**Research Article**

**DHPAC, a novel microtubule depolymerizing agent, suppresses angiogenesis and vasculogenic mimicry formation of human non-small cell lung cancer**

**Running title**: The anti-angiogenic and anti-vasculogenic mimicry effects of DHPAC

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**Abstract**

Angiogenesis and vasculogenic mimicry (VM) are the main causes for the tumor metastasis and recurrence. In this study, we investigated the anti-angiogenesis and anti-VM formation of a novel microtubule depolymerizing agent, DHPAC, as well as combretastatin A4 (CA4, a combretastatin derivate) in NSCLC (non-small cell lung cancer), subsequently elucidating the underlying mechanisms. In HUVECs, DHPAC could enter cells and inhibit the proliferation, migration and angiogenesis in the presence and absence of conditioned medium from H1299 cells. Interestingly, the inhibition was enhanced under the stimulation of conditioned medium. Under hypoxia or normoxia, DHPAC suppressed STAT3 phosphorylation and reduced VEGF expression and secretion from HUVECs, thus impeding the activation of the downstream signal transduction pathway of VEGF/VEGFR2. However, JNK inhibitors reversed the inhibitory effect of DHPAC on the angiogenesis, suggesting that DHPAC regulated angiogenesis through activating JNK. In H1299 cells, DHPAC could inhibit proliferation, migration, invasion, and the formation of VM. What’s more, DHPAC inhibited the phosphorylation of FAK and AKT and decreased the expressions of VEGF, MMP2, MMP9 and Laminin 5, suggesting that DHPAC inhibited VM formation via FAK/AKT signaling pathway. In addition, CA4 showed the similar effect as DHPAC against angiogenesis and VM formation. These new findings will support the microtubule destabilizing agents as a promising strategy for cancer therapy.

**Key words**: Microtubule depolymerizing agent; Angiogenesis; Vasculogenic mimicry; JNK/VEGF; FAK/AKT

**1. Introduction**

The formation of new blood vessels within tumor is fundamentally essential in cancer development. It provides essential oxygen and nutrients to allow rapid growth of tumor cells and also allow rapid disposal of waste (Folkman & Judah, 2002; Morse et al.). Inhibition of angiogenesis is one of the important therapeutic treatments of cancer (Chung, Lee, & Ferrara, 2010; Ho, Wang, Fan, & Yeh, 2017). In comparison to traditional anti-cancer therapies that use chemotherapeutic agents to kill tumor cells, therapies against tumor angiogenesis have a number of advantages. For example, they have stronger effect on blood vessels formation in the fast-growing tumor than in normal tissues in the body thus provide good specificity against tumor; therapeutic drugs targeted angiogenesis often act directly on vascular endothelial cells that are relatively more stable and less susceptible to drug resistance than tumor cells.

In addition to traditional endothelial cell-dependent angiogenesis, highly aggressive tumor cells can form tubular structures by themselves called vasculogenic mimicry (VM). VM channels function similarly as tumor vascular angiogenesis formed by endothelial cells in promoting tumor growth and development (Hendrix, Seftor, & Seftor, 2016). The presence of VM, which is related to tumor stemness and plasticity (Li, Meng, Guan, Guo, & Han, 2016), is mostly seen in highly invasive tumors, including melanoma (Bhattacharyya et al., 2018), NSCLC (Wang et al., 2018), breast cancer (Greco et al., 2018), and glioblastoma (Hallani, Boisselier, Peglion, Rousseau, & Marc, 2010). The presence of VM is considered to be one of the reasons why anti-tumor angiogenesis therapies fail to achieve the desired results in treatment of these highly aggressive tumors (Kirschmann, Seftor, Hardy, Seftor, & Hendrix, 2012).

The development of tumor angiogenesis is believed to attribute partly to the abnormalities of tumor microenvironment (Siemann, 2011). Tumor cells, endothelial cells and stromal cells in tumor microenvironment can secrete many pro-angiogenic factors, of which VEGF (vascular endothelial growth factor) is the most important (De Palma, Biziato, & Petrova, 2017). Binding of VEGF by VEGFR (vascular endothelial growth factor receptors) such as VEGFR2 induces VEGFR2 phosphorylation and initiation of downstream cell signaling in angiogenesis (Goel & Mercurio, 2013). VEGF expression in tumor and endothelial cells is regulated by cytokines, oncogenes and tumor suppressor genes such as STATs (signal transducers and activators of transcription) and c-Jun N-terminal kinases (JNKs) (Krejsgaard et al., 2006; Zhong & Zhong, 2010). STAT3 binds to the VEGF promoter and promotes VEGF gene expression (Niu et al., 2002). Inhibiting VEGF/VEGFR2 pathway is considered to be one of the most effective strategies in anti-tumor angiogenesis therapies.

A distinct feature of the tumor microenvironment is hypoxia. When a tumor reaches a certain volume, oxygen supply becomes less efficient inside the tumor and the tumor become locally hypoxia (Rey, Schito, Wouters, Eliasof, & Kerbel, 2017). Under hypoxic conditions, HIF-1α (hypoxia inducible factor-1α) can be highly expressed and forms a complex with c-Jun through its ODD. The formation of the complex can further inhibit the ubiquitination and degradation of HIF-1α, thereby promoting the expression and secretion of VEGF in the cells (B. Yu et al., 2009).

