Anti-angiogenic activity of ShengMaBieJia decoction *in vitro* and in acute myeloid leukaemia tumour-bearing mouse models

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**ABSTRACT**

**Context:** ShengMaBieJia decoction (SMBJD) is used to treat solid and hematological tumours; however, its anti-angiogenesis activity remains unclear.

**Objective:** This study verified the anti-angiogenic effects of SMBJD *in vitro* and in tumour-bearing acute myeloid leukaemia (AML) mouse models.

**Materials and methods:** *In vivo*, the chicken chorioallantoic membrane (CAM) and BALB/c null mouse xenograft models were treated with SMBJD (0, 2, 4, and 8 mg/mL) for 48 h and for 2 weeks, respectively. Anti-angiogenic activity was assessed according to microvessel density (MVD) and immunohistochemistry (IHC) targeting CD31 and VEGFR2. *In vitro*, proliferation viability, migratory activity and tube formation were measured. Western blots and polymerase chain reaction (PCR) assays were used to examine the levels of PI3K, Akt, and VEGF.

**Results:** HPLC analyses revealed the active constituents of SMBJD such as liquiritin, cimifugin, ferulic, isoflavan, and glycyrrhizic acids. In *vitro*, SMBJD treatment decreased cellular migration, chemotaxis, and tube formation at non-cytotoxic concentrations (2, 4, and 8 mg/mL) in a time- and dose-dependent manner. The dosage of less than IC20 is considered safe. *In vivo*, CAM models exhibited a decrease in MVD, and the tissues of xenografted mice possessed reduced CD31 and VEGFR2 expression. Conditioned media (CM) from AML cells (HL60 and NB4 cells) treated with non-cytotoxic doses of SMBJD inhibited chemotactic migration and tube formation *in vitro*. Both CM (HL60) and CM (NB4) exhibited downregulated expression of PI3K, Akt, and VEGF.

**Discussion and conclusions:** SMBJD inhibited angiogenesis in AML through the PI3K/AKT pathway, which might be combined with targeted therapy to provide more effective treatment.

**Introduction**

Angiogenesis and vasculogenesis are ubiquitous throughout physiology. In the embryo, angioblasts induce vasculogenesis followed by angiogenesis to allow for oxygen transport and tissue remodelling (Boulais and Frenette 2015). In the adult, vessels remain in a quiescent condition until they are activated by certain signals. Angiogenesis, the process by which vessels sprout from existing vascular, has been considered as a hallmark of cancer due to the abnormal hyperplasia observed in tumours (Carmeliet 2003; Hanahan and Weinberg 2011). The concept of angiogenesis in solid tumours was proposed at the beginning of the twentieth century, and it was later confirmed in 1971 in a study examining liquid/hematological diseases (Goldmann 1908; Folkman 1971).

Tumour-derived vessels exhibit an abnormal and imbalanced morphology compared to that of normal vasculature (Folkman 1995; Carmeliet and Jain 2000; Hanahan and Weinberg 2011). This regeneration is mediated by an ‘angiogenic switch’ that improves the imbalance of the internal microenvironment of malignant tumours in humans (Carmeliet and Jain 2000; Weis and Cheresh 2011). The pathological vasculature conveys oxygen and nutrients to neoplasms to promote further growth and invasion, and it even accelerates mutations (Bikfalvi 1995; Szot et al. 2013). The development of tumour-derived vessels indicates a poor prognosis with potential tumour recurrence. This has been confirmed in several solid neoplasms following initial treatment (Li et al. 2018). Moreover, angiogenesis in hematological tumours has also been verified to play a crucial role in stimulating disease procession (Aguayo et al. 2003; Medinger and Mross 2010; Zheng et al. 2016).

