divided into two groups (n = 8) and treated with SC66 at 25 mg/kg by i.p. injection every other 3 days for 18 days. Meanwhile, the control group was intraperitoneally injected with DMSO of the same volume. In addition, we measured tumor volume every 3 days. Tumor volume was calculated using the formula $V = L \times W^2 \times 1/2$ (V, volume; L, length of tumor;

W, width of tumor). Two days after the last drug treatment, the mice were killed and the tumor was stripped. Half of each tumor was fixed with 4% paraformaldehyde and paraffin-embedded (FFPE) sections, the other half was extracted for western blot.

**Statistical analysis**.

Statistical analyses were performed using GraphPad Prism 6.0 software. All experimental results were expressed as the mean ± SD. One-way ANOVA was used to measure differences between various groups. $P<0.05$ was considered statistically significant. Statistical significance was indicated in the figures as follows: *$P<0.05$, **$P<0.01$ and ***$P<0.001$.

# Results

### SC66 suppresses the proliferation of human GBM cells

SC66 is an allosteric inhibitor which displays a dual-inhibitory function toward AKT activity. Its chemical formula is shown in Fig.1A.To investigate the antiproliferative activity of SC66 against GBM cells, U87 and U251 cells viability were detected using Cell Counting Kit-8 (CCK-8). The results showed that SC66 inhibited the proliferation of U87 and U251 cells in a dose-dependent manner. The IC50 values of SC66 in U87 and U251 cells were 10 umol/L and 12 umol/L respectively (Fig. 1B,1C).Besides, the results of colony formation assay also indicated that lesser and smaller colonies were formed as the treatment concentrations of SC66 increased in both GBM cell lines (0,6,10 and 15umol/L)(Fig.1D). In addition, results of EdU-DNA synthesis assay showed that SC66 induced a prominent decrease in the percent of EdU-positive cells in U87and U251 cells (Fig.1E). These results indicated SC66 inhibited the growth of GBM cells in a dose-dependent manner.

### SC66 inhibits the EMT of U87 and U251 cells

We stained the cytoskeleton with phalloidin, SC66 treatment obviously altered the morphology of U87 and U251 cells, and the cytoskeleton was also damaged (Fig.2F). Epithelial-to-mesenchymal transition (EMT) is a biological process that allows epithelial cells to obtain mesenchymal phenotypes. During this process, epithelial cells undergo morphological and biochemical changes to reorganize their cytoskeleton [14].

Furthermore, EMT was confirmed to be important for promoting tumor progression and metastasis in various cancers [15]. Therefore, we hypothesized that SC66 might inhibit EMT processes. In order to further verify our conjecture, wound healing and Transwell assays were performed to assess the inhibitory effect of SC66 on migration and invasion of GBM cells. As shown in Fig.2A, compared to SC66 -treated group, the untreated group demonstrated a rapid increase in the gap distance. The wound healing percentage

also remarkably higher in untreated group after 48 hours (Fig.2B). The result revealed that SC66 impeded the migration of GBM cells in comparison to control SC66-free cells. Similarly, Transwell assays indicated a dose-dependent manner decrease in invasion of SC66-treated GBM cells (Fig.2C-2D). Meanwhile, western blotting showed that the expression of invasion-related protein Matrix metalloproteinase 2(MMP2), snail1 and vimentin was decreased after SC66 treatment (Fig.2E). Simultaneously, immunofluorescence assays demonstrated that the expression of Vimentin and Snai1were reduced in U87 and U251cells after SC66 treatment (Fig.2G-2H). These changes in Vimentin and Snail indicated that a mesenchymal-to-epithelial transition (MET) occurred in U87 and U251cells after SC66 treatment, which is reverse process of EMT. All of results in vitro suggested that SC66 could significantly block EMT of U87 and U25.

### SC66 arrests cell cycle at G0/G1 phase in U87and U251cells

One of the reasons for the infinite proliferation of tumor cells is the deregulation of the cell cycle. We hypothesized that SC66 inhibits cell proliferation of U87 and U251 by blocking the cell cycle. In our study, flow cytometry analysis was utilized to verify whether SC66 affects cell cycle distribution. The results revealed that SC66 treatment resulted in accumulation of cell cycle in G0/G1 phase (Fig .3A-3B). In order to explain

underlying mechanism of G0/G1 arrest induced by SC66, we investigated the expression of key cell cycle regulatory proteins by western blot, the expression of Cyclin D1 was decreased with the increase of the treatment dose of SC66 (Fig.3E-3F).

### SC66 induces cell apoptosis in U87 and U251 cells

We further investigated the effect of SC66 on cell apoptosis. Flow cytometry analysis results demonstrated that a dose-dependent manner increased in U87 and U251 apoptosis cells after SC66 treatment for 24 hours (Fig .4A -4B). Further western bolt analysis indicated that Bax and cleaved caspase3 expression levels were strikingly decreased, and the expression of Bcl2 was remarkably increased (Fig.4C). We evaluated cell apoptosis by performing TUNEL staining assays, and the results showed that TUNEL-positive cells proportion was higher after SC66 treatment (Fig .4D-4E). In addition, immunofluorescence staining assays also demonstrated that Bcl 2 expression level was reduced and cleaved caspase 3 was upregulated (Fig .4F-4G) . These results suggested that SC66 induced cells apoptosis in U87and U251cells.