The formation of VM by tumor cells is a complex process. VEGF, as a pro-angiogenic factor, has been reported to promote the formation of VM in melanoma (Amalia A. Vartanian, Burova, Stepanova, & Baryshnikov, 2007). The formation of VM involves interaction of VE-cadherin with EphA2 on the cell membrane, resulting in EphA2 phosphorylation. This activates FAK and convert premature MMPs into active forms to degrade Laminin 5γ2 into fragments 5γ2' and 5γ2x. These laminin fragments can be released into the tumor microenvironment to promote the formation of VM networks (A. A. Vartanian, 2012). In addition, the FAK/AKT signaling has also been reported to mediate VM formation in NSCLC (Zhou, Gu, Han, Wu, & Liu, 2017).

Angiogenesis inhibitors are potential therapeutic agents for cancer treatment (Miao, Feng, & Ding, 2012). It has been reported that MTAs (microtubule-targeted agents), such as combretastatin and its derivatives, have potential anti-tumor angiogenesis effects (Tozer et al., 2001; Xuan, Mei, Li-Ping, Pui-Kai, & Jian, 2010). Low concentrations of MTAs has appeared to inhibit the migration and capillary-like structure formation of endothelial cells, but not affect endothelial cell proliferation and microtubule structures, by impairing the repositioning of microtubule-organizing center (MTOC) during chemotaxis and cell migration (Pasquier, Stéphane, & Diane, 2006). It has also been found that MTAs can inhibit the expression of HIF-1α by inhibiting the microtubule cytoskeleton (Mabjeesh et al., 2003). In addition, MTAs can exert its anti-tumor angiogenesis by activating JNK (Xu et al.). However, the role of MTAs in VM formation of tumor cells has rarely been reported.

DHPAC, 2 - (6 - ethoxy - 3 - (3 - ethoxyphenylamino) - 1 - methyl -1,4 - dihydroindeno [1,2-c] pyrazol-7-yloxy) acetamide, is a novel microtubule-targeted inhibitor that acts on the colchicine binding site of microtubule through computer-aided drug design. In our previous study, DHPAC exhibited potent anti-tumor activity on NSCLC (Liu et al., 2016). In the present study, we investigated the inhibitory effect of DHPAC on tumor angiogenesis and VM formation in NSCLC.

**2. Materials and Methods**

**2.1. Compounds**

DHPAC was obtained from Institute of Medicinal Chemistry, School of Pharmaceutical Sciences, Shandong University (Liu et al., 2016). Combretastain A4 (CA4) was purchased from Meilun Biotechnology Co, Ltd (Dalian, China). DHPAC and CA4 (Figure 1A1, A2) were dissolved in DMSO (dimethyl sulfoxide) and stored at -20°C.

**2.2. Materials**

Acetonitrile, deionized water, and formic acid (LC/MS grade) were all from Fisher (Fair, NJ). SP600125 was obtained from Selleck Chemicals (Shanghai, China), Matrigel from BD Biosciences (Becton Dickinson Labware, MA, USA) and VEGF ELISA kit from Proteintech (Wuhan, China). Antibodies against HIF-1α (#36169), JNK (#9252), p-JNK (#4668), c-Jun (#9165), p-c-Jun (#9261), STAT3 (#12640), p-STAT3 (#9145) and p-FAK (#8556) were purchased from Cell Signaling Technology (Boston, MA, USA); antibodies against VEGF (19003-1-AP), AKT (10176-2-AP), p-AKT (66444-1-lg) and FAK (66258-1- lg) from Proteintech (Wuhan, China); β-actin (A-09T) from ZS Bio.(Beijing, China); MMP2 (ab37150), MMP9 (ab38898) and Laminin 5 (ab14509) from Abcam (USA).

**2.3. Cell lines**

HUVECs (Human umbilical vein endothelial cells) were purchased from Procell Life Science Technology Co, Ltd (Wuhan, China). Human H1299 cells were purchased from China Cell Bank (Shanghai, China). HUVECs were cultured in F12K medium with endothelial cell growth supplement, heparin and 10% fetal bovine serum. H1299 cells were maintained in DMEM medium supplemented with 10% fetal bovine serum. HUVECs and H1299 cells were incubated at 37°C and in a humidified atmosphere containing 5% CO2.

**2.4. HPLC-MS/MS analysis**

HPLC system (1260, Aglient, USA) with Inertsil ODS-3 (5 μm, 4.6×250 mm) was used in the liquid chromatography in this study. The mobile phase was composed of acetonitrile and water (80:20, v/v) and isocratic elution was used. The injection volume was 5 μL and the analysis was run 6 min with a flow rate at 0.8 mL/min.

Four-stage bar mass analyzer (API 4000, Applied Biosystem Sciex, USA) was used to detect the analytes. For the detection of DHPAC, positive electrospray ionization (ESI) was performed in Multiple Reaction Monitoring (MRM) mode. The related mass spectrometry parameters were spray voltage, 5500 V; TEM: 500 °C; CUR (N2): 20 psi; GS1: 60 psi; GS1: 60 psi. The data processing system was Analyst 1.5.1 (Applied Biosystems, USA).

**2.4.1. Preparation of stock and working solution**

Five mg DHPAC were dissolved in 5 mL acetonitrile to make 1 mg/mL stock solution. The DHPAC stock solution was diluted with acetonitrile for working concentration. All solutions were stored at -20 °C.