Acute myeloid leukaemia (AML) is a hematological malignancy derived from haematopoietic stem cells (HSCs) that manifests as myeloid hypertonphy and mutations (Dohner et al. 2015). Bone marrow angiogenesis, known as sinusoid angiogenesis, possesses unique characteristics compared to solid tumours. The cross-talk between haematopoietic stem cells (HSCs) and endothelial cells (ECs) plays a crucial role in AML due to the autocrine and paracrine signalling that occurs between them (Lo Celso et al. 2009; Haouas 2014; Boulais and Frenette 2015). Clinical data analyses revealed further evidence of angiogenesis...
in AML, and these analyses included microvessel counting in bone marrow biopsies, immunohistochemical staining for CD34, and reverse transcriptase polymerase chain reaction (RT-PCR) assays (Hussong et al. 2000; Padró et al. 2000; Rabitsch et al. 2009). *In vitro* models induced by leukemic cells reveal a stimulating effect on ECs that resulted in angiogenesis in a 3D biomimetic device (Zheng et al. 2016).

Physiological and pathological vessels are both generated based on the balance between pro- and anti-angiogenic factors, among which VEGF and its receptor (VEGFR) play a predominant role (Bikfalvi 1995; Vancopoulou et al. 2000; Shibuya 2013). Clinical data have revealed that a higher level of VEGF-A and VEGF-C is expressed in AML (Hussong et al. 2000; Padró et al. 2002; Mohammadi Najafabadi et al. 2017). Similar findings were noted for the receptors VEGFR1 and VEGFR2 (Hussong et al. 2000; Padró et al. 2002; Mohammadi Najafabadi et al. 2017). Based on this, VEGF has been used as a therapeutic target to achieve a better prognosis in a number of malignancies, and several VEGF-targeting drugs such as Bevacizumab (Prantl) Tanaka, (Ranunculaceae), turtle shell (*Carapax trionycis*), 10 (Xiao et al. 2015), *Marsdenia tenacissima* (Roxb.) Moon. (Apocynaceae), *Indigofera acanthinocarpa* Blatt. (Fabaceae), *Angelica acutiloba* var. *iwatensis* (Kitag.) Hikino. (Apiaceae), and *Liquiritia officinalis* Moench. (Leguminosae). Recent pharmacological studies demonstrated the effects of single herb treatment in regard to cancer and inflammatory diseases, and these effects included cancer cytotoxicity, anti-angiogenesis, and the regulation of immunity (Einbond et al. 2004; Hostanska et al. 2005; Cao et al. 2010; Chen et al. 2016; Han et al. 2017). Previous experiments have revealed the effects of this herbal extract in regard to its lethal effect on leukemic cells and its inhibition of tumour growth both *in vitro* and *in vivo*. It has been demonstrated that SMBJD combined with CAG (Aclacinomycin, Ara-c, G-CSF) chemotherapy can lower the ECOG (Eastern Cooperative Oncology Group) performance status, extend the interval of blood transfusion, and optimize the Traditional Chinese Medicine Syndrome Score Scale (Dai et al. 2016). In addition to its pro-apoptotic effects, we hypothesized that SMBJD exerted anti-angiogenic effects via the PI3K/Akt pathway.

Materials and methods

**Preparation of SMBJD**

SMBJD is a compound composed of certain traditional herbs. The components used are displayed in Table 1. All herbs were purchased and processed from the Jiangsu Province Hospital of Chinese Medicine and the affiliated Hospital of the Nanjing University of CM. The combined materials (100 g) were boiled in 1 L of double distilled water (ddH2O). After 1 h, we collected the supernatant and added another 1 L of ddH2O to the materials with boiling for an additional 1 h. Subsequently, the concentrated fraction of the two extracted supernatants was mixed to a final solution of 100 mL and filtrated using medical gauze. The final storage concentration of SMBJD was 1 g/mL. Prior to its application, the supernatant was autoclaved and filtered through a 0.22 µm size membrane filter to avoid bacterial contamination. Finally, the samples were stored at −20°C until further analysis.

