

Salinomycin exhibits anti-angiogenic activity against human glioma *in vitro* and *in vivo* by suppressing the VEGF-VEGFR2-AKT/FAK signaling axis

YAN-LING BI^{1*}, PEI-YAN MI^{1*}, SHI-JUN ZHAO², HENG-MING PAN³, HUI-JUAN LI¹, FEI LIU⁴,
LU-RONG SHAO², HUI-FANG ZHANG², PU ZHANG¹ and SHI-LIANG JIANG³

¹Department of Cardiology, Taian Central Hospital; ²Key Laboratory of Cerebral Microcirculation in Universities of Shandong, Taishan Medical University, Taian, Shandong 271000; ³Department of Cardiology, Shandong Provincial Hospital Affiliated to Shandong University, Jinan, Shandong 250021;

⁴Department of Breast Surgery, Taian Central Hospital, Taian, Shandong 271000, P.R. China

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Abstract. Tumor angiogenesis plays a crucial role in tumor growth, progression and metastasis, and suppression of tumor angiogenesis has been considered as a promising anticancer strategy. Salinomycin (SAL), an antibiotic, displays novel anticancer potential against several human cancer cells *in vitro* and *in vivo*. However, little information concerning its anti-angiogenic properties is available. Therefore, the anti-angiogenic effect of SAL and the underlying mechanism in human glioma were evaluated in the present study. The results indicated that SAL treatment significantly inhibited human umbilical vein endothelial cell (HUVEC) proliferation, migration, invasion and capillary-like tube formation. Further investigation on intracellular mechanisms showed that SAL markedly suppressed FAK and AKT phosphorylation, and downregulated vascular endothelial growth factor (VEGF) expression in HUVECs. Pretreatment of cells with a PI3K inhibitor (LY294002) and FAK inhibitor (PF562271) markedly enhanced SAL-induced inhibition of HUVEC proliferation and migration, respectively. Moreover, U251 human glioma xenograft growth was also effectively blocked by SAL treatment *in vivo* via inhibition of angiogenesis involving FAK and AKT dephosphorylation. Taken together, our findings validated that SAL inhibits angiogenesis and human glioma growth through suppression of the VEGF-VEGFR2-AKT/FAK signaling axis, indicating the potential application of SAL for the treatment of human glioma.

Introduction

Tumor progression relies on a series of sequential events, including tumor initiation, growth, angiogenesis and metastasis, which occur in a complex and dynamic microenvironment and are regulated by a number of mediators and signaling transduction pathways (1-3). Growing evidence indicates the crucial role of angiogenesis in tumor growth and metastasis (4,5). Tumor cells can develop their microenvironment by the secretion of vascular endothelial growth factor (VEGF) or cytokines to promote abnormal tumor neovasculature formation, which can provide nutrients for tumor growth and a route for metastasis (6). Owing to the key role of angiogenesis, inhibition of tumor angiogenesis has been considered as an effective strategy for suppressing tumor progression (7,8). It has also been demonstrated that agents with anti-angiogenic properties can effectively inhibit tumor cell growth and metastasis (9). Thus, searching for novel agents with anti-angiogenic activity represents an effective strategy for the overall control of human cancers.

Salinomycin (SAL), a polyether antibiotic, isolated from bacterium *Streptomyces albus*, has been used extensively to improve the nutrient absorption and feeding efficiency of poultry (10). SAL was identified as a potent anticancer agent among 16,000 compounds against human breast cancer stem cells (CSCs) for the first time in 2009 (11). It was also demonstrated that SAL displays antitumor activities in other types of human CSCs, including colorectal, lung, gastric, pancreatic and osteosarcoma CSCs (12-16). Recently, several studies have shown that SAL displays broad-spectrum anticancer properties, including inhibition of proliferation, migration and invasion, and induction of autophagy and apoptosis in cancer cells (17-21). Additionally, accumulating evidence indicates that SAL can enhance the cytotoxic effects of several chemotherapeutic drugs, such as doxorubicin and etoposide (22,23). An increasing number of studies have reported that induction of DNA damage (23), mitochondrial dysfunction (24), reactive oxygen species (ROS) accumulation (20), signal regulation of FOXO3a, Wnt/ β -catenin,

Correspondence to: Dr Shi-Liang Jiang, Department of Cardiology, Shandong Provincial Hospital Affiliated to Shandong University, 324 Jingwu Weiqi Road, Jinan, Shandong 250021, P.R. China
E-mail: slyyjsl@126.com

*Contributed equally

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STAT3/Skp2 and Akt/NF- κ B/mTOR (25-28), all contribute to SAL-mediated anticancer mechanisms. However, little information concerning the anti-angiogenic potential of SAL is available. In this study, SAL-mediated angiogenic activity was examined, and the underlying molecular mechanisms in human glioma were also evaluated.

