



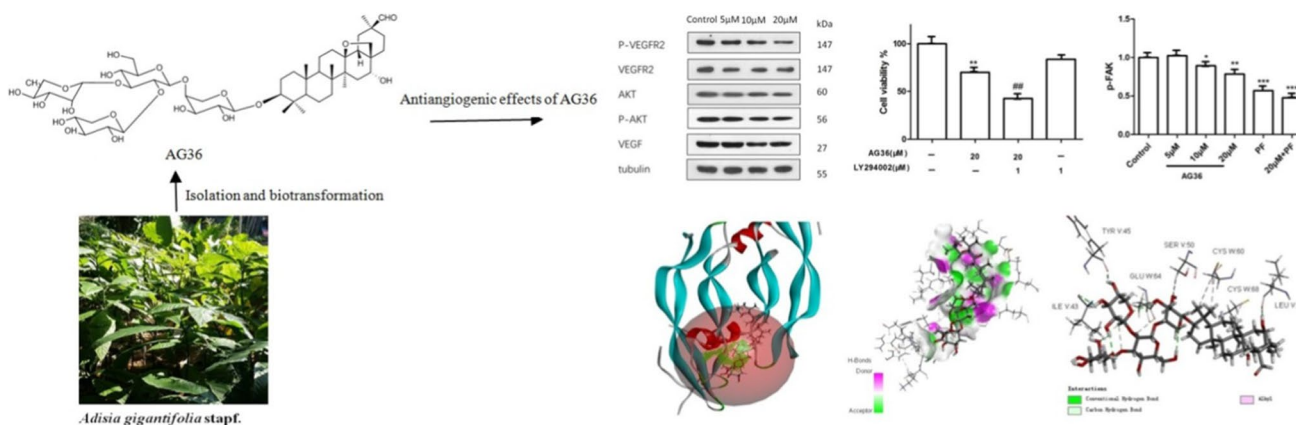
# Antiangiogenic effects of AG36, a triterpenoid saponin from *Ardisia gigantifolia* stapf.

Li-Hua Mu<sup>1</sup> · Li-Hua Wang<sup>1</sup> · Yu-Ning Wang<sup>2</sup> · Ping Liu<sup>1</sup> · Can Yan<sup>3,4</sup>

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## Abstract

AG36 is a triterpenoid saponin from *Ardisia gigantifolia* stapf. Our recent studies proved that AG36 displayed prominent cytotoxicity against breast cancer cells both in vitro and in vivo. However, whether AG36 has antiangiogenic properties is unknown. Therefore, in the present study, we evaluated the antiangiogenic effect of AG36 and the underlying mechanism. The results indicated that AG36 could significantly inhibit the proliferation, migration and invasion of human umbilical vein endothelial cells (HUVEC). Further antiangiogenic molecular mechanism investigation showed that AG36 significantly suppressed phosphorylated FAK and AKT, and downregulated the expressions of vascular endothelial growth factor (VEGF) and vascular endothelial growth factor receptor 2 (VEGFR2) in HUVECs. PI3K inhibitor (LY294002) and FAK inhibitor (PF562271) pretreatment could markedly enhance AG36-induced inhibition of HUVEC proliferation and p-FAK suppression, respectively. In addition, AG36 inhibited the tumor growth in xenograft model and expressions of p-VEGFR2 and p-AKT in vivo. Molecular docking simulation indicated that AG36 formed hydrogen bonds and hydrophobic interactions within the ATP binding pocket of VEGFR2 kinase domain. The present study firstly revealed the high antiangiogenic potency and related underlying molecular of AG36, demonstrating that AG36 maybe a potential antiangiogenic cancer therapy agent or lead candidate.



**Keywords** *Ardisia gigantifolia* · Triterpenoid saponin · Human umbilical vein endothelial cells · Antiangiogenic activity · Molecular docking

Li-Hua Mu and Li-Hua Wang contributed equally to this work.

✉ Ping Liu  
cpi301@163.com

✉ Can Yan  
gzyancan@hotmail.com

Extended author information available on the last page of the article

## Introduction

The rhizome of *Ardisia gigantifolia* stapf. is a traditional Chinese medicine used to treat traumatic injury, rheumatism, muscles, and bones pain. The antitumor activities about this plant were reported recently [1, 2]. As part of our ongoing

work to discover new anticancer lead compounds, we have isolated a series of triterpenoid saponins from *A. gigantifolia* stapf., most of them showed cytotoxicity against different kinds of cancer cells [3–6]. One of the isolated triterpenoid saponin AG4 was biotransformed by *Alternaria alternata* AS 3.6872 to obtain AG36 (Fig. 1a) [7]. Our previous study showed that AG36 exerted prominent cytotoxicity against breast cancer cells both in vitro and in vivo [8]. Recently, several studies have shown that some natural triterpenoid saponins display antiangiogenic properties, including inhibition of proliferation, migration, invasion and tube formation in cancer cells and the microvessels formation in animal models [9–12]. However, it remains unknown whether AG36 can suppress angiogenesis, a crucial step in tumor growth and metastasis.

Angiogenesis plays key role in the process of tumor growth and metastasis [13]. Angiogenesis inhibition is considered as an effective cancer therapeutic target and several agents have been successfully translated into cancer clinic [14, 15]. In view of the prominent antitumor values of natural products from medical herb, more and more natural products are currently being focused on their antiangiogenic activity [16–19].