**High-performance liquid chromatography (HPLC) analysis for SMBJD**

The six main ingredients in SMBJD (1 g/mL) were determined using an Agilent 1260 liquid chromatography system (Agilent

| Herbs name                        | Part used   | Traditional effect                                      | Dosage (g) |
|-----------------------------------|-------------|---------------------------------------------------------|------------|
| *Cimicifuga acerina* (Prantl) Tanaka | root        | Clearing heat, removing toxicity, promoting Yang Qi, treating papule | 35         |
| *Marsdenia tenacissima* (Roxb.) Moon | vine        | Clearing heat, removing toxicity, promoting lactation, relieving cough and asthma | 40         |
| *Carpapax trionycis*              | carapace    | Nourishing Yin, anti-febrile, softening hardness         | 10         |
| *Indigofera acanthinocarpa* Blatt | powder from indigo plant | Clearing heat, removing toxicity, cooling blood, Purging Fire, arresting convulsion | 5          |
| *Angelica acutiloba* var. *iwatensis* (Kitag.) Hikino | roots       | Supplementing blood, relieving pain, regulating menstruation, relaxing the bowel to relieve constipation | 5          |
| *Liquiritia officinalis* Moench. | roots       | Relieving cough and asthma, relieving pain, reinforcing Qi, Clearing heat, removing toxicity | 5          |
Technologies, Palo Alto, CA). Briefly, 10 µL SMBJD was injected into the apparatus using an auto sampler. The chromatographic separation was achieved at a flow rate of 1 mL/min through an Agilent Zorbax SB-C18 column (4.6 x 250 mm, 5 µm). The mobile phase was composed of solvent A (0.1% phosphoric acid) and solvent B (acetonitrile). A linear gradient was set from 2% to 18% consisting of solvent B for 0–8 min, and this was followed by isocratic inflow of 18% solvent B for 8–20 min, 18–25% solvent B for 20–30 min, 25–45% solvent B for 30–40 min, 45–60% solvent B for 40–50 min, and 60–2% solvent B for 50–51 min. The separation temperature was maintained at a constant 40°C with a detection wavelength of 230 nm. All major constituents in the decoction are shown in Figure 1.

Cell culture and the preparation of conditional medium (CM)
The human acute leukaemia (HAL) cell lines-HL60 and NB4 and human umbilical vein endothelial cells (HUVECs) were supplied by the Central Laboratory of the Jiangsu Province Hospital of Chinese Medicine and Affiliated Hospital of Nanjing University of Chinese Medicine. Both cell types were cultured under humidity conditions at 37°C in the presence of 5% CO2. HL60 and NB4 cells were cultured in Roswell Park Memorial Institute medium (RPMI-1640) with 10% foetal bovine serum (FBS) (Gibco, Carlsbad, CA), while HUVECs were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% FBS. HUVECs were digested by trypsin (Beyotime, Shanghai, China) and washed using phosphate buffer solution (PBS) (Gibco, Carlsbad, CA). The cells at the second to sixth passages were used for this experiment. All experiments were repeated in triplicate.

AML cells (5 x 10^5/mL) were cultured in RPMI-1640 complete medium with or without SMBJD (2, 4, or 8 mg/mL) for 24 h. The conditioned medium (CM) was collected and centrifuged at 2000 rpm for 10 min twice to obtain the supernatant. The samples were stored at 4°C for use in subsequent experiments.

The formation of chicken chorioallantoic membrane (CAM) models
The human acute leukaemia (HAL) cell lines-HL60 and NB4 and human umbilical vein endothelial cells (HUVECs) were supplied by the Central Laboratory of the Jiangsu Province Hospital of Chinese Medicine and Affiliated Hospital of Nanjing University of Chinese Medicine. Both cell types were cultured under humidity conditions at 37°C in the presence of 5% CO2. HL60 and NB4 cells were cultured in Roswell Park Memorial Institute medium (RPMI-1640) with 10% foetal bovine serum (FBS) (Gibco, Carlsbad, CA), while HUVECs were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% FBS. HUVECs were digested by trypsin (Beyotime, Shanghai, China) and washed using phosphate buffer solution (PBS) (Gibco, Carlsbad, CA). The cells at the second to sixth passages were used for this experiment. All experiments were repeated in triplicate.