**2.4.2. Cell sample preparation**

HUVECs were seeded into cell culture dishes, and after overnight culture, they were exposed to DHPAC at final concentration 40 nM. Cells cultured with culture medium without DHPAC were used as negative control. Cells were harvested and resuspended in acetonitrile, followed by repeated freezing and thawing in liquid nitrogen and 37°C. The cells were lysed in acetonitrile, sonicated for 30 min and then centrifuged (12000 rpm, 4°C) for another 30 min. The supernatant obtained were used as cell sample fluid or blank control fluid.

**2.5. Cell proliferation assay**

**2.5.1. Preparation of conditioned medium from H1299 cells**

H1299 cells were seeded into cell culture dishes. After the cells were attached, the serum-free medium was replaced instead of the original medium. After 24 h, the cell supernatant was collected and centrifuged (1000 rpm, 5 min). The supernatant was then collected and stored at -80°C. At the time of use, the conditioned medium was mixed with F12K medium at 1:1 (v/v), and serum (Gibco) was added to 10% or 1% for use. H1299 cells conditioned medium was also used in cell migration assays, capillary-like tube formation assay and Western blot.

**2.5.2. MTT assay**

The MTT assay is a common experimental protocol for detecting cell proliferation. Briefly, cells (2000-3000 per well) were seeded into 96-well plates, and different concentrations of DHPAC and CA4 were allowed to treat for 24 h, 48 h and 72 h after cell attachment. After that, 20 μL MTT solution (5 mg/mL) was added to each well for further 4 h. Finally, the culture medium was removed and DMSO (150 μL per well) was used to dissolve the formazan crystals. The absorbance of the wells was measured by Thermo Multiskan GO microplate reader (Thermo-1510, CA, USA) at 570 nm (Y. Zhang et al., 2017).

**2.6. Wound scratch assay**

HUVECs were seeded in 6-well plates and incubated until the cell monolayers were tightly confluent and then gently scratched by a sterile pipette. After 0 h, 12 h and 24 h of DHPAC and CA4 treatment, cell images in the scratch area were captured under the inverted microscope at 100 × magnification (NIKON ECLTPSE, Tokyo, Japan). The migration rate was calculated by measuring the gap width in each group.

**2.7. Transwell migration, invasion assays**

Cells were seeded into the upper chamber containing serum-free medium and complete culture medium (10% serum) was added to the lower chamber as chemoattractant. After treating the cells with DHPAC or CA4 for 24 h, the cells that migrated to the bottom of the chamber membrane were fixed with methanol and glacial acetic acid, followed by staining with crystal violet and quantification. For the Transwell invasion experiment, Matrigel diluted in medium was added to the upper chamber, and the remaining steps were the same as the migration experiments.

**2.8. Capillary-like tube formation, VM formation assays**

Matrigel was used to simulate the basement membrane for the detection of endothelial cell angiogenic ability. After Matrigel polymerization for 1 h, 50 μL HUVECs suspension (90-100×104/mL) were added into 96-well plates with or without DHPAC or CA4 for 6 h. The cell morphogenesis was visualized with a microscope (NIKON ECLTPSE). The vascular networks were quantified by calculating the length of branches. For the VM formation, H1299 cell suspension was added into 96-well plates (90-100×104/mL), and the remaining steps were the same as the tube formation assay.

**2.9. CD31 immunofluorescence staining assay**

After administration of DHPAC or CA4 for 14 days to mice, the mice were sacrificed, and the tumor tissue pieces were taken out and frozen sectioned. Tissue sections were incubated with 0.5% Triton X-100 for 20 min. After blocking the sections with PBS containing 10% goat serum for 1 h, an appropriate amount of CD31 antibody (diluted with blocking solution) was added and incubated overnight at 4°C. The sections were washed with PBS, and FITC-conjugated secondary antibody was applied at 37°C for 1 h. After addition of appropriate amount of Hoechst for 5 min, the sections were observed by fluorescence microscopy and photographed.

**2.10. VEGF secretion and quantification**

VEGF secretion by HUVECs and H1299 cells were analyzed using a human VEGF ELISA kit. After treating the cells with of DHPAC or CA4 for 24 h, the cell supernatant was collected. The concentration of VEGF in the supernatant was then determined using the VEGF ELISA kit according to the instructions for use.

**2.11. Western blot assay**

After treating the cells with DHPAC or CA4 for 24 h, the cells were harvested and lysed with cell lysis buffer (RIPA: PMSF=99:1, v/v). This was followed by centrifugation at 14000 rpm for 15 min at 4°C, and the protein concentration of the lysate was measured using a BCA Protein Assay Kit (Thermo Science, USA). Lysates were subjected to 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to PVDF (polyvinylidene difluoride) membranes (Millipore, USA). After blocking the membrane for 4 h at room temperature, it was incubated with specific antibodies overnight at 4°C, and then incubated with HRP-conjugated secondary antibody for 1 h. Binding was finally detected by ECL (enhanced chemiluminescence) detection system and quantiﬁed by densitometry using a ChemiDoc XRS (Bio-Rad, Hercules, California, USA) (H. H. Zhang et al., 2017).