The present study firstly researched the antiangiogenic activities of AG36 on human HUVEC including cell proliferation, motility and migration. Moreover, to further investigate the antiangiogenic molecular mechanism of AG36, the effects of AG36 on VEGF-VEGFR2-AKT signaling axis were explored by western blot and xenograft model, the interactions between AG36 and VEGFR2 kinase were researched by molecular docking assay.

## Materials and methods

### Materials

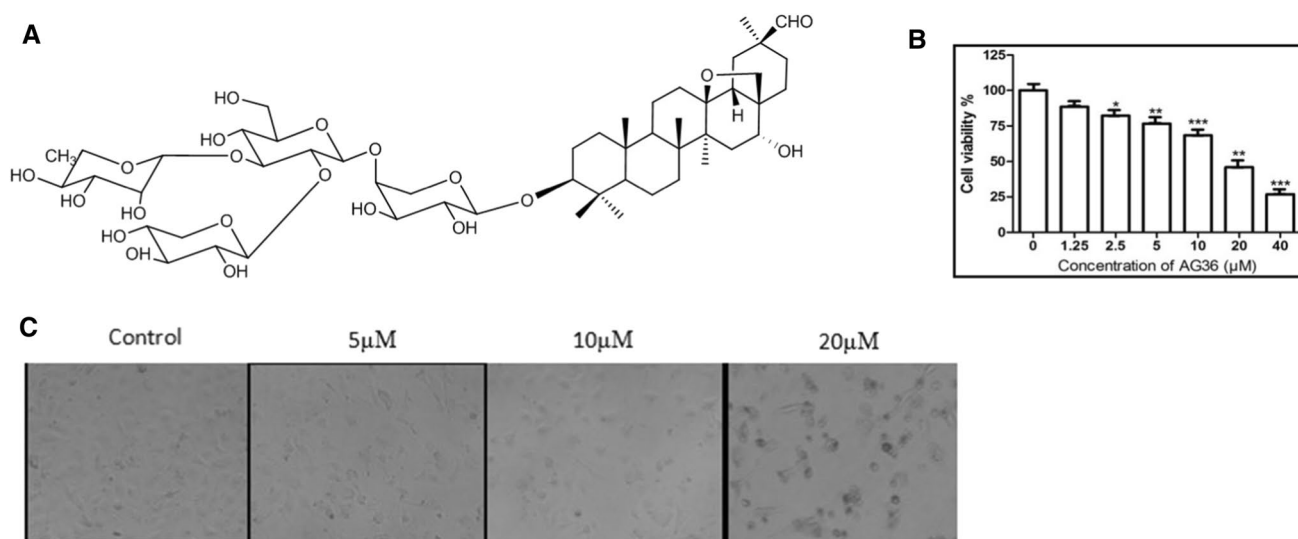
AG36 was obtained as described previously [7]. Dulbecco's modified Eagle's medium (DMEM)-F12 and fetal bovine serum (FBS) were purchased from Beyotime (Beijing, China). The antibodies p-FAK, VEGF and  $\beta$ -tubulin used in this study were purchased from Bioss (Beijing, China). p-VEGFR2, VEGFR2, AKT and p-Akt were purchased from Abcam (Cambridge, MA, USA). The inhibitors LY294002 and PF562271 were purchase from Selleck (Houston, TX, USA) and MCE (Monmouth Junction, NJ, USA) respectively. All solvents used were of high-performance liquid chromatography (HPLC) grade.

### Cell culture

The HUVECs were obtained from Cell Culture Collection of Chinese Academy of Medical Sciences (Beijing, China) HUVECs were cultured in DMEM-H supplemented with FBS (10%) and 1% penicillin/streptomycin at 37 °C in a humidified incubator with 5% CO<sub>2</sub> atmosphere.

### Cell viability measurement (MTT assay)

The effect of AG36 on HUVEC viability was determined by MTT assay. Briefly, HUVECs (5,000–10000 cells/well) were seeded in 96-well culture plates for 24 h and incubated with different concentrations of AG36 for 48 h. Then, 20  $\mu$ l/well



**Fig. 1** Anti-proliferation effects of AG36 on human umbilical vein endothelial cells (HUVECs). **a** Structure of AG36. **b** HUVECs were treated with different concentrations of AG36 for 48 h, and the cell viability was detected by MTT assay. Each value represents the

mean  $\pm$  SD of three independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$  vs. control. **c** Morphologic changes of HUVECs. After AG36 treatment for 48 h cells were examined by phase-contrast microscopy (magnification  $\times 200$ )

of MTT solution (5 mg/mL) was added and incubated for 4 h. The medium was aspirated and replaced with 150  $\mu$ L/well of DMSO to dissolve the formazan salt formed. The absorbance was measured at 492 nm by a microplate spectrophotometer. The cell viability was expressed as % of the control (as 100%).