### SC66 inhibits AKT/β-catenin signaling pathways in U87 and U251 cells

SC66 is a novel small molecule allosteric inhibitor. To further confirm whether SC66 exerts anti-tumor effects by affecting the AKT signaling pathway, the related proteins of AKT pathway was verified by western blot, including AKT, GSK3-β, β-catenin and their corresponding phosphorylated forms. The results demonstrated that p-AKT, p-GSK-3βand β-catenin were remarkably reduced with a dose- dependent

manner, and p-β-catenin was notably accumulated. However, total AKT and GSK3-β expression were not significant change (Fig.5A). Furthermore, immunofluorescence assay revealed that nuclear transfer level of β-catenin was remarkably decreased after SC66 treatment compared with control group (Fig.5B). TCF/LEF luciferase reports showed that the TCF/LEF luciferase activity in U87 and U251 cells were significantly reduced after SC66 treatment (Fig.5C). The results may demonstrate that SC66 inhibits AKT/β-catenin signaling pathways in U87 and U251 cells.

**Elevating β-catenin activity rescued SC66 inhibition-mediated GBM cell proliferation and metastasis.**

In order to further confirm SC66 exerts antineoplastic effect through the AKT/β-catenin signaling pathways. IM12 which an inhibitor of GSK-3β that has been confirmed to enhances canonical Wan/β-catenin signaling [16].IM-12 was added to cells after 24 hours of SC66 treatment. CCK 8 assays showed that IM12 rescued cell proliferation ability inhibited by SC66, and colony formation assay also confirmed the same results (Fig. 6A -6C). In addition to, Transwell assays indicated the SC66 inhibited function of migration and invasion was partly abrogated after IM12 elevated Wan/β-catenin signaling in U87 and U251cells (Fig .6D-6E). These results indicated that SC66 mediated β-catenin signaling activation to inhibit GBM malignancy.

**SC66 suppressed tumor growth in nude mouse models**

To assess the anti-tumor ability of SC66 in vivo, we established a U87 xenograft tumor model in nude mouse. The results demonstrated that SC66 remarkably suppressed tumor growth, as tumor weights and volume were notably smaller in SC66 treatment group than control group (Fig.7A-7C). Furthermore, western blot results showed that β-catenin,P-AKT,P-GSK-3β、Cyclin D1,Vimentin,and Bcl-2 protein expression in xenograft tumor were obviously reduced in SC66 treatment group (Fig 7D). Moreover, immunohistochemical staining revealed that the expression of P-AKT、P-GSK-3β、β-catenin,Snai1,Vimentin and Bcl-2 were remarkably decreased, while the expression of BAX, and P-β-catenin was significantly increased（Fig.7E）.

# Discussion

Gliomas are the most common malignant brain tumors in adult, and it is estimated that the annual incidence in the United States is 6.6 per 100,000 individuals. About half of newly diagnosed gliomas are classified as glioblastoma (GBM), which is the most aggressive subtype with the worst prognosis among all gliomas [17]. Therapeutic options are limited, including surgery and treatment with radiotherapy plus TMZ chemotherapy. Due to benefits from TMZ chemotherapy, survival of GBM patients prolong for ~ 2.5 months [18]. Nevertheless, the median survival time of GBM patients is less than 16 months even with optimal treatments [19]., more effective drugs were needed to improve prognosis.

Previous studies have shown that AKT activation can stimulated cell proliferation and affected cell cycle regulation through multiple downstream targets. AKT dependent phosphorylation of target proteins such as GSK3 may promote cell proliferation by regulating the stability and synthesis of proteins involved in

cell-cycle entry. GSK3-mediated phosphorylation of cyclin D and cyclin E, which play a significant role in the transformation of G1 to -S phase cell-cycle transition [20]. In this study, we demonstrated that SC66 notably inhibited U87 and U251 cells proliferation in a dose-dependent manner. Meanwhile, SC66 suppressed tumor growth also was confirmed in nude mouse models. Nevertheless, flow cytometry analysis showed that treatment with SC66 induced remarkably cell cycle arrest at the G0/G1 phase in U87 and U251 cells. The results of western blot proved that cell cycle related protein cyclin D1 was downregulated after SC66 treatment. Based on these results, SC66 may arrest the cell cycle in G0/G1phase to exert antitumor effects.

The EMT is a process during which cells lose their epithelial characteristics and obtain characteristics of mesenchymal cell phenotypes, as well as upregulated migratory and invasive properties. The EMT converts innocent tumors into invasive, metastatic tumors and exerts a vital role in regulating tumor progression and metastasis [21–22]. In this study,we demonstrated that the expression of EMT related protein snail1 and vimentin were decreased after SC66 treatment. Treatment with SC66 obviously damaged the architecture of cytoskeleton. Moreover, we confirmed the GBM cell's migration and invasion capacities were notably downregulated after SC66 treatment. These data indicate that SC66 suppressed EMT-mediated cell migration and invasion in GBM cells.

Cell apoptosis is the basic mechanism that maintains the homeostasis between cell proliferation and cell death, and also considered as a defense mechanism against tumorigenesis [23]. Apoptosis was mediated by the death-receptor-induced extrinsic pathway and the mitochondria-mediated intrinsic pathway, and both pathways ultimately activate caspase-3 to execute apoptosis. Besides, the members of the anti-apoptotic and pro-apoptotic families, such as Bcl-2 and BAX were exerted significantly effect to mediate the activation of caspases in the mitochondria-mediated pathway [23–24]. Hence, it is a major direction that may enhance GBM cell apoptosis for development of effective therapeutic drugs. Previous study had demonstrated that SC66 could significantly induced apoptosis by accompanied by inactivating AKT. The expression of BAX and Bcl-XL were dramatically decreased when colon cancer cells were treated with SC66[13]. Consistent with these findings, we found that SC66 treatment induced U87 and U251 cells apoptosis in a dose-dependent manner detected by flow cytometry.