**2.12. In vivo human NSCLC xenografts**

Five-week-old female Balb/C nude mice were purchased from Animal Center of China Academy of Medical Science (Beijing, China) and housed under pathogen-free conditions. All animal experiment protocols were conducted in strict accordance with the Institutional Guidelines of Animal Care and Use Committee of Shandong University. Under sterile conditions, H1299 xenografts were established by inoculating 10×106 H1299 cells into armpit of nude mice. After about one month, when the xenograft volume was grown to about 1000 mm3, the mice were sacrificed by cervical dislocation, and the tumor tissues were taken out and cut into 1 mm3 fragments. Each fragment was transplanted s.c. into the right flank of each mouse with a trocar. Once the transplanted tumor volume increases to 100-200 mm3, remove the oversized or undersized ones and randomly divide the remaining mice into five groups (n=5): blank (normal saline, i.p.), vehicle (castor oil: ethanol: 5% dextrose = 1:1:4, i.p.), DHPAC (15 mg/kg, 30 mg/kg, i.p.) and CA4 (30 mg.kg, i.p.). All the mice were administrated daily with DHPAC, or CA4, for 14 days. The body weight and tumor volume of all of the animals were measured every 2 days. V=L\*W2/2 is used to calculate tumor volume (V), in which W refers to the short axis and L refers to the long axis. After DHPAC or CA4 treatment was completed, the mice were sacrificed, and the xenografts, lungs and hearts were removed and weighed. Finally, the effects of DHPAC and CA4 on the growth of tumor tissue in nude mice were observed by comparison with the vehicle group.

**2.13. Statistical analysis**

All experiments were performed at least three times. All quantitative data are presented as mean ± SD or mean ± SEM. Statistical comparison were analyzed by one -way analysis of variance. P value < 0.05 was considered statistically significant and P value < 0.01 exhibited an extremely distinct difference. SPSS/Win 13.0 software was used for statistical analysis.

**3. Results**

**3.1. DHPAC enters cells and inhibits HUVECs proliferation**

DHPAC is a MTA which targets the colchicine binding site (Liu et al., 2016). In previous studies, we did not investigate whether DHPAC can enter cells. In this study, we first used HPLC-MS/MS to assess whether DHPAC can enter cells. As shown in Figure 1, no peaks in blank solvent group (Figure 1B1) and blank cell lysate (Figure 1B3) were seen. A slightly delayed retention time of cellular DHPAC occurred in sample cell fluid (4.36 min, Figure 1B4) in comparison to DHPAC standard working solution (4.45 min, Figure 1B2), may be due to the influence of intracellular substances, suggesting possible DHPAC cell entry.

The presence of DHPAC showed to produce a time-and DHPAC concentration- dependence to inhibit HUVECs proliferation. Compared with DHPAC, CA4 also showed a similar, albeit stronger, inhibition of HUVECs proliferation (Figure 1C1, C2). To examine the effect of DHPAC on the proliferation of endothelial cells under the condition of tumor microenvironment, we used the culture supernatant of H1299 cells to mimic the tumor microenvironment and assessed the DHPAC effects. As shown in Figure 1C3 and C4, the inhibitory effect of DHPAC on HUVECs proliferation was significantly enhanced in the presence of the tumor microenvironment mimicry condition.

**3.2. DHPAC inhibits HUVECs migration and capillary-like tube formation**

DHPAC caused a slower migration of HUVECs (slower closure of the cell gaps than the vehicle control group) in the wound scratching experiments (Figure 2A1). There were fewer floating cells in the DHPAC-treated culture in comparison to CA4-treated cells. The cell migration rate was significantly accelerated, and DHPAC-mediated inhibition on migration was enhanced when the cells were cultured in the conditioned medium from H1299 cells (Figure 2A2). Analysis of the effect of DHPAC on the migration of HUVECs in the Transwell migration experiment showed similar results (Figure 2B1, B2).

In the capillary-like tube formation analysis, HUVECs cultured on Matrigel for 6 h formed a distinct network structure, and the length of branching was increased in the conditioned medium. Application of DHPAC showed to reduce HUVECs tube formation, an effect of DHPAC which was more prominent in cells cultured in the conditioned medium (Figure 2C1, C2). Consistent with these effects of DHPAC on angiogenesis in these *in vitro* angiogenesis experiments, CD31 immunofluorescence staining of tumor tissue sections showed that the microvessel density in the DHPAC-treated groups was significantly lower than that in the vehicle group (Figure 2D). Together, the results of the above experimental results revealed that DHPAC effectively inhibits HUVECs migration and angiogenesis in the tumor microenvironment.

**3.3. DHPAC activates JNK and inhibits STAT3/VEGF/VEGFR2 signaling in HUVECs in tumor microenvironment**

As a major pro-angiogenic factor in tumor angiogenesis, VEGF binds to VEGFR2 and activate multiple downstream cell signal transduction pathways including FAK/AKT pathway to promote tumor angiogenesis. To understand the mechanism of DHPAC-mediated inhibition of tumor angiogenesis, we investigated the effect of DHPAC on the expression and secretion of VEGF in HUVECs. As shown in Figure 3A and B, treatment with DHPAC at the concentration of 20 nM and 40 nM dramatically reduced VEGF expression as well as VEGF secretion in HUVECs. DHPAC also inhibited the phosphorylation of AKT in normal medium (Figure 3A), and DHPAC showed to inhibit the phosphorylation of both FAK and AKT in the tumor microenvironment-mimicry medium (Figure 3C1, C2).