Animal xenograft models
Female nude mice (n = 20) that were 6 weeks-old were grown in a laboratory animal environment according to the Experimental Animal Ethics Committee of the Nanjing University of Chinese Medicine. HL60 cells were collected and injected into the armpit of the right forelimb of the mice subcutaneously at a density of 5 x 10^7. Following two weeks of growth, the neoplasms exhibited dimensions of 3 mm x 3 mm. At this stage, the tumour-bearing model was considered successful. For different interventions, tumour-bearing mice were divided into four groups (4 mice per group) that included a control saline group (0.2 mL/10 g) and low-, middle-, and high-dosage SMBJD groups (0.2 mL/10 g, 20, 40, and 80 g/kg). The animals possessing their respective xenografts were gavaged daily for 2 weeks. The tumour width and weight of the mice was measured twice per week. The tumour tissues were collected for immunohistochemical analysis.

Immunohistochemical staining
The tissues from the animal xenograft models were fixed with paraformaldehyde, cut into blocks, and dewaxed. According to the used protocol, the sections of the tissues were incubated with rabbit monoclonal primary antibodies against CD31 and VEGFR2 (Proteintech Group, Chicago, IL), and this was followed by treatment with peroxidase-conjugated secondary antibodies.
The images were acquired using an Olympus microscope (Olympus, Tokyo, Japan).

**Cell proliferation and viability assays**

HUVECs (2 × 10^4/mL) were seeded into 96-well-plates and allowed to adhere. Subsequently, the supernatants from the CM of HL60 or NB4 cells were added, and the samples were incubated for 24 and 48 h. A final volume of 15 μL/well of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was added at 37°C in the presence of 5% CO2. Subsequently, 150 μL/well of DMSO was added, and this was followed by a 4 h of the cells in the presence of MTT. The samples were shaken for 10 min, and the OD value was measured by spectrophotometry (BIO-RAD) at 570 nm according to the manufacturer’s protocol (Sigma, St. Louis, MO). At least three independent experiments were performed, and each sample was assessed five times. The results were measured according to the ratio of the absorbance values corresponding to the test and the control groups.

**Wound healing assay**

HUVECs were transferred at a density of 6 × 10^4/well into 6-well plates in the presence of complete medium. The samples were incubated at 37°C in the presence of 5% CO2. Following a 24 h incubation period, the cells adhered to the surface, and a scratch was made at the bottom of the plates using a 200 μL pipet tip. The cells were washed 2 times with PBS to remove non-adherent cells. The culture medium was altered to CM for HL60 or NB4 cells. The observation of the differences at 0, 12, and 24 h allowed for the measurement of the gap area by Image J. The data were processed to allow for the observation of an increased or decreased rate.

**Transwell assay**

Prior to the experiment, Matrigel (BD, Franklin Lakes, NJ) was prepared at 4°C, and the 200 μL pipette tips were precooled. The bottom of the upper compartment of the transwell insert was covered using 70 μL Matrigel (diluted for 1:29) and cultured at 37°C. The wells were incubated for 30 min. HUVECs (2 × 10^4/well) were seeded and cultured in DMEM without FBS. The lower compartment was prepared with complete medium containing 2% FBS or CM and incubated at 37°C in the presence of 5% CO2 for 24 h. Subsequently, the non-chemotactic cells were fixed with methanol, washed with PBS, and stained for 30 min. The cell number was counted using a microscope.

**Tube formation assay**

The previously described prepared matrigel samples were stored at 4°C overnight and were thawed the following day, while the 96-well plates and tips were precooled at 4°C for 30 min prior to the preparation. Matrigel (70 μL/well) was coated onto 96-well plates and cultured in a 37°C incubator for 30 min. HUVECs were seeded into a 96-well plate at a density of 4 × 10^4/well. The cells (HL60 and NB4) were cultivated with CM. Each experiment was repeated in triplicate. The assessment of tube formation was performed by counting the number of nodes, junctions, segments, and branches using Image J software (NIH Image J system, Bethesda, MD).