SC66 treatment of U87and U251cells induced the expression of cleaved-caspase3 and increased BAX expression. Concurrently, Bcl-2 protein expression was decreased, which revealed that apoptotic effects of SC66 might be associated with the mitochondrial pathway.

We further investigated the effect of SC66 on pathway through which SC66 regulated proliferation, EMT and apoptosis of glioma cells. SC66 suppressed colon cancer growth through AKT /GSK-3β/Bax axis in vivo and vitro [13]. Meanwhile, SC66 induced changes in cytoskeletal organization and ROS production, resulting in phosphorylated AKT level was notably decreased, and demonstrated that SC66 remarkably inhibit tumor growth via AKT/mTOR/β-catenin pathway in hepatocellular carcinoma [25].

GSK-3β was inactivated by phosphorylation at Ser9 site after Wnt signal pathway activation, leading to the accumulation of β-catenin was in the cytoplasm. Cytoplasmic β-catenin subsequently transferred into nuclear, and bond with TCF/LEF, which resulted in transcriptional expression of target genes, such as CyclinD1, c-Myc, etc. [26]. After treatment with SC66, the expression of P-GSK-3β and β-catenin were decreased, and P-β-catenin expression was increased. IF assay demonstrated that the level of β-catenin nuclear translocation was prominently downregulated in SC66 treated group. Meanwhile, TCF/LEF luciferase report assay indicated that the activity of TCF/LEF was remarkably suppressed. Moreover, elevating β-catenin activity by IM12 rescued SC66 inhibition-mediated GBM cell proliferation and metastasis. These results indicated that SC66 could suppressed the development of GBM cells by downregulated AKT/β pathway.,

# Conclusion

In this study, we verified that SC66 exerts prominently antitumor efficiency in GBM cells in vivo and vitro by deregulated AKT/β-catenin pathway. We also demonstrated that SC66 inhibited EMT-mediated cell migration and invasion. Our findings may contribute to its potential applications for the valid treatment of GBM.

# Abbreviations

TMZ
Temozolomide
GBM
Glioblastoma
EMT
Epithelial-to-mesenchymal transition

# Declarations


### Acknowledgements

This research was supported by the National Natural Science Foundation of China (NO.81572489 and 81372683)


### Conflicts of interest

The authors declare no potential conflicts of interest.

### Consent for publication

All authors approved publication of the manuscript

### Ethics declarations

All animal experiments were approved by the Institutional Animal Care and Use Committee at Renmin Hospital of Wuhan University and complied with the internationally recognized Animal Research: Reporting of In vivo Experiments guideline

### Author information

Lun Gao, Junhui Liu and Pengfei Xu contributed equally to this work

### Contributions

LG and JHL performed cell biology experiments, western blot, flow cytometry analysis, immunofluorescence, immunohistochemistry, manuscript preparation and analyzed the statistical data. PFX□GD and BHL participated in flow cytometry analysis. FEY, YQT, QS and, YX participated in western blot. HKZ, YZQ, HXJ, KY, RXG and MSH participated in immunofluorescence. QXC participated in manuscript preparation.