Materials and methods

Materials. SAL and conventional chemicals were all obtained from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM)-F12 and fetal bovine serum (FBS) were purchased from Beyotime (Beijing, China). All of the antibodies and inhibitors (LY294002 and PF-562271) used in this study were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). All solvents used were of high-performance liquid chromatography (HPLC) grade. Water used in this study was obtained from a Milli-Q from Millipore (Billerica, MA, USA).

Cell culture. The U251 human glioma cell line and human umbilical vein endothelial cells (HUVECs) were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). HUVECs were cultured in DMEM-F12 supplemented with FBS (10%) at 37°C in a humidified incubator with 5% CO₂ atmosphere.

Measurement of cell viability (MTT assay). The effect of SAL on HUVEC growth was determined by MTT assay. Briefly, HUVECs (6,000 cells/well) were seeded in 96-well culture plates for 24 h and incubated with different concentrations of SAL. In the preliminary experiments, SAL treatment for 12, 24, 48 and 72 h showed time-dependent effects on cell growth inhibition. However, treatment for 48 h was the optimal time and was selected for further mechanism evaluation. After SAL treatment for 48 h, 20 μ l/well of MTT solution (5 mg/ml) was added and incubated for 5 h. The medium was aspirated and replaced with 200 μ l/well of DMSO to dissolve the formazan salt formed. The color intensity of the formazan solution was measured at 570 nm by a microplate spectrophotometer. The cell viability was expressed as % of the control (as 100%).

Scratch motility (wound-healing) assay. HUVECs were incubated in 6-well plates and allowed to grow to full confluence. After serum starvation for 4 h, the cells were scratched using pipette tips, washed with phosphate-buffered saline (PBS) and photographed by using a phase-contrast microscope. Fresh medium supplemented with 1% FBS and 4 μ M SAL was added into the well. VEGF (200 ng/ml) and 5 μ M cisplatin were employed as the positive and negative control, respectively. After 48 h of treatment, cells were photographed again at three random areas. The migrated cells were quantified by manual counting and the migration ratio was calculated.

Transwell invasion assay. HUVECs were placed on a Transwell Boyden chamber (8- μ m pore; Corning Inc., Lowell, MA, USA) pre-coated with Matrigel for 4 h at 37°C. One hundred microliters of cell suspension (2x10⁵ cells/ml) in FBS-free medium was added into the upper compartment of the chamber. The bottom chambers were supplemented with 500 μ l complete

medium (10% FBS) containing the indicated concentrations of SAL with or without 200 ng/ml VEGF. After 24 h of treatment, the non-invaded cells from the upper face were scraped using a cotton swab. The invaded cells on the lower face were fixed with methanol, stained with Giemsa and photographed by a phase-contrast microscope. The invaded cells were quantified by manual counting and the invasion ratio was expressed as % of the control.

Tube formation assay. HUVECs (1x10⁴ cells/well) were seeded in a Matrigel pre-coated 48-well plate. VEGF (200 ng/ml) and 5 μ M cisplatin were employed as the positive and negative control, respectively. After 24 h of treatment, the tube formation was visualized with an inverted microscope and the tube number was quantified by manual counting.

Western blotting. After treatment, the cells were harvested and incubated with cell lysis buffer overnight at -20°C. The protein concentrations were detected using a BCA protein assay kit. After electrophoresis, the separated proteins were transferred onto nitrocellulose membrane for 75 min at 110 V and blocked with 5% non-fat milk in TBS buffer for 1 h. Subsequently, the membranes were washed with TBST buffer and incubated with primary antibodies [p-FAK (#8556), VEGF (#2463), p-VEGFR2 (#2478s), VEGFR2 (#9698), p-AKT (#4058), AKT (#4691), Ki67 (#9027) and CD34 (#3569); Cell Signaling Technology, Inc.] overnight at 4°C and then secondary antibody [IgG (#3452), Cell Signaling Technology, Inc.] for 2 h at room temperature. The target proteins were detected on X-ray film using a chemiluminescence reagent. β -actin was used to confirm the equal loading and transfer of proteins.