### Scratch motility assay (wound-healing)

HUVECs ( $5 \times 10^5$  cells/well) were incubated in 6-well plates and allowed to grow to more than 90% confluence. After being scratched using pipette tips, the cells were washed with phosphate-buffered saline (PBS) three times and photographed using a phase-contrast microscope. Fresh medium supplemented with 1% FBS and different concentrations of AG36 (5, 10 and 20  $\mu$ M), positive control VEGF (50 ng/mL) and negative control cisplatin (5  $\mu$ M) were added into the well. After 48 h of treatment, cells were photographed. The migrated cells were quantified and the migration ratio was calculated.

### Transwell invasion assay

HUVECs were placed on a Transwell Boyden chamber (8- $\mu$ m pore; Corning Inc., Lowell, MA, USA) and precoated with Matrigel for 4 h at 37 °C. 800  $\mu$ L of cell suspension ( $1 \times 10^5$  cells/ml) in FBS-free medium was added into the upper compartment of the chamber. The bottom chambers were supplemented with 2 mL complete medium (10% FBS) containing different concentrations of AG36 (5, 10 and 20  $\mu$ M), VEGF (50 ng/mL) and cisplatin (5  $\mu$ M) are used as positive and negative control, respectively. After 48 h of treatment, the non-migrated cells on the upper face were scraped by a cotton swab. The migrated cells on the lower face were fixed with 4% paraformaldehyde for 20 min, stained with Hematoxylin. After being washed 3 times by PBS, the cells were photographed by a phase-contrast microscope and the migrated cells were quantified and counted.

### Western blotting

After AG36 treatment for 48 h with or without inhibitors LY294002 (1  $\mu$ M) and PF562271 (1.5 nM), the HUVECs were harvested and lysed in total protein extraction reagent with proteinase inhibitors. The protein concentrations were detected using a BCA protein assay kit. Protein samples from treated TNBC cells were separated by SDS-PAGE and further transferred onto PVDF membranes, which were washed and blocked in 5% nonfat dry milk in TBST for 1 h at room temperature. Subsequently, the membranes were washed with TBST buffer and incubated with primary antibodies p-VEGFR2 (#38473), VEGFR2 (#39638), AKT (#8805) and p-Akt (#81283) from Abcam (Cambridge,

MA, USA) and p-FAK(#3159R), VEGF (#1313R) from Bioss (Beijing, China) overnight at 4 °C and then secondary antibody [ $\beta$ -tubulin (#4511R), Bioss] for 1 h at room temperature. The load protein bands were performed using the enhanced chemiluminescent detection reagent (Pierce, Rockford, Illinois, USA).

### In vivo antitumor effects

Female BALB/c nude mice (5 weeks old, 18–19 g) were supplied by Beijing Vital River Laboratory Animal Co. Ltd. (Beijing, China). All care and procedures of all animal experiments were in accordance with the national guideline for the care and use of laboratory animals. MDA-MB-157 cells ( $2 \times 10^7$  cells, 0.1 ml/mouse) were subcutaneously inoculated into the right anterior armpit of nude mice for 7 days, the mice were assigned to 3 groups ( $n = 8$ ) randomly. AG36 was administered i.p. at doses of 0.75 and 1.5 mg/kg/day every 2 days. The control group was injected with the same volume of PBS instead. The tumor volumes were calculated using the following formula: tumor volume ( $\text{mm}^3$ ) =  $0.56 \times \text{length (mm)} \times \text{width}^2$  (square mm).

Mice were sacrificed on the 19th day and the isolated tumors were weighed. The tumor samples were then formalin-fixed and paraffin-embedded. The Sections (4  $\mu$ m) were stained with hematoxylin and eosin (Lengene Biotechnology, Beijing, China) or immunostained with antibodies targeting mouse AKT (1:200), p-AKT (1:200), VEGFR2 (1:200), and p-VEGFR2 (1:200). The immunostaining procedures were performed according to the manufacturer's instructions.

### Docking study

In an effort to elucidate the binding modes of AG36 with VEGFR, it was constructed with Sybyl/Sketch module and optimized using Powell's method with the Tripos force field with convergence criterion set at 0.05 kcal/( $\text{\AA}$ mol), and assigned with Gasteiger-Hückel method. The docking study performed using Sybyl/FlexX module, the residues in a radius of 5.0  $\text{\AA}$  around the VEGFR (PDBID: 1VPP) were selected as the active site. Other docking parameter simplified in the program were kept default.

### Molecular docking

By using Discovery Studio (DS) 3.5 (Molecular Operating Environment), computational docking was performed to elucidate binding modes between VEGFR2 and AG36. X-ray structure (PDB ID: 1VPP) of VEGFR2 kinase domain and its ligand were obtained from Protein Data Bank (<http://www.rcsb.org>). To initiate docking study in DS 3.5, water molecules and heteroatoms were manually removed out

from the protein structures. The Chemistry at HARvard Macromolecular Mechanics (CHARMM) force field was applied and the active site was identified using cavity-based method from receptor cavities. The 3D structure of AG36 was generated through energy minimization using MMFF force field. Then, AG36 was subjected to docking process and the molecular interactions were analyzed and visualized by based on DS consensus scoring function. Hydrogen bond interactions between the ligands and active site residues were also assessed.

### Statistical analysis

All data are presented as mean  $\pm$  SD from three independent experiments. Statistical analysis of the data was performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA). Differences between groups were examined using Student's *t* test. Differences were considered significant if *p* value was less than 0.05.