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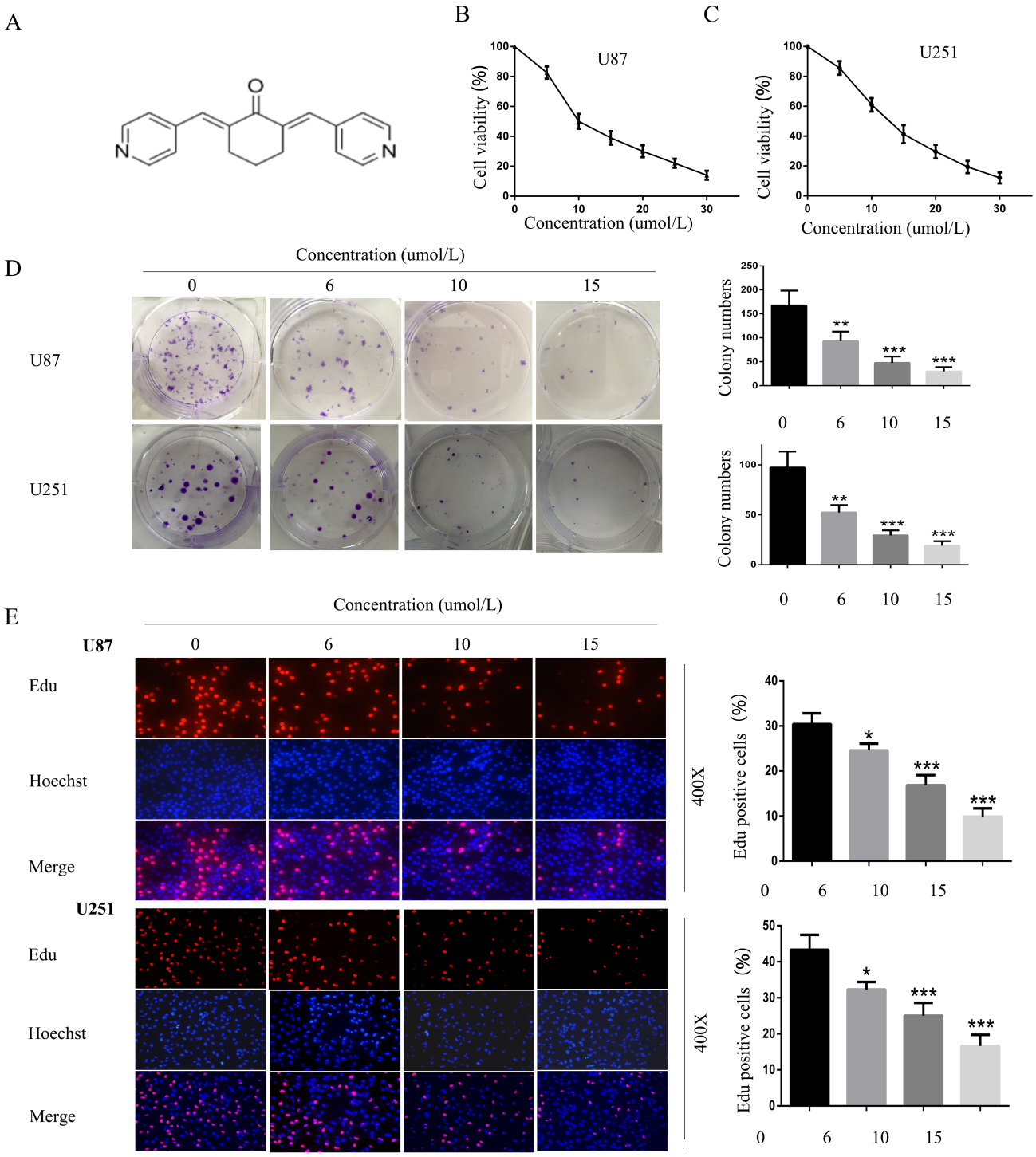
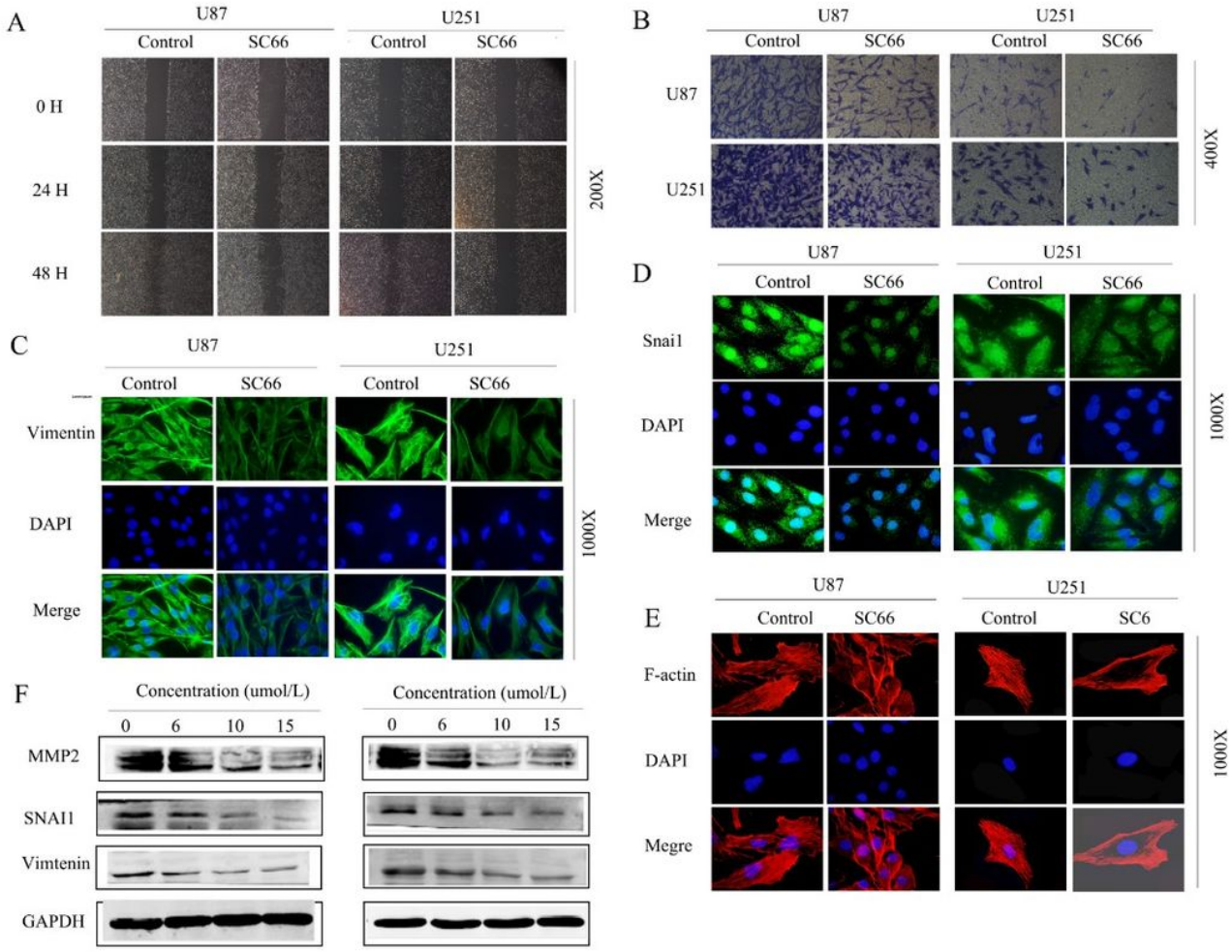
# Figures