In vivo study. Human glioma U251 cells (1x10⁷) suspended in 100 μ l PBS were injected into the right lower hind flank of each 6-week-old male nude mouse. The mice were then randomly assigned into three groups of 10 mice in each group. After one week, SAL (5 and 10 mg/kg) was administered into the caudal vein every other day for 16 days. Control mice received an equal volume of vehicle (saline) only. Body weight and tumor volume were monitored every two days. At the end of the experiments, tumors were excised, photographed, and weighed. Tumors from each group were used for western blotting and immunohistochemical (IHC) assay. All animal experiments were approved by the Animal Experimentation Ethics Committee of Taishan Medical University (no. 2015026).

Statistical analysis. Experiments were carried out at least in triplicate and the results are expressed as mean \pm SD. Statistical analysis was performed using SPSS version 13 (SPSS, Inc., Chicago, IL, USA). A difference between two groups was analyzed by two-tailed Student's t-test. Difference with $p < 0.05$ or $p < 0.01$ was considered statistically significant.

Results

SAL inhibits the proliferation of human HUVECs. Due to the essential role of HUVECs in angiogenesis, in the present study, HUVECs were chosen to evaluate the anti-angiogenic potential of SAL. Firstly, the inhibitory effect of SAL on the growth of HUVECs was determined by MTT assay. As shown

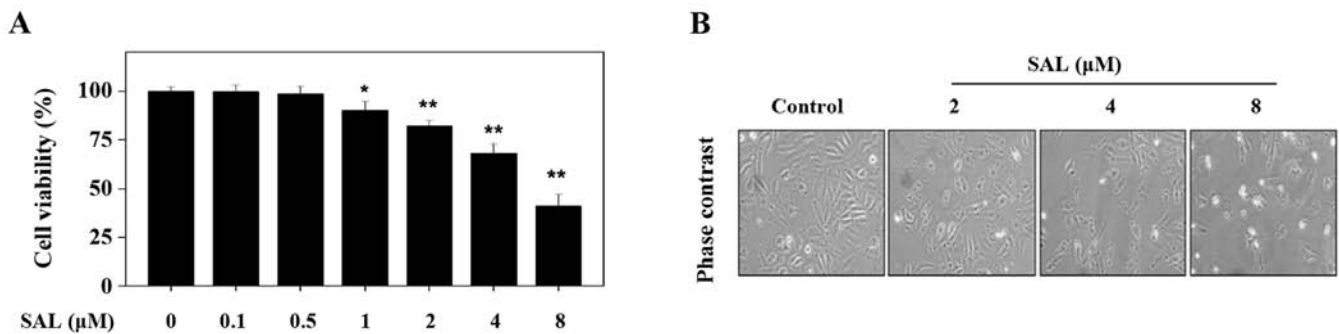


Figure 1. Salinomycin (SAL) inhibits the cell proliferation in human umbilical vein endothelial cells (HUVECs). (A) HUVECs seeded in 96-well plates (6,000 cells/well) were treated with the indicated concentrations of SAL for 48 h, and the cell viability was detected by MTT assay. Each value represents the mean \pm SD of three independent experiments. * $p < 0.05$; ** $p < 0.01$ vs. the control. (B) Changes in the morphology of HUVECs. Cells after treatment with the indicated concentrations of SAL for 48 h were examined by phase-contrast microscopy (magnification, $\times 200$).

in Fig. 1A, treatment of HUVECs with the indicated concentrations (0.1–8 μ M) of SAL resulted in a dose-dependent inhibition of cell growth, accounting for 32.1 and 59.2% inhibition at 4 and 8 μ M, respectively. In the phase contrast observation (Fig. 1B), HUVECs exposed to 2, 4 and 8 μ M of SAL for 48 h showed a dose-dependent reduction in cell number and a change in cell morphology, such as cell shrinkage and cell rounding. These results demonstrated that SAL could inhibit HUVEC growth *in vitro*.