## Results

### AG36 inhibits the proliferation of human HUVECs

HUVECs play an essential role in angiogenesis, so, we chose HUVECs to evaluate the anti-angiogenic activities of AG36. Firstly, the inhibitory effect of AG36 on HUVECs was investigated by MTT assay. As shown in Fig. 1b, at the concentrations of 1.25–40  $\mu$ M, AG36 inhibited the viability of HUVECs in a dose-dependent manner and the  $IC_{50}$  value was 17.32  $\mu$ M. In the phase contrast observation (Fig. 1c), after AG36 treatment at 5, 10 and 20  $\mu$ M for 48 h, the cell number of HUVECs reduced in a dose-dependent manner and the cell morphology including cell shrinkage and cell rounding was changed. These results indicated the inhibiting activity of AG36 on HUVEC growth.

### AG36 suppresses migration and invasion of HUVEC

Migration and invasion of HUVECs are crucial for tumor-induced angiogenesis. Thus, the effects of AG36 on the metastatic potential of HUVECs were evaluated. We used scratch motility and transwell invasive assays to test the migration and invasion of AG36 on HUVECs, respectively. As shown in Fig. 2a–d, VEGF (positive control) could increase the migration and invasion of HUVEC obviously. In Fig. 2a and c, after AG36 treatment for 48 h, HUVEC migration was suppressed significantly in a dose-dependent manner. As shown in Fig. 2b and d, AG36 dose-dependently inhibited vertical migration to the bottom chamber in the transwell test. In all the scratch motility and transwell assays,

cisplatin (negative control, 5  $\mu$ M) inhibited the migration and invasion of the HUVECs significantly.

### Effects of AG36 on FAK phosphorylation in HUVECs

FAK is a kind of cytoplasmic protein tyrosine kinase which plays vital role in cell proliferation, survival and metastasis. To further investigate the underlying mechanisms, the effects of AG36 on p-FAK in HUVECs were examined. In this experiment, FAK inhibitor (PF562271) was used to confirm the role of FAK inactivation in cell migration inhibition of AG36. As shown in Fig. 3a, b, AG36 could significantly decrease the levels of p-FAK of HUVECs in a dose-dependent manner. PF562271-treatment (1.5 nM) and AG36 treatment (20  $\mu$ M) alone for 48 h both displayed notable inhibitory effects on FAK phosphorylation. Notably, PF562271 pretreatment could markedly enhance AG36-induced inhibition against FAK phosphorylation indicating that FAK dephosphorylation contributed to inhibiting effects of AG36 on HUVEC migration.

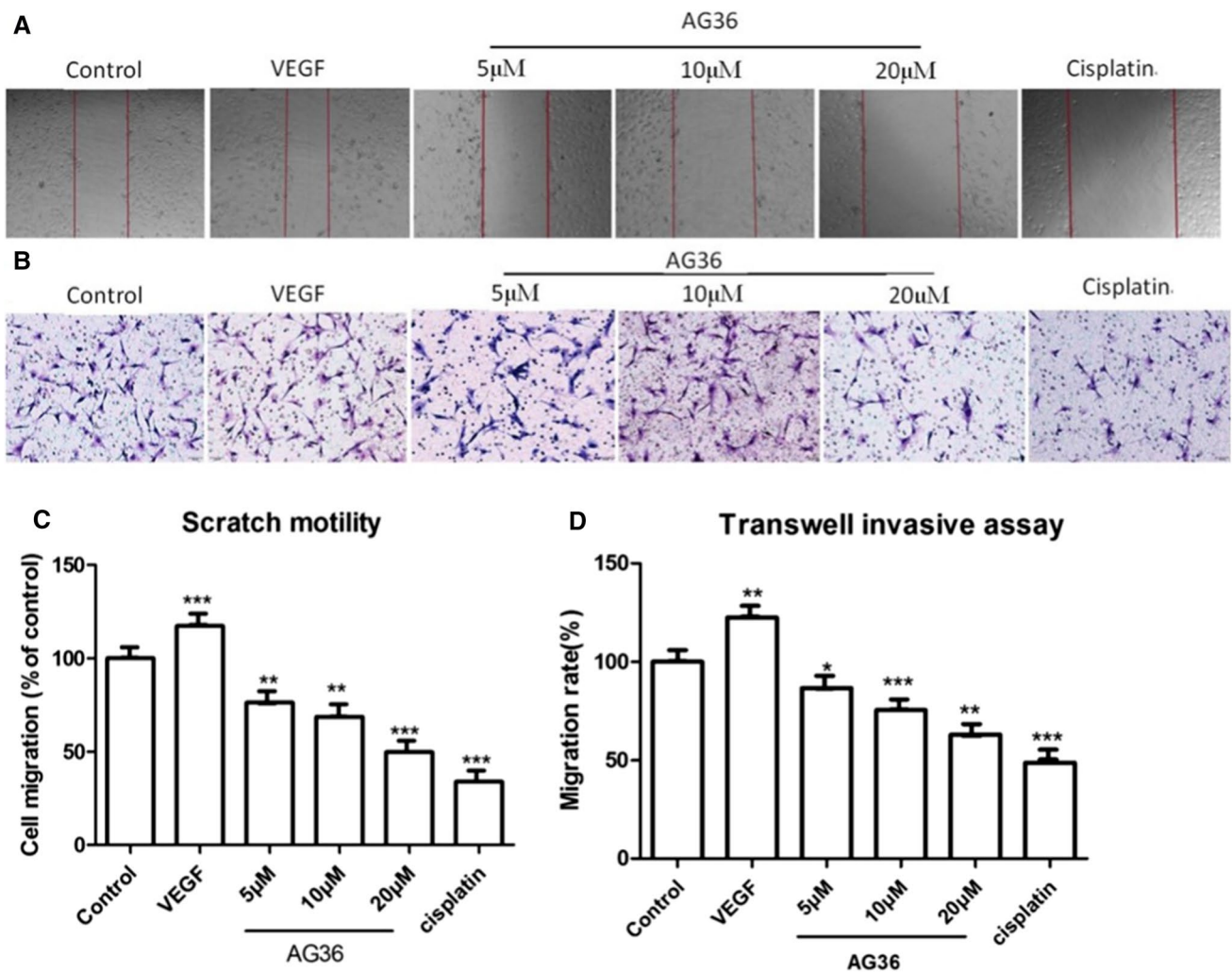
### Effects of AG36 on the VEGF-VEGFR2-AKT in HUVECs