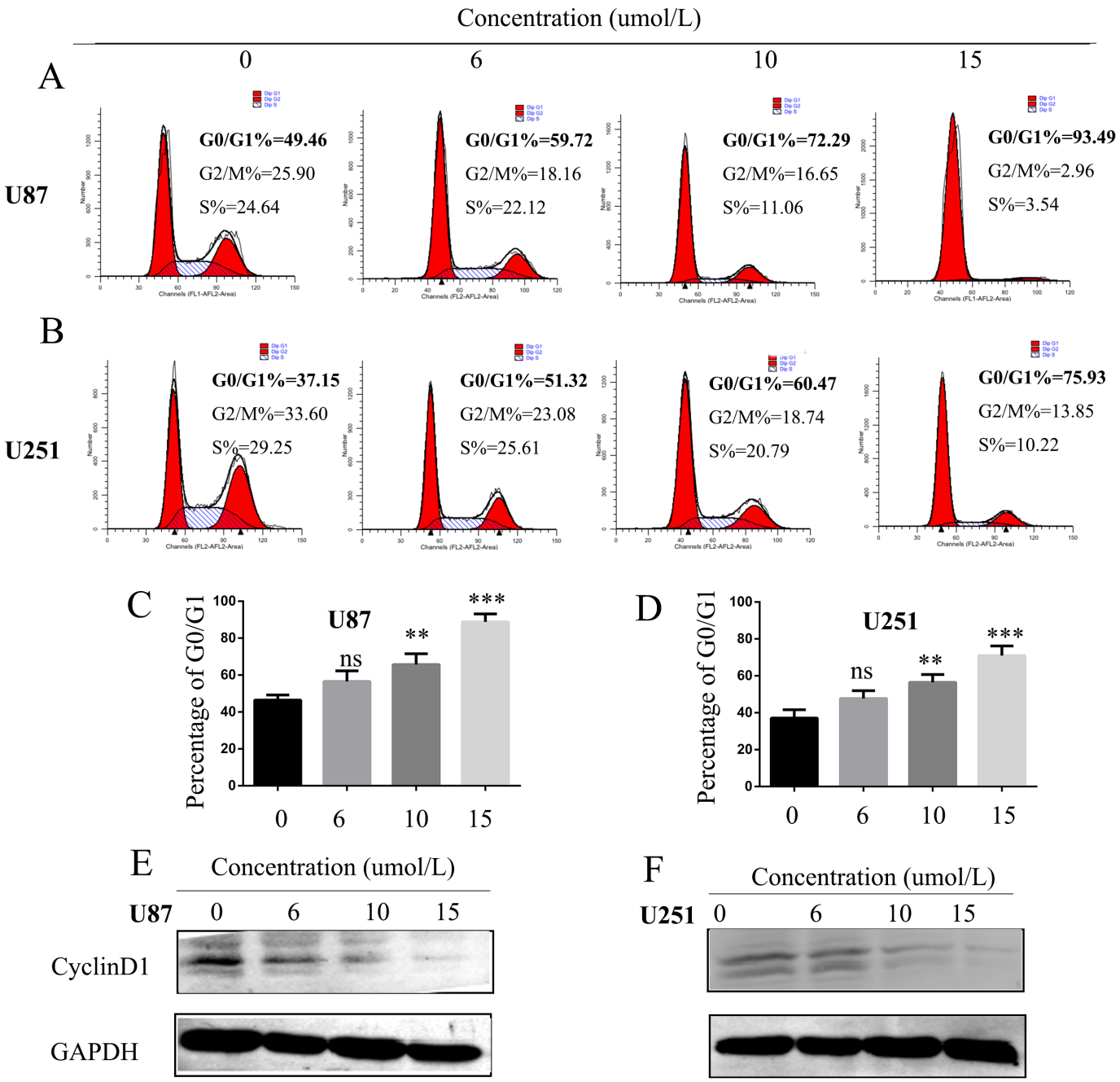

## Figure 1

SC66 inhibited the proliferation of GBM cells in vitro. (A) The molecular structure of SC66. (B and C) Cell viability was measured by CCK8 assay after SCC66 treatment with at various concentrations (0,5,10,15,20,25,30umol/L). (D) SC66 inhibited U87 and U251 cells colony formation and results were statistically analyzed. (E)EDU assay shown that SC66 inhibited DNA synthesis in U251 and U87 cells. For at least 3 independent experiments. * P<0.05, ** P<0.01, *** P<0.001.