It has been reported that constitutive activation of STAT3 promotes VEGF transcription and expression. To further clarify the mechanism of DHPAC on inhibition of angiogenesis, we examined the effects of DHPAC on STAT3, JNK and c-Jun proteins expression and activation. DHPAC showed to effectively inhibit the phosphorylation of STAT3 under normal culture conditions and tumor microenvironment-mimicry medium, an effect was also observed with CA4 (Figure 3A, C3). Moreover, DHPAC presence also significantly increased JNK and c-Jun phosphorylation (Figure 3C4, C5), suggesting the involvements of both STAT3 and JNK signaling in the anti-angiogenic effect of DHPAC.

**3.4. DHPAC activates JNK and inhibits HIF-1α/VEGF/VEGFR2 signaling in HUVECs at hypoxia**

Hypoxia is an important feature of tumor microenvironment. HIF-1α, which is highly expressed under hypoxia, promotes VEGF transcription in tumor angiogenesis and cause hypoxia adaptation. We next explore the effect of DHPAC on tumor angiogenesis and signaling under CoCl2 (100 μM)-created hypoxic environment. As shown in Figure 3D1, HIF-1α expression was significantly increased as well as HIF-1α regulated protein VEGF in HUVECs under such hypoxic condition. Approximately three-fold higher secretion of VEGF was seen under hypoxia than normoxia. The presence of DHPAC inhibited HIF-1α expression and the expression and secretion of VEGF (Figure 3B, D1). Similar to that under normoxic conditions, DHPAC presence also inhibited the phosphorylation of STAT3 and FAK under hypoxic conditions (Figure 3D2, D3).

The formation of a complex of HIF-1α with c-Jun in the cytoplasm is known to prevent HIF-1α degradation. We found here that DHPAC promotes phosphorylation of c-Jun and JNK under hypoxic conditions (Figure 3D4, D5). To investigate the relationship of DHPAC-mediated effect on HIF-1α expression and c-Jun phosphorylation, we examined the effects of DHPAC on HIF-1α, c-Jun and p-c-Jun proteins expression at different time intervals. As shown in Figure 3D6, without the presence of DHPAC, the expressions of HIF-1α and p-c-Jun were rapidly increased up to 6 h, and then decreased at 12 h. In response to DHPAC, the c-Jun phosphorylation of HUVECs was increased from 2 h, reached a plateau at 6 h, and decreased from 12 h. No significant change of the expression of HIF-1α occurred within 6 h and a decrease appeared only after 12 h in cells response to DHPAC. These results demonstrated that DHPAC induced-increase of c-Jun phosphorylation occurred prior to the decrease of HIF-1α expression under hypoxia. This suggests that under hypoxic conditions, DHPAC is likely to exert its anti-angiogenic effect by activating JNK.

**3.5. DHPAC inhibits VEGF/VEGFR2 signaling through activating JNK under both normoxia and hypoxia conditions**

To further understand the role of JNK in DHPAC-mediated effect on endothelial cells migration and angiogenesis, we assessed the presence of SP600125, a JNK phosphorylation inhibitor, on cell migration and tube formation with and without DHPAC. Transwell migration assay showed that the presence of SP600125 caused 29.6% reduction of DHPAC-mediated cell migration (Figure 4A). It also caused significant reduction (23.4%) of DHPAC-mediated tube formation in capillary tube formation analysis (Figure 4B). These results indicated that JNK inhibitor could substantially inhibit DHPAC-mediated inhibition on cell migration and angiogenesis.

Next, we also examined the effects of DHPAC on VEGF secretion, STAT3 phosphorylation, and expression of related proteins in the FAK/AKT signaling under the presence of SP600125. Under normoxic and hypoxic conditions, the inhibition of DHPAC (20 nM) on VEGF secretion was 38.81% and 28.92%, respectively, and the inhibition decreased to 16.70% and 10.42%, respectively, after the exposure to SP600125 (Figure 4C, D). Under normoxic conditions, SP600125 partially reversed the activation of JNK induced by DHPAC, and reversed the phosphorylation promoting effect of DHPAC on c-Jun (Figure 4E, F). Moreover, in the presence of SP600125, the phosphorylation inhibition of STAT3 and FAK by DHPAC was also partially inhibited (Figure 4G, H). Similar to the results under normoxic conditions, this JNK inhibitor partially reversed the activation of JNK by DHPAC and its inhibition of STAT3 and FAK phosphorylation under hypoxic conditions (Figure 4I, J, K). These results indicate that JNK is located upstream of STAT3 during cell signaling of DHPAC-mediated anti-tumor angiogenesis, supporting a sequence of DHPAC-mediated VEGF/VEGFR2 signaling through activation of JNK then inhibition of STAT3.

**3.6. DHPAC inhibits the growth of H1299 cells in vitro and in vivo**

The administration of DHPAC showed to inhibit the growth of H1299 cells *in vitro* (Figure 5A). Compared with CA4, DHPAC at lower concentrations (5, 10 nM) showed weaker effect than CA4 on the inhibition of H1299 cells proliferation, probably due to the stronger cytotoxic effects of CA4 (Figure 5B).