SAL blocks HUVEC migration, invasion and capillary-like tube formation. Migration, invasion and tube formation of HUVECs are crucial components in the process of tumor-induced neovascularization. Thus, we evaluated the effects of SAL on the metastatic potential of HUVECs *in vitro*. Scratch motility and Transwell invasive assays were used to detect the migration and invasion of HUVECs after SAL treatment, respectively. As shown in Fig. 2A–D, VEGF treatment obviously promoted HUVEC migration and invasion. However, SAL treatment effectively inhibited HUVEC migration and invasion at the concentration of 4 μ M after 48 h of treatment (Fig. 2A–D). A similar effect was also observed in the HUVECs exposed to cisplatin (negative control).

To further confirm the effect of SAL on angiogenesis, a tube formation assay was carried out to investigate whether SAL can inhibit the capillary-like tube formation of HUVECs. After pretreatment with VEGF, elongated and robust tube-like structures were observed, while a significant disruption in capillary-like tube formation was observed in the HUVECs exposed to 4 μ M of SAL for 24 h (Fig. 2E and F). These results indicated that SAL inhibited angiogenesis of HUVECs *in vitro*.

SAL suppresses FAK phosphorylation in HUVECs. Further investigation of the underlying mechanisms was evaluated. FAK, a cytoplasmic protein tyrosine kinase, plays a vital role in cell proliferation, survival and metastasis. To illustrate whether the inactivation of FAK is involved, we examined the levels of phosphorylated level of FAK in HUVECs after SAL treatment. As shown in Fig. 3A and B, exposure of HUVECs to SAL significantly suppressed the expression levels of phosphorylated (p)-FAK in a time- and dose-dependent manner. In addition, PF562271 (FAK inhibitor) was used to confirm

the role of FAK inactivation in SAL-mediated inhibition of cell migration. As shown in Fig. 3C–E, treatment with 10 nM PF562271 or 4 μ M SAL alone for 24 h both displayed notable inhibitory effects on FAK phosphorylation and HUVEC migration. Notably, pretreatment with PF562271 markedly enhanced SAL-induced inhibition against FAK phosphorylation and HUVEC migration. Taken together, these results indicated that FAK dephosphorylation contributed to SAL-mediated inhibition against HUVEC migration.

SAL disturbs the VEGF-VEGFR2-AKT signaling axis. As a crucial mediator of angiogenesis in the tumor microenvironment, VEGF promotes tumor angiogenesis via interacting with VEGFR to regulate downstream signaling transduction. In the present study, the expression of VEGF, VEGFR2, AKT and their phosphorylated levels in HUVECs were detected by western blotting. As shown in Fig. 4A, little change was observed in the expression levels of total VEGFR2 and AKT after SAL treatment. However, HUVECs exposed to SAL showed a significant decrease in VEGF expression, leading to the reduction of phosphorylated VEGFR2 and AKT. To further study the role of AKT in SAL-mediated anti-angiogenesis *in vitro*, we examined the effects of AKT-upstream inhibitor (LY294002) on HUVEC growth by MTT assay. As shown in Fig. 4B, pretreatment with LY294002 resulted in a marked decrease in cell viability, which supports the key role of AKT inactivation in SAL-induced cell growth inhibition. Taken together, our results indicated that SAL inhibited HUVEC angiogenesis by disturbing the VEGF-VEGFR2-AKT signaling axis.

SAL inhibits glioma growth in vivo by inhibiting angiogenesis. To evaluate the anticancer and anti-angiogenic potential of SAL *in vivo*, we treated tumor-bearing nude mice with 5 and 10 mg/kg of SAL. As shown in Fig. 5A–C, the average tumor volume and tumor weight were significantly suppressed by SAL treatment. The body weight of the mice showed no significant change (Fig. 5D). Moreover, consistent with the *in vitro* model, we also found that SAL moderately decreased FAK and AKT phosphorylation in the tumor model (Fig. 5E). IHC staining showed that the expression of Ki-67, a biomarker of proliferation, was significantly inhibited by SAL treatment (Fig. 5F). CD34, an important