VEGF is crucial for tumor proliferation and angiogenesis, it can interact with VEGFR and regulate the downstream signaling proteins. In this study, we tested the protein levels of VEGF, VEGFR2, p-VEGFR2, AKT and p-AKT in HUVECs by western blotting. As shown in Fig. 4a, AG36 showed little effects on the expression levels of total VEGFR2 and AKT. However, AG36 could decrease the VEGF expression levels significantly, which led to the reduction of p-VEGFR2 and p-AKT. To further verify the role of AKT in the antiangiogenic activities of AG36, the effects of AKT-upstream inhibitor (LY294002) on HUVEC growth were tested. As shown in Fig. 4b, LY294002 didn't show significant effects on cell viability, however in the LY294002 + AG36 group, the cell viability resulted in a significant decrease compared with the AG36 alone group, indicating the key role of AKT inactivation in AG36-induced cell growth inhibition of HUVECs. These results indicated that AG36 inhibited the angiogenesis of HUVECs through the VEGF-VEGFR2-AKT associated pathways.

### AG36 inhibits tumor growth in nude Mice

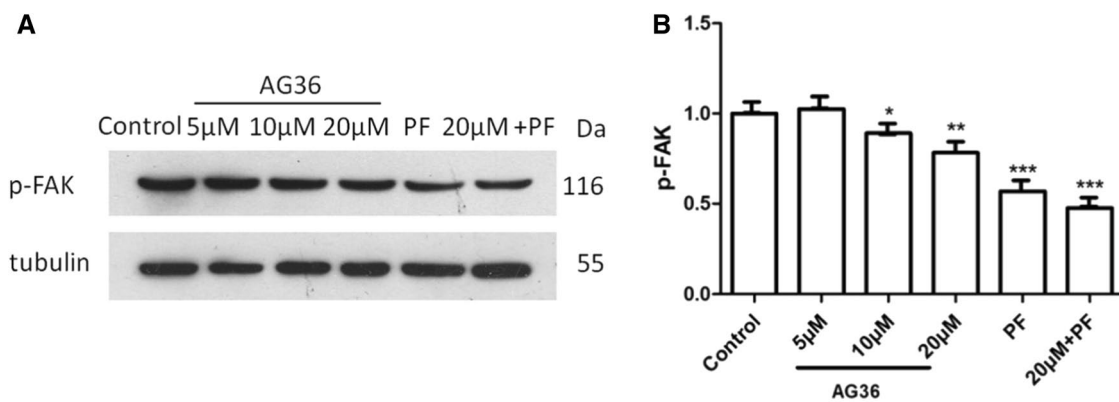
The tumors of the AG36-treated groups were significantly smaller, as compared with those in the control group (Fig. 5a). At the dose of 1.5 mg/kg, AG36 significantly decreased the mean tumor weight ( $p < 0.01$ ) (Fig. 5b). To further assess the inhibitory effects of AG36 on VEGF-VEGFR2-AKT associated pathways in vivo, the hematoxylin and eosin (H & E) analysis of AG36 treatment groups and model group of tumor samples demonstrated tumor growth was inhibited.





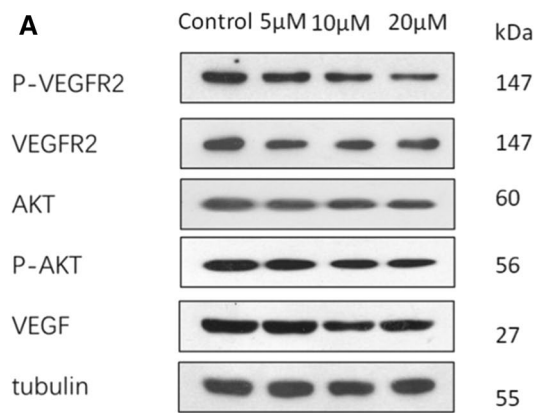
**Fig. 2** AG36 blocked HUVEC migration and invasion. Effects of AG36 on (a) HUVEC migration and (c) invasion. VEGF and cisplatin were used as positive control and negative control, respectively. Experiments details were provided in materials and methods. **b** The

migration ratio and (d) invasion rate were quantified by manual counting. All data are expressed as mean  $\pm$  SD of triplicate experiments. \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$  vs. the control group