In a mice xenograft model, treatment with DHPAC led to significant inhibition of tumor growth of H1299 cells (Figure 5C, E). Compared with vehicle control group, DHPAC at 15 mg/kg and 30 mg/kg caused 35.56% and 52.24% reduction of tumor growth, while the administration of CA4 at 30 mg/kg resulted in 41.30% tumor reduction in mice (Table 1). No significant change of the animal body weight, lung weight and heart weight were seen among the five groups (Figure 5D, F and G), indicating little lung- and cardio-toxicity of DHPAC.

**3.7. DHPAC suppresses H1299 cells migration, invasion and the VM formation**

In the Transwell migration analysis, the presence of DHPAC at 5 nM, 10 nM and 20 nM reduced H1299 cells migration by 25.18%, 32.63%, 49.33%, respectively, compared with the vehicle group (Figure 6A1). In the Transwell invasion assay, H1299 cells displayed high invasive activity in the vehicle group. After exposure to DHPAC at 5 nM, 10 nM and 20 nM, cell invasion was suppressed by 18.94%, 35.60% and 49.49% respectively (Figure 6A2).

VM formation occurs mostly in highly invasive tumors which is attributed to tumor cells migration and invasion. Using Matrigel as basement matrix we assess the effect of DHPAC on VM formation of H1299 cells. From Figure 6A3, we are not difficult to find that tumor cells showed to connect to each other and form a network structure with a large number of small tubes and a complete structure in the vehicle group. The presence of DHAPC at 20 nM caused almost complete collapse of VM structure with an inhibition of 88.09%.

**3.8. DHPAC inhibits VEGF expression and secretion and FAK/AKT signaling in H1299 cells in vitro and in vivo**

VEGF expression and FAK/AKT signaling are both known to be involved in VM formation (A. A. Vartanian, 2012). To better understand the molecular mechanism of DHPAC-mediated effect on tumor vasculature, the effect of DHPAC on the expression and secretion of VEGF and FAK/AKT signals was investigated. The presence of DHPAC significantly inhibited the expression and secretion of VEGF in H1299 cells (Figure 6B1, B2). It also caused significant reduction of FAK and AKT phosphorylation in H1299 cells (Figure 6B3 and B4). Consistent with these *in vitro* discoveries, administration of DHPAC in H1299 xenograft mice inhibited the expression of VEGF (Figure 6C1), the phosphorylation of FAK (Figure 6C2) and AKT (Figure 6C3).

It has been reported that in the final stage of signaling cascade associated with VM formation, tumor cells express MMPs, which degrade laminin to aid vascular channel formation. Therefore, we next examined the expression of MMPs and laminin 5 and FAK/AKT signaling pathway in cell response to DHPAC. Results showed that the expression of MMP2, which is known to be associated with tumor cell invasion, was reduced by DHPAC (Figure 6B1). In H1299 xenograft model, administration of DHPAC also reduced the expressions of MMP2, MMP9 and laminin 5(Figure 6C4).

**4. Discussion**

Microtubule-targeted inhibitors are known to have potential anti-angiogenic effects (Lu, Jianjun, Min, Wei, & Duane, 2012). The colchicine site-targeted microtubule inhibitors have been reported to have more pronounced anti-angiogenic effects than other tubulin binding sites–targeted inhibitors (taxane binging site or vinca alkaloid binding site) (Dumontet & Jordan, 2010; Su et al., 2016). Our previous studies have found that DHPAC induces the apoptosis of NSCLC through G2/M phase arrest and inhibit the growth of vincristine-resistant oral cancer cells. This research focused on the effects of DHPAC on endothelial cell angiogenesis and tumor cell VM formation.

The angiogenic process by vascular endothelial cells and the formation of VM by tumor cells are both critically important in tumor development and metastasis (Seftor, Hess, Seftor, Kirschmann, & Hendrix, 2012). Both tumor angiogenesis and VM formation are associated with the unique microenvironment of tumors. Factors such as cytokines secreted by tumor cells contributes to the tumor microenvironment that affect the angiogenesis. Therefore, the traditional way of angiogenesis induction in vitro by the addition of VEGF does not fully reflect the true tumor microenvironment. In this study, we used the culture supernatants from tumor cells to mimic the tumor microenvironment and assessed the influence of DHPAC on endothelial cell angiogenesis and VM formation.

During tumor angiogenesis, VEGF is a key angiogenic promoter. It can bind to VEGFR2 on the endothelial cells membrane and activate multiple downstream pathways to initiate angiogenesis in which FAK/AKT signaling is important in the migration and adhesion of endothelial cells (Sulzmaier, Jean, & Schlaepfer, 2014). A variety of inhibitors targeting VEGF/VEGFR2 signaling pathway have been developed in the past, of which bevacizumab is the first VEGF inhibitor that was approved by the US FDA (Food and Drug Administration) as first-line treatment for colorectal cancer in 2004 (Ferrara & Adamis, 2016). The expression and secretion of VEGF are regulated by a variety of cytokines and carcinogenic factors, such as STAT3, JNK (Shen, Lili, Bin, & Zhengtao, 2012). In this study, we found that DHPAC presence promotes phosphorylation of JNK and c-Jun but inhibits STAT3 phosphorylation under tumor microenvironment-mimicry medium. The presence of a JNK inhibitor SP600125 partially reduced DHPAC-mediated inhibition of STAT3 phosphorylation, suggesting STAT3 activation is located downstream of JNK. VEGF secreted to the outside of cells activates the downstream FAK/AKT signal transduction pathway by acting on VEGFR2 on the endothelial cell membrane in an autocrine manner. In the tumor microenvironment, reduction of VEGF secretion of endothelial cells by DHPAC would be expected to reduce VEGF interaction with VEGFR2 angiogenesis.