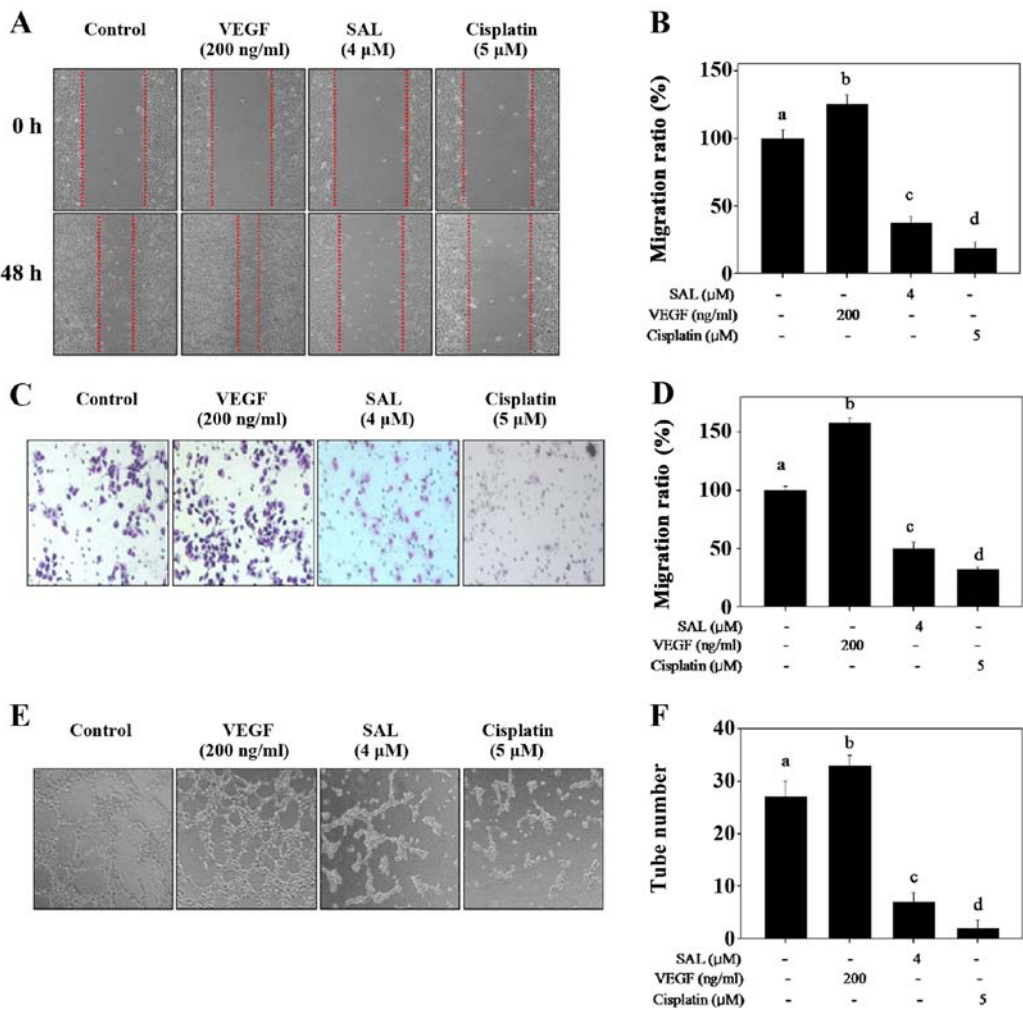


Figure 2. Salinomycin (SAL) blocks human umbilical vein endothelial cell (HUVEC) migration, invasion and capillary-like tube formation *in vitro*. Effects of SAL on (A) HUVEC migration, (C) invasion and (E) tube formation. Positive control [vascular endothelial growth factor (VEGF)] and negative control (cisplatin) were employed to evaluate the potential of SAL. The details of the experiments is provided in Materials and methods. (B) The migration ratio, (D) invasion rate and (F) the tube formation number were quantified by manual counting. All data are expressed as means \pm SD of triplicate experiments. Bars with different characters (a, b and c) are statistically different at $p<0.05$ level.