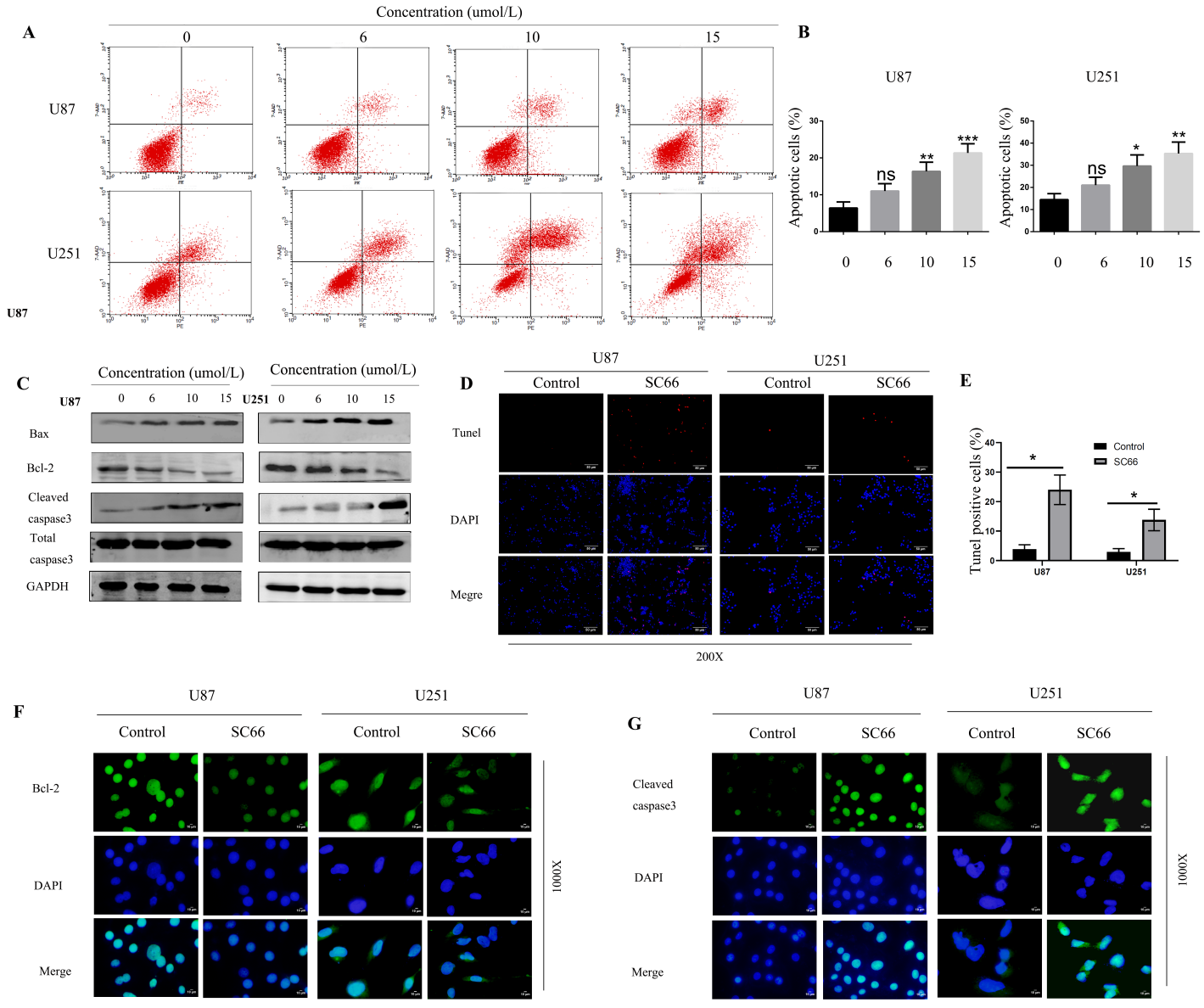

## Figure 2

SC66 suppressed the metastatic biological functions of GBM cells in vitro. (A-B) Wound healing was demonstrated that SC66 suppressed the migration of U87 and U251 cells. (C-D) Tanswell assay showed that SC66 inhibited the invasion of U87 and U251 cells. (E) The expression of MMP2, Vimentin and Snai1 after SC66 treatment (G-H) Representative fluorescent images of Vimentin and Snai1 in U87 and U251 cells. (F) Cells were stained of F-actin with Phalloidin. For at least 3 independent experiments. *P<0.05, ** P<0.01, *** P<0.001, ****P<0.0001.