Hypoxia is an important feature of the tumor microenvironment which promotes tumor angiogenesis and metastasis (Chappell, Payne, & Rathmell, 2019). When a solid tumor grows to a certain volume, an oxygen-deficient microenvironment is formed inside the tumor which results in HIF-1α overexpression. HIF-1α overexpression induces hypoxia adaptation through altering the expressions of molecules such as VEGF, glucose transporters (GLUT) and various glycolytic enzymes to support the glycolysis of tumor cells (Al Tameemi, Dale, Al-Jumaily, & Forsyth, 2019). The complex formation of HIF-1α with c-Jun, a transcription factor associated with cell proliferation, differentiation, apoptosis, oxidative stress, can protect HIF-1α from degradation and thus contributes to the transcription and expression of VEGF and MDR1 (multidrug resistance genes) (Bing Yu et al., 2012). DHPAC was shown in this study to reduce the content of unphosphorylated c-Jun in the cytoplasm. This would be expected to reduce the HIF-1α/c-Jun complex formation and enhance HIF-1α degradation, leading to inhibition of VEGF transcription and secretion. Thus, in the tumor hypoxia microenvironment, DHPAC can reduce the expression of HIF-1α by activating JNK to decrease the expression and secretion of VEGF to prevent VEGF/VEGFR2 interaction in angiogenesis.

Although the incidence of tumor VM is small, its presence in tumor tissues is associated with higher risk of metastasis and decreased efficacy of anti-tumor therapies (Sun, Zhang, Zhao, Wei, & Hao, 2004). VEGF, in addition to its angiogenic promoting effect through paracrine way, as well as VE-cadherin, EphA2, Nodal/Notch, FAK/AKT, are believed to play important role in the VM formation process (A. A. Vartanian, 2012; Amalia A. Vartanian et al., 2007). In NSCLC, activation of FAK/AKT signaling can upregulate the expression and activation of matrix metalloproteinases (MMPs) that activate the cleavage of laminin and promote VM formation. This study showed that DHPAC suppresses phosphorylation of FAK and AKT and decreases the expression of MMP2, MMP9 and laminin 5, indicating the involvement of FAK/AKT inhibition in DHPAC–mediated reduction of VM formation.

In conclusion, in addition to its anti-mitotic effect, the microtubule-targeted inhibitor DHPAC, can also inhibit tumor angiogenesis and VM formation in the tumor microenvironment. The mechanism of its anti-endothelial angiogenesis effect is proposed in Figure 7. In the tumor microenvironment, DHPAC enters cells and inhibits STAT3/VEGF/VEGFR2/FAK/AKT signal transduction by activating JNK. In hypoxic microenvironment, DHPAC inhibits angiogenesis by activating JNK, thereby activating c-Jun, and inhibiting the HIF-1α/VEGF/VEGFR2/FAK signaling pathway. In addition, DHPAC can down-regulate the expression levels of MMP2, MMP9 and laminin 5 by inhibiting the activation of FAK/AKT signaling pathway in tumor cells to exert its anti-VM formation activity.

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**Conflict of Interest**

The authors declare that they have no conflict of interest.

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**Figure legends**

**Figure 1.** DHPAC enters cells and inhibits HUVECs proliferation. The chemical structures of compound DHPAC (A1) and CA4 (A2). Representative chromatograms of acetonitrile (ACN, B1), DHPAC 0.2 μg/mL (B2), blank cell lysate (B3) and cell samples (B4) collected from HUVECs incubated with or without 40 nM DHPAC for 24 h. Cell proliferation was determined by MTT assay after being treated with DHPAC (C1) or CA4 (C2) for 48 h and 72 h. HUVECs were treated with DHPAC in the presence of conditioned medium from H1299 cells for 48 h (C3, C4). All data are presented as mean ± SD, n=3. \*P<0.05, \*\*P<0.01 versus vehicle (NM) or vehicle (CM).

**Figure 2.** DHPAC inhibits migration and tube formation of HUVECs. Representative images (100×) of scratch wound-healing migration in HUVECs treated with DHPAC and CA4 for 12 h and 24 h in normal medium (A1) and conditioned medium (A2). Cell migration was determined by Transwell assay after treatment with DHPAC or CA4 for 24 h in the normal medium (B1, 200×, bar=100 μm) and conditioned medium (B2, 200×, bar=100 μm). Representative images (60×, bar=200 μm) of tube formation after treatment with DHPAC for 6 h in the normal medium (C1) and the conditioned medium (C2). Representative images of CD31 immunofluorescence staining in tumor tissues were shown for different groups (D). All data are presented as mean ± SD, n=3. \*P<0.05, \*\*P<0.01 versus vehicle (NM) or vehicle (CM).