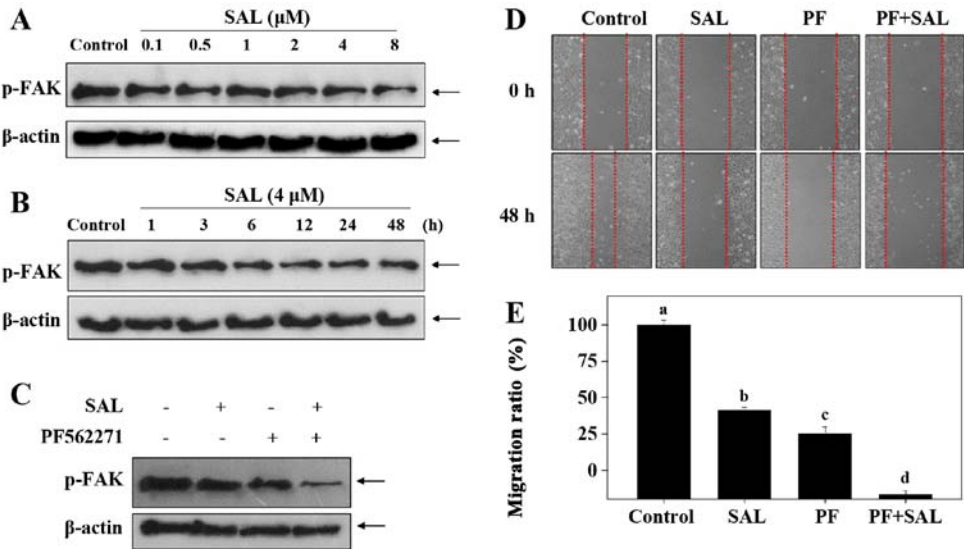


Figure 3. Salinomycin (SAL) suppresses FAK-dependent cell migration in human umbilical vein endothelial cells (HUVECs). (A) Effects of SAL on the expression level of phosphorylated FAK (p-FAK). Cells were treated with the indicated concentrations of SAL for 48 h. (B) Western blotting of the time-dependent regulation of p-FAK in HUVECs. Cells were treated with 4 μ M SAL for various periods of time. (C-E) Inhibitory effects of SAL and/or FAK inhibitor (PF562271) on the expression levels of p-FAK and cell migration in HUVECs. Cells were pretreated with or without 10 nM PF562271 for 1 h and co-treated with 4 μ M of SAL for 48 h. All data are expressed as means \pm SD of triplicate experiments. Bars with different characters (a, b and c) are statistically different at $p<0.05$ level.

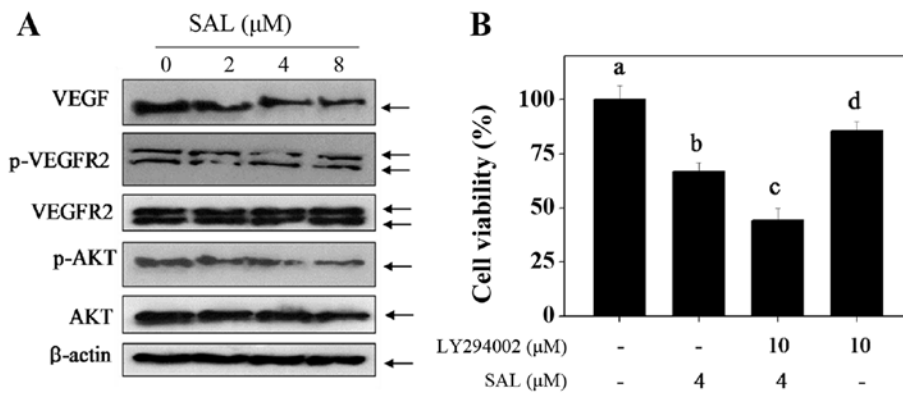


Figure 4. Salinomycin (SAL) disturbs the vascular endothelial growth factor (VEGF)-VEGFR2-AKT signaling axis. (A) Western blotting of expression levels of VEGF, VEGFR2, phosphorylated VEGFR2 (p-VEGFR2), AKT and p-AKT in human umbilical vein endothelial cells (HUVECs) exposed to different concentrations of SAL for 48 h. (B) Inhibitory effects of SAL and/or LY294002 on HUVEC growth. Cells were pretreated with or without 10 μ M LY294002 for 1 h and co-treated with 4 μ M of SAL for 48 h. Cell viability was determined by MTT assay. All data are expressed as means \pm SD of triplicate experiments. Bars with different characters (a, b and c) are statistically different at $p < 0.05$ level.