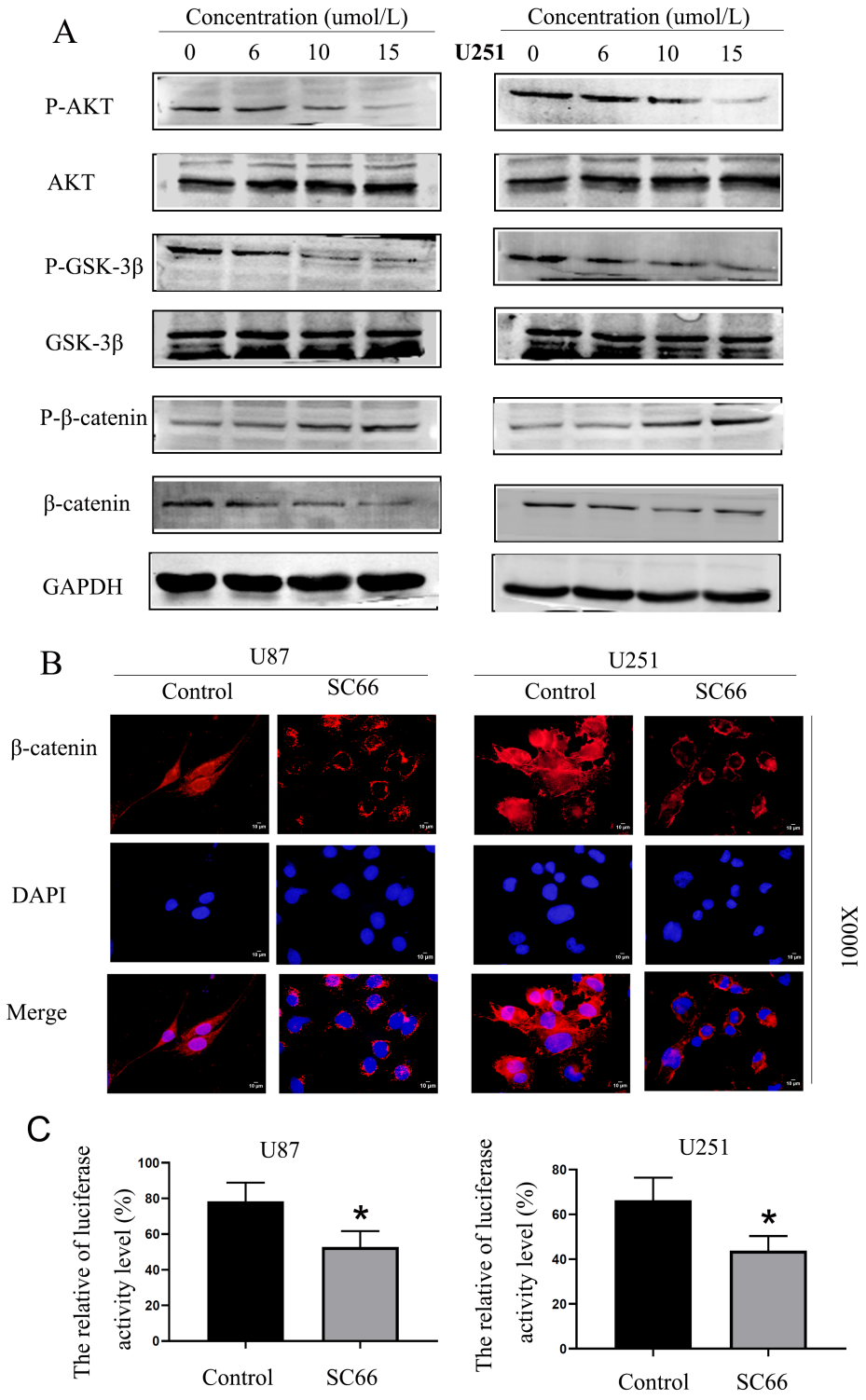

## Figure 3

SC66 arrests cell cycle at G0/G1 phase in U87and U251cells. (A-D) Cells accumulated at the G0/G1 phase after SC66 treatment for 24 h measured by flow cytometry and results were statistically analyzed. (E) Cell cycle protein expression quantified with western blot. For at least 3 independent experiments. ** P<0.01, *** P<0.001.




## Figure 4

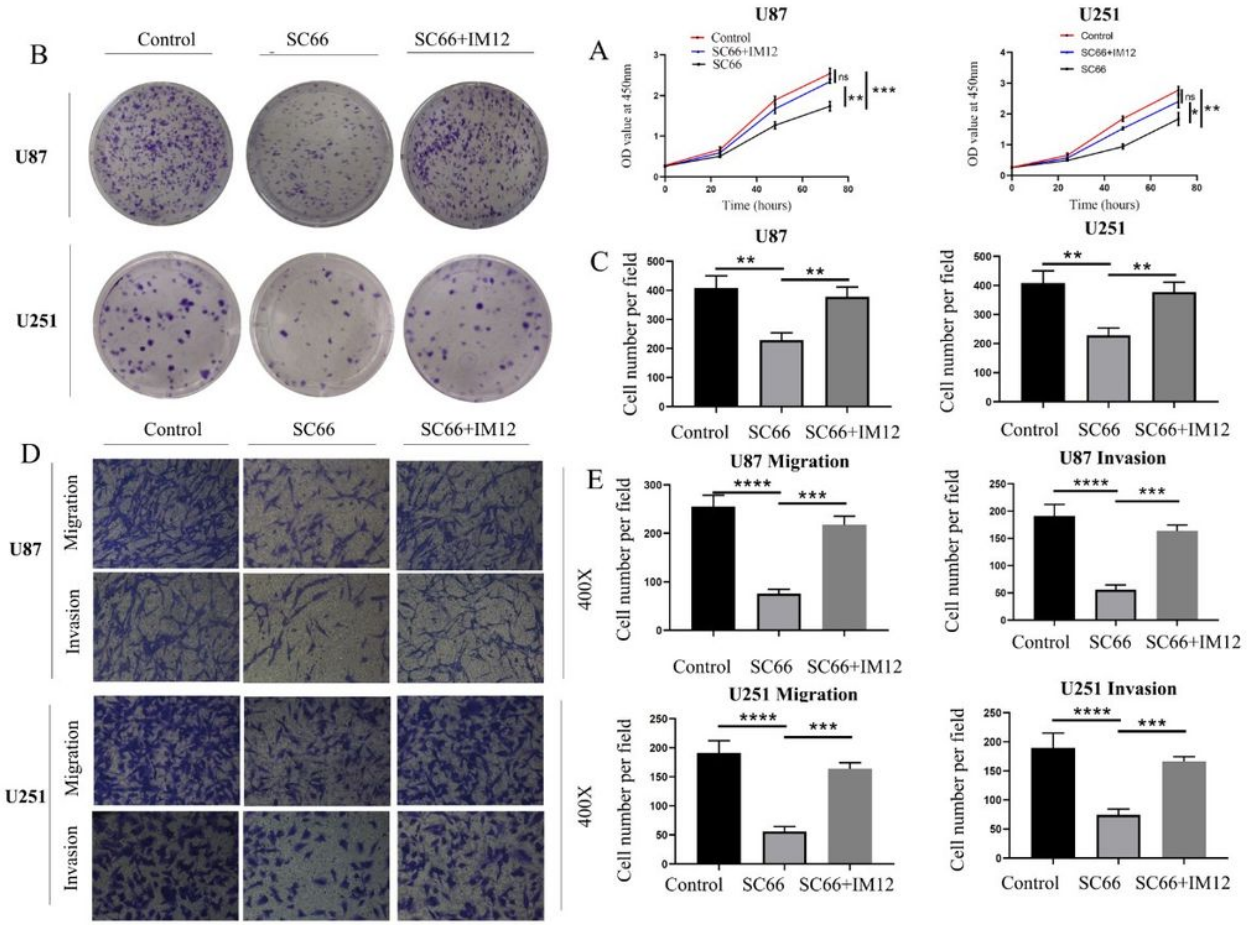
SC66 induced apoptosis of GBM cells in vitro. (A-B) Cells apoptosis were remarkably enhanced after SC66 treatment for 24 h measured by flow cytometry and results were statistically analyzed. (C) Cell apoptosis-related protein expression quantified with western blot. (D-E) U251 and U87 cells death were detected by TUNEL staining. (F-G) Representative fluorescent images of Bcl-2 and Cleaved- caspase3 in U87 and U251 cells. For at least 3 independent experiments. *P<0.05, ** P<0.01, *** P<0.001.