**Western blot**

HUVECs were seeded into 6-well plates with CM at a density of 6 × 10^4/well. Following 24 h of incubation, RIPA lysis buffer that contained protease inhibitor cocktail was used for cell lysis. The procedure was performed for 15 min. The lysates were subjected to ultrasonication for 60 s (4 times of 15 s each) and centrifuged at 20 g for 20 min at 4°C. The protein concentration was measured, and equal amounts of protein were added to the 10% SDS gel. The proteins were separated by PAGE, and the membranes were transferred for 1 h at 250 mA on ice. The membranes were blocked in TBST containing 5% skim milk for 1 h at room temperature on a shaking platform. Subsequently, the membranes were incubated with primary antibodies against VEGF (1:500–1000, Proteintechn, Rosemont, IL), PI3K (1:1000, CST, DeKalb, IL), phosphorylated PI3K (1:2000, CST, DeKalb, IL), AKT (1:1000, CST, DeKalb, IL), and phosphorylated AKT (1:1000, CST, DeKalb, IL) at 4°C overnight. The next morning, the membranes were washed in TBST, incubated with rabbit secondary antibody for 1 h at room temperature, and visualized using ChemiDOC XRS+ (BIO-RAD, Hercules, CA). Measurement of the OD value was achieved using Image Lab. The values from the different bands were expressed as fold of control after normalization to β-actin.

**Real time PCR analysis**

HUVECs were seeded at a density of 3 × 10^4/mL into 6-well plates with complete medium or CM (HL60 or NB4 cells) for 24 h. Total RNA was isolated using the Trizol (Ambion, USA) reagent and chloroform. RNA was used for reverse-transcription to synthesise the cDNA. Quantitative real-time PCR analysis was performed using the 7500 Fast Real-Time System (Thermo Fisher Scientific, USA). The mixed reaction was composed of SYBR Green dye and master mix (Takara, Japan), forward primers, reverse primers (Invitrogen, CA, USA), and DEPC H2O. The thermal cycling conditions included the following steps: initial denaturation at 50°C and 95°C for 10 min each; 40 cycles of initial denaturation at 95°C for 1 min. β-Actin was used to normalise gene expression levels. The mRNA abundance was analysed using the comparative threshold cycle (2^−ΔΔCT) method. The primer sequences used included the following: actin: TCACCCACACTGTGCCCATCTACGA (F), CAGCGGAACCGCTCA TTGCCAATGG(R); PI3K: TTGTTTCATAGCAGCATGGTC(F), ATGGAAGACGGGAGATTCC(R); AKT: TCACCATCACACCAGCTCAG(R); VEGF: CCACTGGAGGAGTCCACAT(F), TGCCCTTCTGGAACTGATT(R).

**Statistical analysis**

The two-tailed Student’s t-test was used to compare the differences between the two experimental groups. One-way analysis of variance (ANOVA) and factorial analysis were used to evaluate differences in more than two groups. SPSS 24.0, Image J, and Graph Pad 7.0 software packages were used for image processing. In all cases, a p-value lower than 0.05 (p < 0.05) was considered to be indicative of statistically significant differences.
Results

**SMBJD reduces vasculogenesis in vivo**

In CAM models, vascular growth was decreased and exhibited a trend of dose-dependent inhibition (Figure 2(A)). Additionally, xenograft models were established and analysed. We established mouse xenograft models and demonstrated that SMBJD decreased tumour width on days 7, 10, and 13 compared to that of the control. However, there was no significant difference between the control group and the intervention group. The weight of the experimental mice also exhibited a significant difference compared to that noted in the control group. (C) The width of tumour did not exhibit a significant difference among the different groups. (D,E) SMBJD suppressed the expression levels of CD31 and VEGFR2 in mouse xenograft models treated with or without SMBJD (2, 4, and 8 mg/mL). *p < 0.05, **p > 0.05.