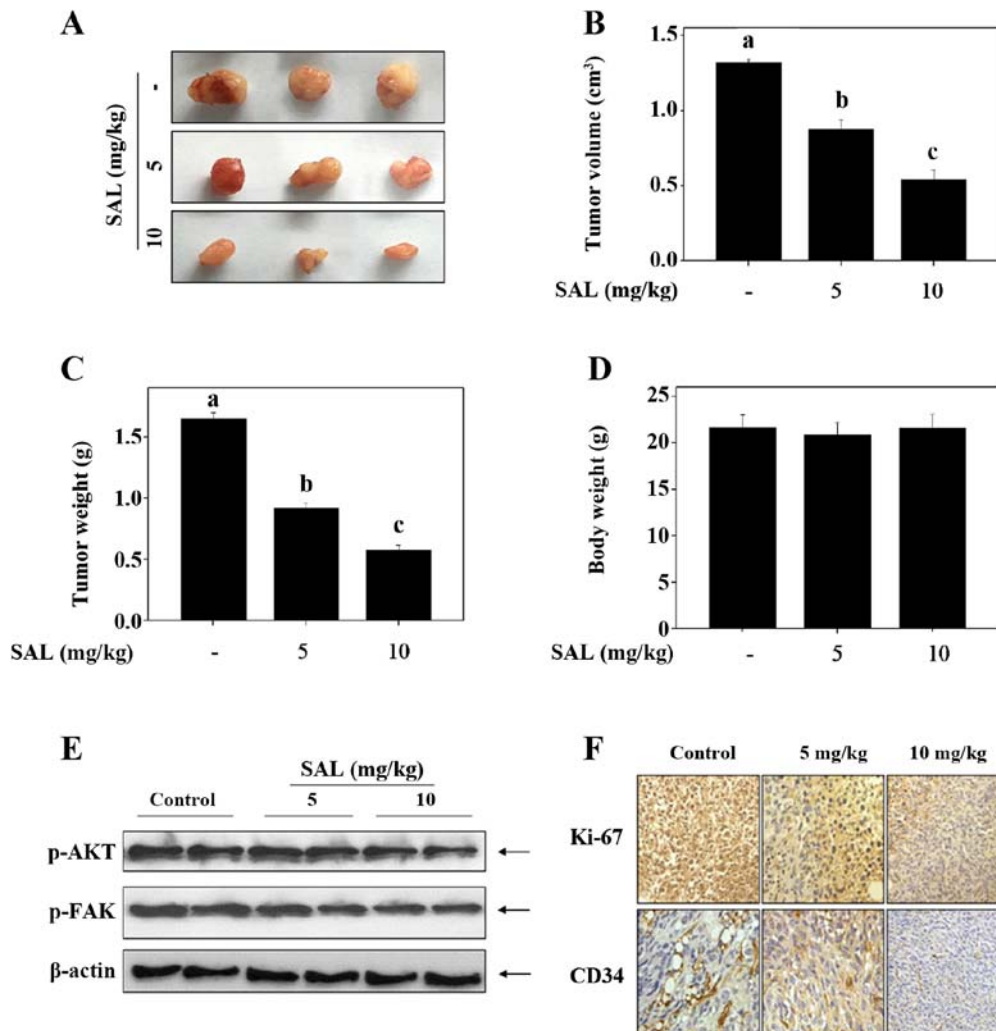


Figure 5. Salinomycin (SAL) suppresses the U251 human glioma cell growth *in vivo* via inhibition of angiogenesis. (A and B) Tumor volume, (C) tumor weight and (D) body weight of the mice were all assessed at the end of the experiment. U251 tumor xenograft model was established as described in Materials and methods. (E) AKT and FAK expression. Homogenized cell lysate from each group were prepared for western blotting of expression levels of phosphorylated AKT (p-AKT) and p-FAK. (F) Immunohistochemical (IHC) analysis of the expression levels of Ki-67 and CD34 in SAL-treated U251 tumor xenografts.

marker of hematopoietic progenitor cells and the small vessel endothelium was highly expressed in the control

tumor tissue (Fig. 5F). However, SAL treatment significantly inhibited the *in vivo* angiogenesis (Fig. 5F). Taken together,

these findings revealed that SAL hindered the U251 human glioma cell growth *in vivo* via inhibition of angiogenesis with involvement of AKT and FAK dephosphorylation.

Discussion

Glioma is one of the most common malignant brain cancers worldwide and has become a great threat to human health (29). Glioma is highly aggressive and is associated with a very poor prognosis, and the median survival rate is ~12-15 months (30,31). Even though the current clinical treatments for glioma have achieved satisfactory results, adjuvant chemotherapy after surgical resection and radiotherapy are still irreplaceable (32-34). This forms a rationale for the development of novel chemotherapeutic agents for the treatment of glioma. Preclinical data indicate that angiogenesis is essential for the proliferation and survival of glioma cells (35,36). Thus, searching for novel anti-angiogenic agents to treat human glioma is urgently needed. Herein, in the present study, we investigated the anti-angiogenic potential of SAL in the treatment of human glioma and clarify the underlying mechanisms.