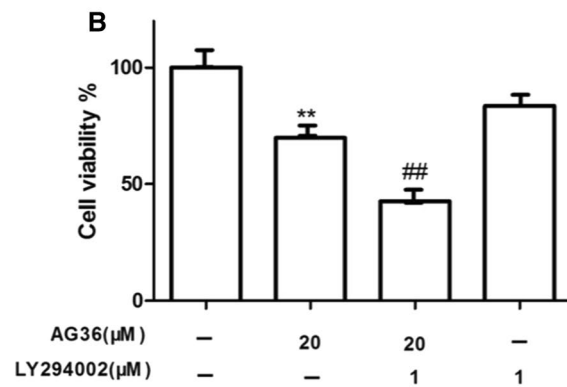


**Fig. 3** Inhibitory effects of AG36 and/or FAK inhibitor (PF562271) on the p-FAK levels of HUVECs. **a** HUVECs were treated with AG36 (5, 10 and 20  $\mu$ M) with or without PF562271 (1.5 nM) for 48 h

and protein expression was analyzed using Western blotting. **b** Bars of p-FAK were expressed as mean  $\pm$  SD of triplicate experiments. \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$  vs. the control group



**Fig. 4** Effects of AG36 on the VEGF-VEGFR2-AKT signaling axis. **a** HUVECs were treated with AG36 (5, 10 and 20 μM) for 48 h and VEGF, VEGFR2, p-VEGFR2, AKT and p-AKT protein expressions were analyzed by Western blotting. **b** Effects of AG36 and/or LY294002 on the HUVEC cell viabilities. Cells were pretreated



with or without 1 μM LY294002 for 1 h and co-treated with 20 μM of AG36 for 48 h. All data are expressed as mean ± SD of triplicate experiments. \*\* $p < 0.01$  vs. the control group and ## $p < 0.01$  vs. the AG36 group

(Figure 5c). Furthermore, At the doses of 0.75 and 1.5 mg/kg, AG36 significantly decreased the expression levels of p-VEGFR2 and p-Akt (brown-stained field) in the xenograft model (Fig. 5d), while AG36 almost showed no effects on the expression levels of total VEGFR2 and AKT, as compared with the control group. These results consist with the in vitro results, suggesting that AG36 is a potent angiogenic inhibitor.

### AG36 docked into the ATP binding pocket of VEGFR2 kinase domain

As AG36 downregulated the p-VEGFR2, we hypothesized that AG36 may interact with VEGFR2 kinase domain. Then, to investigate the possible binding pattern between AG36 and VEGFR2 kinase domain, molecular dockings was performed. As shown in Fig. 6, AG36 docked into the ATP binding pocket of VEGFR2 kinase domain. We can see that glycosyl group extends to one hole of the active site (Fig. 6a). Figure 6b indicates the hydrogen bond density on the surface of VEGFR. As shown in Fig. 6c, there were hydrogen bonds between the saccharide moiety of AG36 and ILE V: 43 and TYR V: 45 residues of ATP binding pocket. Hydrogen bond between the 16-OH of aglycone and LEU V: 32 residue of VEGFR was also observed. In addition, AG36 also moderately interacted with other amino acid residues, including CYS W: 60, CYS W: 68 and GLU W: 64 through the hydrophobic interaction and alkyl bond (Fig. 6c).