**Figure 3**. DHPAC suppresses VEGF signaling in HUVECs. Representative Western blots show the expressions of VEGF, AKT, p-AKT, STAT3, p-STAT3 in HUVECs treated with DHPAC and CA4 for 24 h in the normal medium (A). Secretion of VEGF in culture medium of HUVECs after treated with DHPAC was measured by ELISA assay (B). The expressions of FAK and p-FAK (C1), AKT and p-AKT (C2), STAT3 and p-STAT3 (C3), JNK and p-JNK (C4), c-Jun and p-c-Jun (C5) after being treated with DHPAC and CA4 under the stimulation of conditioned medium were measured by Western blot. Representative blots show the expressions of VEGF and HIF-1α (D1), STAT3 and p-STAT3 (D2), FAK and p-FAK (D3), JNK and p-JNK (D4), c-Jun and p-c-Jun (D5) in HUVEC cells treated with DHPAC and CA4 for 24 h at hypoxia induced by 100 μM CoCl2. The expressions of HIF-1α, c-Jun and p-c-Jun in HUVECs treated with 20 nM DHPAC at hypoxia for 2 h, 6 h and 12 h were determined by Western blot (D6). All data are presented as mean ± SD, n=3. \*P<0.05, \*\*P<0.01.

**Figure 4.** DHPAC inhibits migration, tube formation and VEGF signaling through activating JNK in HUVECs. The JNK inhibitor SP600125 decreased the inhibitory effect of DHPAC on migration (A, 200×, bar=100 μm) and tube formation (B, 60×, bar=200 μm) of HUVECs. ELISA assay was used to detect the secretion of VEGF from HUVECs in culture medium at normoxia (C) and hypoxia (D). The cellular expressions of JNK and p-JNK (E), c-Jun and p-c-Jun (F), STAT3 and p-STAT3 (G), FAK and p-FAK (H) in HUVECs were measured by Western blotting assay. Representative bands of Western blot show the expressions of JNK and p-JNK (I), STAT3 and p-STAT3 (J), FAK and p-FAK (K) after HUVEC cells being treated with DHPAC and SP600125 at hypoxia. All data are presented as mean ± SD, n=3. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001.

**Figure 5.** DHPAC inhibits proliferation of H1299 cells *in vitro* and *in vivo*. Cell proliferation was determined by MTT assay after treatment with DHPAC (A) or CA4 (B) for 24 h, 48 h and 72 h. The data (A, B) are presented as mean ± SD, n=3. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001 versus vehicle. H1299 xenografts were established by inoculating H1299 cells subcutaneously. DHPAC and CA4 were administrated every day for 14 days by intraperitoneal injection. The body weight and tumor volume were measured every 2 days (D, E). At the end of the experiment, all of the mice were sacrificed and the tumors (C), the lungs (F) and the hearts (G) were weighed. The data (C) are presented as mean ± SEM, n=5. \*\*P<0.01 versus vehicle.

**Figure 6.** DHPAC suppressed VM formation via FAK/AKT pathway. Cell migration (A1, 200×, bar=100 μm) and invasion (A2, 200×, bar=100 μm) was determined by Transwell assay after treatment with DHPAC. Representative images (A3, 60×, bar=200 μm) of VM formation after treatment with DHPAC for 6 h. The cellular expressions of VEGF and MMP2 in H1299 cells after treatment with DHPAC or CA4 for 24 h were measured by Western blot (B1). The secretion of VEGF in H1299 cells was measured by ELISA assay (B2). The expression of total and phosphorylated FAK (B3), AKT (B4) was measured by Western blot. Representative bands of Western blot showd the expressions of VEGF (C1), FAK and p-FAK (C2), AKT and p-AKT (C3), MMP2, MMP9 and Laminin 5 (C4) in H1299 cells xenografts treated with DHPAC or CA4 for 14 days. All data are presented as mean ± SD, n=3. \*P<0.05, \*\*P<0.01 versus vehicle.

**Figure 7.** Proposed mechanisms of the inhibitory effects of DHPAC on tumor angiogenesis and VM formation. In endothelial cells, DHPAC promotes the phosphorylation of JNK, which in turn inhibits phosphorylation of STAT3. Under hypoxic conditions, DHPAC also inhibits the formation of c-Jun/HIF-1α complex by activating JNK, thereby hindering the accumulation of HIF-1α in the cytoplasm. Decreased expression of both p-STAT3 and HIF-1α inhibits the VEGF/VEGFR2 signal pathway, further impeding angiogenesis. In tumor cells, DHPAC reduces the expression and secretion of VEGF. In addition, DHPAC inhibits the expression of MMP2, MMP9 by reducing FAK/AKT signal pathway to prevent laminin cleavage by MMPs.

**Table 1.** DHPAC inhibited the growth of H1299 cells xenografts in athymic mice (mean ± SD, n=5).

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Groups | Mice (n)  Initial/end | Body weight (g)  Initial/end | Tumor weight  (g) | Inhibition  rate (%) |
| Blank | 5/5 | 18.8 ± 0.3/20.1± 0.7 | 1.65 ± 0.51 | / |
| Vehicle | 5/5 | 18.9 ±1.0/20.5± 0.7 | 1.63 ± 0.54 | / |
| DHPAC-15 mg/kg | 5/5 | 18.5 ± 0.4/19.5± 0.3 | 1.05 ± 0.55 | 34.56 |
| DHPAC-30 mg/kg | 5/5 | 18.2 ± 0.5/19.9± 0.8 | 0.76 ± 0.44 | 52.24 |
| CA4-30 mg/kg | 5/5 | 18.5 ± 0.6/19.5± 0.9 | 0.94 ± 0.41 | 41.30 |

Fig 1

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Fig 7

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