Tumor cells can release growth factors and cytokines into the microenvironment, which activate the sprouting and proliferation of formerly quiescent endothelial cells on nearby blood vessels (3). VEGF, as one of the most crucial mediators in angiogenesis, can effectively promote the metastasis of endothelial cells. HUVEC migration, invasion and tube formation were significantly enhanced by the addition of VEGF. However, this tendency was significantly suppressed by SAL, indicating the anti-angiogenic potential of SAL *in vitro*. Growing evidence suggests that the interaction of VEGF and receptors (VEGFR1/2/3) promote the activation of downstream signaling transduction cascades (37,38). Among these VEGF receptors, VEGFR2 plays a major role in the transduction of angiogenic signaling. The activation of VEGFR2 by VEGF stimulation subsequently results in activation of several downstream pathways, including Ras/MEK/ERK and PI3K/Akt, which can positively regulate pro-survival and pro-angiogenic signals (3,37). Recent research has shown that SAL decreases VEGF-induced phosphorylation of VEGFR2 in HUVECs (17). Consistent with the previous data, we found that the expression levels of VEGF and phosphorylated VEGFR2 were both downregulated by the treatment of SAL, leading to the inactivation of the pro-survival PI3K/Akt signaling pathway (Fig. 4). In addition, pretreatment with the PI3K inhibitor (LY294002) markedly enhanced the growth inhibitory effects of SAL, indicating that the VEGF-VEGFR2-AKT signaling axis plays an essential role in SAL-induced HUVEC growth inhibition *in vitro*.

FAK, a cytoplasmic protein tyrosine kinase, plays a vital role in cell proliferation, survival and migration, and is associated with integrin-mediated signal transduction (39). Integrin clustering-mediated activation of FAK results in the phosphorylation of Tyr397, which is a binding site for PI3K (40). Previous studies have shown that the activation of FAK by integrin engagement or growth factor stimulation both promote the activation of downstream PI3K/Akt signaling pathways, which leads to the activation or overexpression of pro-metastatic proteins, such as matrix metalloproteinases, urokinase-type plasminogen activator and VEGF (39,41).

Inactivation of FAK was involved in the SAL-induced suppression of metastatic potential in HUVECs (Fig 3). Moreover, the introduction of FAK inhibitor (PF562271) further confirmed the above result.

Accumulative studies have demonstrated that SAL has the ability to inhibit cancer cell growth in nude mouse xenograft models, including human gastric cancer (17), human nasopharyngeal carcinoma (18) and human hepatocellular carcinoma (19) through induction of apoptotic cell death and anti-angiogenesis. To make the *in vitro* data more convincing, we detected the inhibitory effect of SAL on U251 tumor xenografts. The results revealed that SAL effectively inhibited human glioma growth by inhibiting FAK and AKT phosphorylation and angiogenesis, which validated that the anti-angiogenic potential of SAL contributes to SAL-induced growth inhibition of U251 human glioma cells *in vivo*.

SAL is a potential anticancer agent, and its action mechanism has been extensively explored. For example, Qin *et al* found that SAL inhibited human glioma cell growth by ROS/p53/cyclophilin-D signaling-mediated necrosis (42). Zhang *et al* found that SAL can inhibit the growth of colorectal carcinoma by targeting tumor stem cells (43). Kim *et al* reported that SAL can induce apoptosis and autophagy, and also acts as a sensitizer to enhance the effects of doxorubicin on human cancer cells (20,23). Researchers also found that ROS-mediated oxidative damage (20,23), mitochondrial dysfunction (24), and several signaling pathway, such as FOXO3a, Wnt/ β -catenin, STAT3/Skp2 and Akt/NF- κ B/mTOR (25-28), all contribute to the anti-cancer mechanisms of SAL. However, we provide new evidence that SAL can act as a potent anti-angiogenic agent *in vitro* and *in vivo* to inhibit human glioma growth via suppression of the VEGF-VEGFR2-AKT/FAK signaling axis. Our findings validate the potential of SAL as a promising anti-angiogenic candidate for the treatment of human glioma in clinical trials.

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