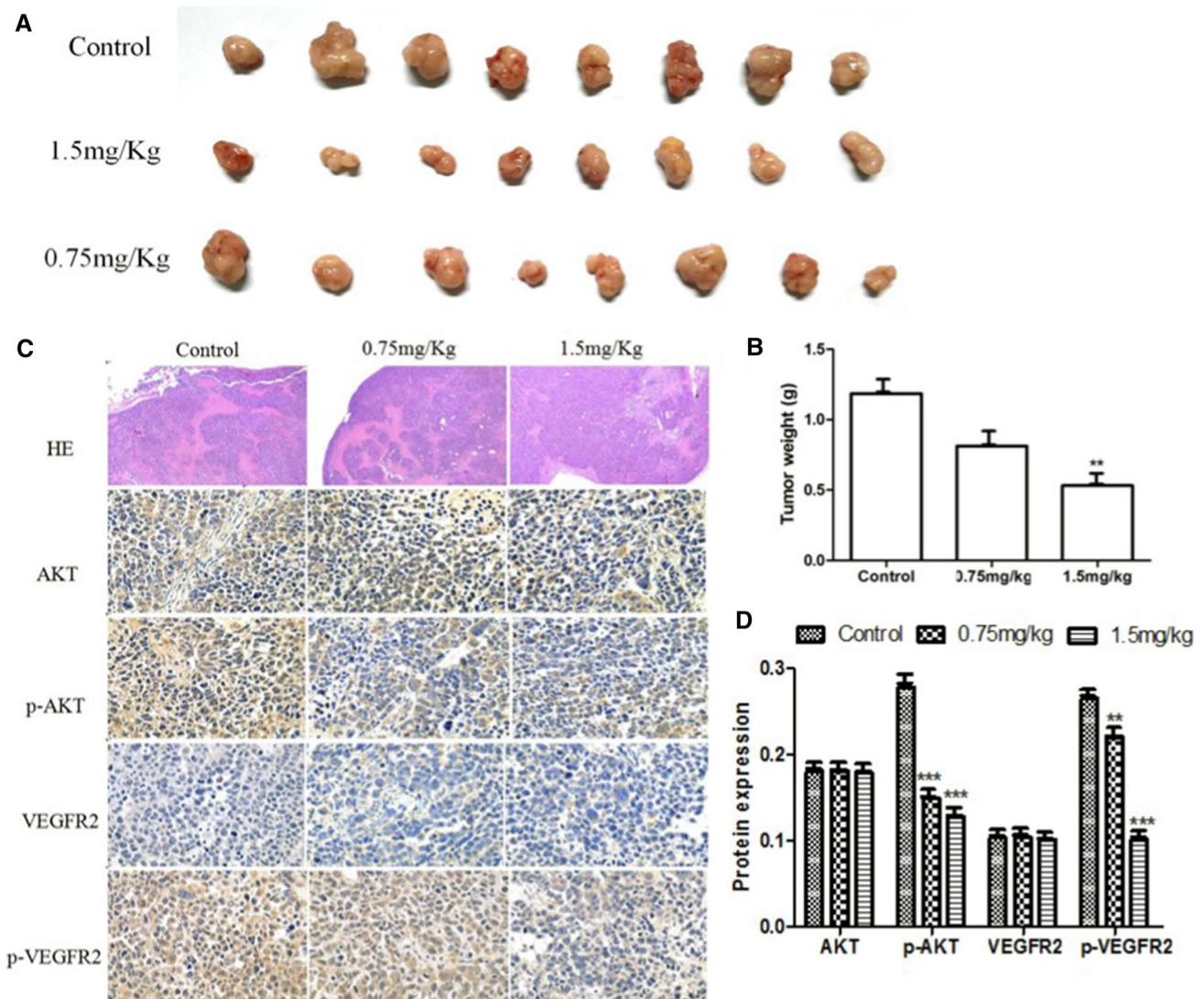
## Discussion

Angiogenesis is crucial for tumor growth and metastasis and is a potential target in cancer treatment [20, 21]. The natural products with antiangiogenic intervention activities could

provide effective anticancer strategy and are now a promising research area. Many natural products have been reported to have antiangiogenic activity in vitro and in vivo. AG36 exerted prominent cytotoxicity against breast cancer cells both in vitro and in vivo [22]. However, it remains unknown whether AG36 can suppress angiogenesis so far. Herein, in this study, we firstly evaluated the antiangiogenic efficacy and associated mechanisms of AG36. AG36 inhibited the progress of angiogenesis including growth, survival and migration of HUVECs. These results indicated that AG36 could suppress the VEGF and/or bFGF axis in endothelial cells.

FAK plays a key role in cellular functions including proliferation, survival and migration and is associated with integrin-mediated signal transduction [23]. The clustering of integrin will cause activation of FAK which result in phosphorylation of the PI3K binding site Tyr397 [24]. The activated FAK triggers the downstream PI3K/Akt signaling pathways, which leads to the activation or over expression of prometastatic proteins including VEGF [23, 25]. In our research, AG36 suppressed the metastatic potential of HUVECs (Fig. 3). In addition, pretreatment of FAK inhibitor (PF562271) could markedly enhance AG36-induced inhibition against FAK phosphorylation, which further confirmed the above result.

VEGF is one of the most crucial mediators in angiogenesis and can effectively promote the metastasis of endothelial cells. VEGF can enhance the migration, invasion and tube formation of HUVEC. However, AG36 significantly suppressed the enhance effects of VEGF, indicating the antiangiogenic potential of AG36. It is reported that the interaction of VEGF and receptors (VEGFR1/2/3) will activate the downstream signaling transduction cascades [26, 27]. Among these VEGF receptors, VEGFR2 plays a major role



**Fig. 5** AG36 inhibited tumor growth and VEGFR2 signaling pathway in xenograft tumor model. **a** Representative images of solid tumor tissues following treatment for 19 days. **b** Tumor weight was measured after treatment for 19 days in each group. **c** Serial sections were processed for hematoxylin and eosin (HE) staining, and immunohis-