The results revealed that SMBJD decreased the positive expression of both markers (Figure 2(D,E)). Our findings illustrated the anti-tumour and anti-angiogenic effects of SMBJD in vivo.

**SMBJD impeded the angiogenic ability of HUVECs in vitro**

The inhibition of cell viability caused by SMBJD treatment in HUVECs and AML cells was assessed using (Figure 3(A,C,D)) the MTT assay, and our results indicated that SMBJD treatment resulted in a dose-independent inhibition of cell growth. Moreover, exposure of HUVECs to the CM derived from HL60 and NB4 cells resulted in an increase in cell viability (Figure 3(B)). Cell viability was decreased in response to increasing concentrations of SMBJD within the CM (Figure 3(E,F)). To exclude false-positive results due to cell death, specific concentrations of
SMBJD (2, 4, and 8 mg/mL) were used for further experiments. The rate of HUVECs migration was considered significant. Chemotactic analysis indicated that the higher dose of SMBJD reduced the movement of the cells from the upper compartment of the transwell insert to the bottom (Figure 4(A–C)). Wound healing assays indicated that the wound of the control group was almost healed, while the wounds from the other groups were not visibly altered (Figure 4(D,E)). The results indicated a concentration-dependent regulation of angiogenesis by SMBJD. The tube formation assay demonstrated that increasing concentrations of SMBJD suppressed tube formation by HUVECs (Figure 5), and at a higher dosage of the herbal extract, the decrease in the formation of vascular tubes was higher. Vascular endothelial cells were negatively regulated by SMBJD treatment. This suppression was indicated by a lower number of nodes, junctions, and branches.

**The protein and mRNA expression levels of VEGF and PI3K/akt are downregulated in HUVECs**

Western blot and Real Time PCR analyses were used to measure the expression levels of VEGF protein and mRNA, respectively. The results indicated that the protein expression levels of VEGF were decreased in HL60 and NB4 cells treated with a single dose of SMBJD. We also cultured HUVECs using CM from HL60 and NB4 cells to observe the effect of the supernatant lacking SMBJD, and our results revealed only a small promotion of VEGF (Figure 6(A)). This indicated that CM promoted the proliferation of HUVECs. To verify the impact of SMBJD on the cross-talk between AML cells and endothelial cells, we cultured HUVECs in CM supplemented with or without SMBJD. Interestingly, we observed that the activity of HUVECs in the CM groups was inhibited following an increase in the concentration of SMBJD treatment. The results indicated that in addition to VEGF, PI3K and Akt were also down-regulated (Figure 6(B,C)). Moreover, the mRNA levels of VEGF, PI3K, and Akt were down-regulated (Figure 6(D)). Taken together, these data indicate that SMBJD inhibited angiogenesis by down-regulating the relative protein and mRNA levels in AML cells and HUVECs.

**Discussion**

Anti-angiogenic therapy has been developed in response to the incidence of angiogenesis in both solid and liquid cancers. Over the last few decades, anti-angiogenic drugs have been considered...
as an appropriate treatment for certain solid tumours and for hematological malignancies. Various compounds have been designed and evaluated for their ability to inhibit tumour vessel formation. VEGF has been widely studied by various research groups. Since 2004, novel drugs have been assessed through in vitro experiments or clinical trials. Chinese herbal

Figure 4. SMBJD suppresses the chemotactic activities and migratory of HUVECs in vitro. (A–C) SMBJD and CM decreased the chemotactic activity of HUVECs. (D–E) Wound healing assay demonstrated that the CM (0 mg/mL) group exhibited lower gap area, whereas CM with 2, 4, and 8 mg/mL SMBJD indicated a larger gap. *p < 0.05.
prescriptions have been used to treat neoplastic disease as a supplementary therapy for thousands of years. According to traditional Chinese medicine, cancer belongs to the category of neoplasms based on its anatomy or to the category of consumptive diseases based on the associated symptoms. Consequently, identifying the role of prescriptions that could provide a beneficial choice for the treatment of various malignancies is necessary.