## Figure 5

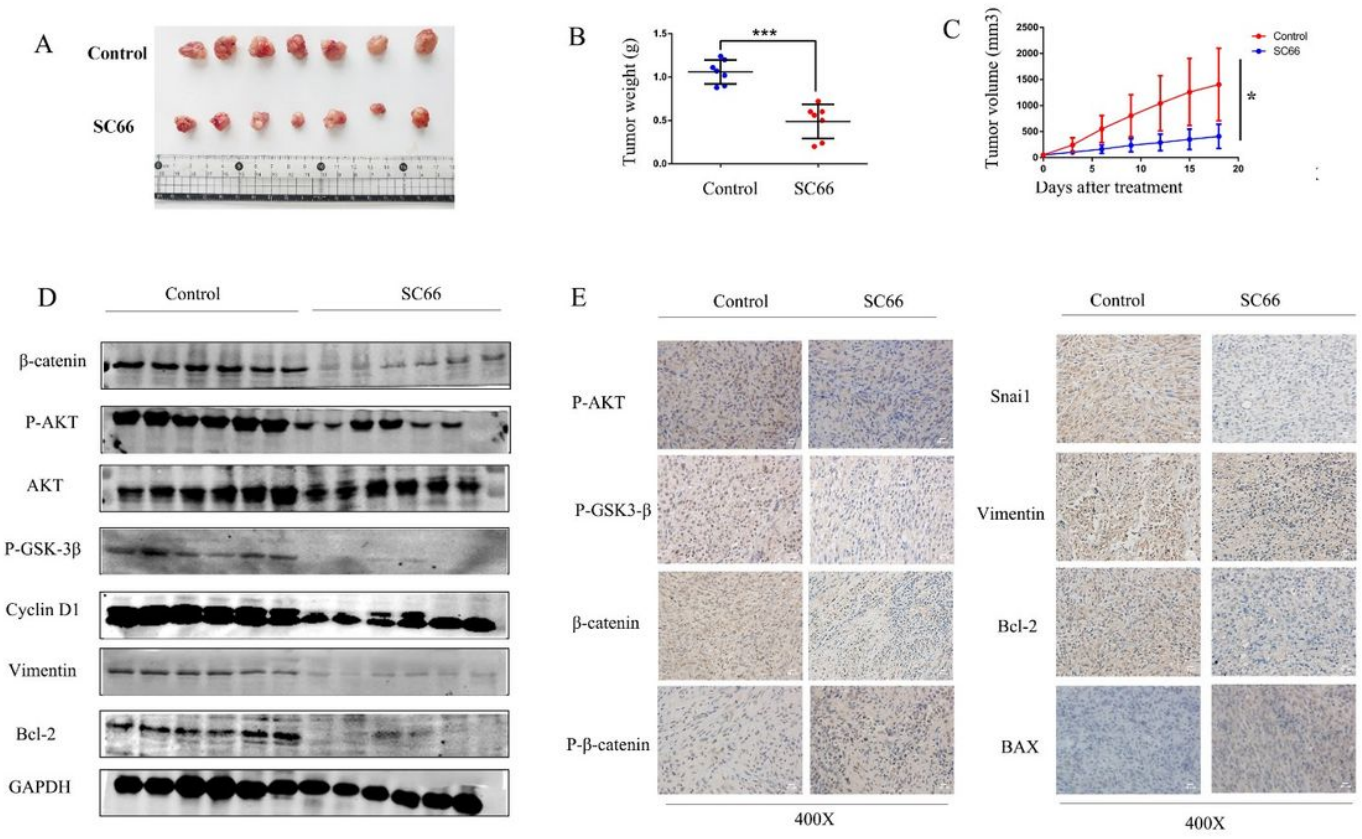
SC66 inhibits AKT/β-catenin signaling pathways in U87 and U251 cells. (A) Western blot assay of main protein expression of signaling pathways after SC66 treatment. (B) Representative fluorescent images of β-catenin in U87 and U251 cells. Nuclear transfer level of β-catenin was remarkably decreased after SC66 treatment compared with control group. (C) The relative TCF/LEF luciferase activity is shown as the

percentage of relative light units of firefly luciferase to Renilla luciferase. For at least 3 independent experiments. *P<0.05




## Figure 6

Elevating β-catenin activity rescued SC66 inhibition-mediated GBM cell proliferation and metastasis. (A-C) IM12 was used to enhance β-catenin signaling pathways. CCK 8 and Clone formation assays were performed to investigate the proliferation in U87 and U251 cells. (D and E) Transwell assay was performed to detect the migration and invasion in GBM cells. For at least 3 independent experiments. **P<0.01, ***P<0.001, ****P<0.0001.




## Figure 7

SC66 suppressed tumor growth in nude mouse models. (A) Image of the xenograft tumors formed in nude mouse models. (B and C) Tumor weight and tumor volume were calculated. (D) Western blot assay of main protein expression of control group and SC66-treated group. (E) Representative images of IHC staining of P-AKT,P-GSK-3β□β-catenin, P-β-catenin, Snai1,MMP2,Bax,Bcl-2 ,and Cleaved-caspase3.*P<0.05, ***P<0.001.