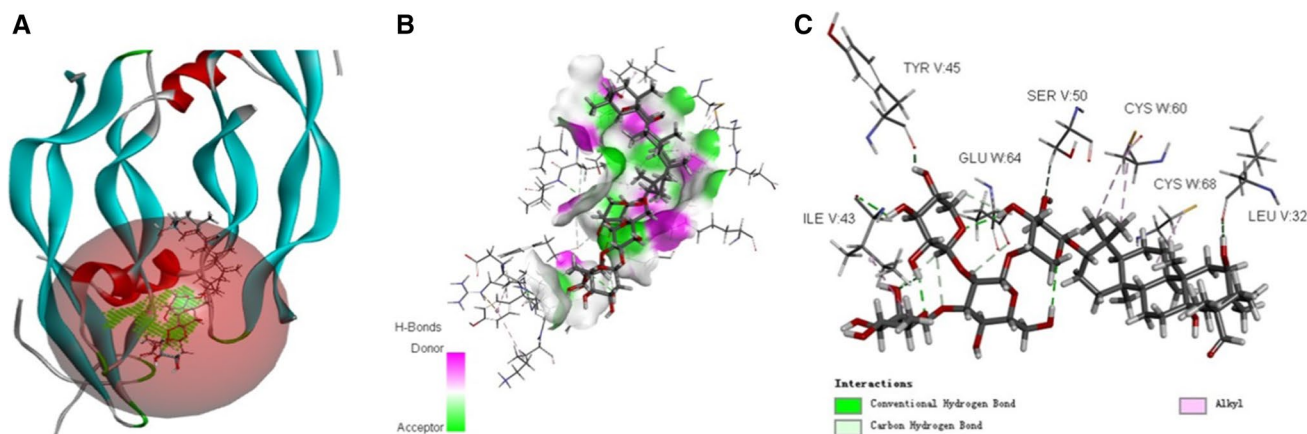
tochemistry with antibodies targeting AKT, p-AKT, VEGFR2 and p-VEGFR2. **d** Bars of AKT, p-AKT, VEGFR2 and p-VEGFR2 in solid tumor tissues were expressed as mean  $\pm$  SD of triplicate experiments. \*\* $p < 0.01$  and \*\*\* $p < 0.001$  vs. the control group

in the angiogenic process. VEGF stimulate VEGFR2 and subsequently activate the downstream pathways, including Ras/MEK/ERK and PI3K/Akt, which can further upregulate the proangiogenic signals [26, 28]. In this research, we found that AG36 significantly downregulated the expression levels of p-AKT and p-VEGFR2 in HUVEC and tumor-bearing mice (Figs. 4 and 5). In addition, pretreatment with the PI3K inhibitor (LY294002) markedly enhanced AG36-induced cell growth inhibition of HUVEC, indicating AG36 inhibited the angiogenesis of HUVECs through the VEGF-VEGFR2-AKT signaling axis.

Some triterpenoid compounds showed antiangiogenic activity through various signaling pathway, including

VEGF/VEGFR2, bFGF/FGFR1 and mTOR/S6K pathways [29–31]. Moreover, some of the saponins showed both antitumor and antiangiogenic activity [32–34]. Molecular docking test indicated that AG36 could stably dock into the ATP binding pocket of VEGFR2 kinase domain (Fig. 6a). Hydrogen bonds between the saccharide and aglycone moiety of AG36 with residues of ATP binding pocket were essential for the stable conformation of VEGFR2-RA complex (Fig. 6b, c). AG36 also moderately interacted with other amino acid residue through the hydrophobic interaction and alkyl bond (Fig. 6c). It seems that the interactions between the saccharide moiety and VEGFR2 contributed more to the stability of VEGFR2-RA complex than that of





**Fig. 6** FlexX docked conformation of AG36 in the active site of VEGFR (PDBID: 1VPP). Molcad surface cavity depth and docking conformation between the AG36 and residues are shown by ribbon

structure (a). Molcad surface H-acceptor/donor density (b). Interactions between the AG36 and residues (c)

the saponin moiety of AG36. The saccharide moiety of AG36 may help improve the hydrophilicity of the compound and facilitate distribution.

AG36 is a potential anticancer triterpenoid saponin, we previously found that it inhibited MCF-7, MDA-MB-231, and SK-BR-3 cells proliferation by the intrinsic mitochondrial and the extrinsic death receptor pathways and significantly inhibited the growth of MCF-7 xenograft tumors in BALB/c nude mice. In this research, our findings demonstrated for the first time that AG36 showed potent antiangiogenic activity through VEGF–VEGFR2–AKT/FAK signaling axis inhibition *in vitro* and *in vivo*, which means it may be an applicable as a treatment for antiangiogenic cancer.

## Conclusions

The present work firstly demonstrated that AG36 inhibit the proliferation, migration and invasion of HUVEC through suppressing phosphorylated FAK and AKT, down-regulating the expressions of VEGF and VEGFR2. Molecular docking simulation further confirmed the interactions between AG36 and VEGFR2. Our finding provides a new insight into the molecular mechanism of AG36 for cancer intervention and suggests that AG36 may be a potential agent for translational antiangiogenic cancer therapy.

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## Compliance with ethical standards

**Conflict of interest** All the authors declare no conflict of interest regarding the publication of this paper.

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## Affiliations

Li-Hua Mu<sup>1</sup> · Li-Hua Wang<sup>1</sup> · Yu-Ning Wang<sup>2</sup> · Ping Liu<sup>1</sup> · Can Yan<sup>3,4</sup>

<sup>1</sup> Medical Supplies Center, Chinese PLA General Hospital, Beijing 100853, China

<sup>2</sup> First Medical Center, Chinese PLA General Hospital, Beijing 100853, China

<sup>3</sup> Department of Basic Theory of Chinese Medicine, School of Pre-Clinical Medicine, Guangzhou University of Chinese Medicine, Guangzhou 510060, China

<sup>4</sup> The Research Centre of Basic Integrative Medicine, Guangzhou University of Chinese Medicine, Guangzhou 510060, China