Figure 5. SMBJD impedes tube formation of HUVECs in vitro. (A) The tube of HUVECs treated with single SMBJD is inhibit compared to control group. (B,C) Increasing concentrations of SMBJD in CM mediated different suppressive activities in tube formation. The statistical comparisons were performed on the number of nodes, junctions, segments and branches. The number of tube points was also quantified. *p < 0.05.
SMBJD has long been used for the treatment of not only dermatosis and immunological diseases, but also for neoplastic disease. A preliminary study by our group revealed in vitro anti-leukemic and cancer growth inhibitory effects of SMBJD. We demonstrated that this treatment induced AML cell apoptosis via the MAPK signalling pathway in a concentration- and time-dependent manner. Subsequently, we explored if this regimen exerted anti-angiogenic effects on AML in vivo and in vitro. The expression of specific cytokines that exhibit crosstalk with the VEGF signalling pathway was investigated. VEGF is widely known as a significant contributor to angiogenesis, notably in tumour neovascularization. Our present results demonstrated that SMBJD inhibited the secretion of VEGF in HUVECs and AML cells at a non-cytotoxic dosage. Furthermore, we examined if SMBJD could reduce the migratory and chemotactic activities of HUVECs and also their tube formation in vitro at a non-cytotoxicity dosage. Moreover, specific functions of HUVECs cultured with CM from HL60 and NB4 cells were inhibited, including metastatic activity and angiogenesis. Interestingly, the group that received CM intervention was more effective in

Figure 6. SMBJD down-regulates the expression of VEGF, PI3K and Akt. (A) SMBJD decreases the level of VEGF in vitro. The secretion of VEGF is inhibited in a non-cytotoxicity concentration on AML cell lines. HUVECs intervened by conditional medium (CM) of HL60 and NB4 cells shows an upgrade proliferation. (B,C) According the dosage of SMBJD increased, the relative protein expression of HUVECs cultured with CM shows a down-regulated trend. Additionally, (D) the relative mRNA level is also decreased compared with that without SMBJD. *p < 0.05.
regard to anti-neovascularization compared to that in the HUVECs cultured with 1640 medium alone. Hence, a better understanding of the results may aid in developing more effective treatments, as AML cells activate endothelial cell proliferation and migration by secreting endothelial growth factors. In vivo, the MVD of CAM models was decreased in a dose-dependent manner. The expression of VEGFR2 and CD31 was down-regulated in mice xenograft models. However, the tumour width did not decrease as much as did the animal weight, and this may provide a direction for further research. These findings may be due to an inadequate duration of treatment or may result from the phenomenon of progression-free survival (PFS).

Furthermore, we assessed the expression levels of specific proteins associated with angiogenesis. The VEGF signalling pathway is a classical pathway in angiogenesis, and it has been demonstrated to be important in the mechanism of SMBJD action (Chin et al. 2018; Deng et al. 2015; Olsson et al. 2006). Numerous studies have shown that the regulation of the expression levels of angiogenesis-associated proteins such as VEGF, PI3K, and AKT can influence tumour angiogenesis and further reduce tumour metastasis (Gerber et al. 1998; Han et al. 2018; Sun et al. 2018). The protein levels and mRNA expression levels of VEGF and PI3K/AKT were downregulated by SMBJD treatment. Based on this, we speculated that SMBJD interrupted relevant signalling pathways to achieve anti-angiogenic and anti-tumour functions. Tumour growth is intensified under hypoxic and/or ischaemic conditions that further stimulate the up-regulation of PI3K and AKT and the release of VEGF. Potential cross-talk between these pathways can be investigated in future studies.

Our data suggested that SMBJD decreased the proliferation, metastasis, and tube formation of HUVECs by downregulating the expression levels of certain proteins involved in key signalling pathways. These findings confirmed the anti-angiogenic effects of SMBJD in AML both in vitro and in vivo, and these findings can be further investigated in solid tumours or other hematological diseases.

Disclosure statement

All authors declare that they have no any conflict of interests.

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Data availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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