

# 7-Difluoromethyl-5,4'-dimethoxygenistein exerts anti-angiogenic effects on acute promyelocytic leukemia HL-60 cells by inhibiting the TLR4/NF- $\kappa$ B signaling pathway

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**Abstract.** Angiogenesis plays an important role in the development and metastasis of tumors, and anti-angiogenesis agents are used to treat tumors. For example, the acute promyelocytic leukemia (APL) may be treated with arsenic trioxide. Angiogenesis in APL is a multi-step dynamic equilibrium process coordinated by various angiogenic stimulators and inhibitors, which play key roles in the occurrence, progression and chemosensitivity of this disease. Our research group previously synthesized 7-difluoromethyl-5,4'-dimethoxygenistein (DFMG), and found that it inhibits angiogenesis during atherosclerotic plaque formation. In the present study, the effect and mechanism of DFMG in angiogenesis induced by APL HL-60 cells was investigated using a chick embryo chorioallantoic membrane model and Matrigel tubule formation assays. The results obtained revealed an anti-angiogenesis effect of DFMG towards HL-60 cells. When the Toll-like receptor 4/nuclear factor- $\kappa$ B (TLR4/NF- $\kappa$ B) signaling pathway was inhibited, the anti-angiogenic effect of DFMG was further enhanced. However, when the TLR4/NF- $\kappa$ B signaling pathway was activated, the anti-angiogenic effect of DFMG was attenuated. These results demonstrated that DFMG inhibits angiogenesis induced by APL HL-60 cells, and provides insights into the mechanism by which DFMG inhibits the TLR4/NF- $\kappa$ B signaling pathway. In conclusion, in the present study, the anti-angiogenesis effect of DFMG on APL has been reported, and the mechanism by which DFMG

induced the anti-angiogenesis effect was explored. These findings have provided a potential new drug candidate for the treatment of patients with APL.

## Introduction

Acute promyelocytic leukemia (APL) is one of the most common types of adult acute leukemia (1), in which variations in myeloid-derived hematopoietic stem cells in the bone marrow prevent the differentiation of myeloid cells. Primordial or naïve myeloid cells are in a state of clonal hyperplasia, while normal hematopoiesis is inhibited (2). Numerous studies have shown that angiogenesis plays an important role in the pathogenesis of APL (3-5). Bone marrow angiogenesis is a complex process in which new blood vessels form from the original blood vessel to create a stable vascular network in the bone marrow microenvironment after extensive reconstruction (6). Bone marrow microvessel density is closely associated with the occurrence, development and chemotherapy sensitivity of APL (7,8). Currently, the main treatments for APL are chemotherapy and stem cell transplantation. However, drug tolerance or recurrence can develop. In addition, chemotherapy drugs can cause serious side effects due to their cytotoxic effects on normal cells in addition to tumor cells (9). Therefore, in recent years, molecularly targeted drugs for acute leukemia, such as targeting factors related to angiogenesis, have gained attention (9).

The bone marrow is the major microenvironment of APL cells; various cytokines secreted by APL cells can promote the proliferation of endothelial cells and formation of tubular structures (10), as well as enhance the angiogenesis of the bone marrow microvessels (11). Additionally, changes in the bone marrow provide suitable growth conditions for APL cells (12). Vascular endothelial growth factor (VEGF) is the most widely studied pro-angiogenic factor in acute myeloid leukemia. VEGF not only promotes bone marrow angiogenesis, but also stimulates APL cell proliferation (13). VEGF is highly expressed in patients with acute leukemia, and is an effective indicator for evaluating patient prognosis (14). Previous research has shown that targeting VEGF is effective in treating acute leukemia (15,16). Moreover, thalidomide and arsenic trioxide,

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which have been widely used in clinical practice and achieved successful therapeutic effects, are involved in the inhibition of angiogenesis in APL. These agents can significantly reduce the expression of VEGF in patients and delay the development of the disease; however, some patients experience side effects, such as peripheral neuritis, vascular embolism and skin damage (17-20). Therefore, the search for low-toxicity, safe drugs targeting angiogenesis is important for treating APL.

7-Difluoromethyl-5,4'-dimethoxygenistein (DFMG), designed and synthesized by our research group, is a compound derived from legume plants, and causes few adverse reactions in the human body compared with other molecularly targeted drugs (21). In our previous studies, DFMG was shown to prevent human umbilical vein endothelial (HUVE-12) cells from hydrogen peroxide-induced impairment *in vitro* (21), decrease the release of cell adhesion molecules and inflammatory factors by downregulating the expression of Toll-like receptor 4/nuclear factor  $\kappa$ B (TLR4/NF- $\kappa$ B) and decrease the adhesion of circulating monocytes to endothelial cells (22). Additionally, DFMG was found to inhibit angiogenesis during atherosclerotic plaque formation (23). Based on this information, it was predicted that DFMG may have an anti-angiogenesis role in APL. Therefore, in the present study, it was investigated whether DFMG affects angiogenesis induced by APL HL-60 cells, and the underlying mechanism of DFMG was explored.

## Materials and methods

**Cell culture.** Human promyelocytic leukemia cell line HL-60 cells (preserved in our laboratory) and human umbilical vein endothelial cell line HUVE-12 cells (cat. no. GDC166; China Center For Type Culture Collection) were cultured in RPMI-1640 (Biological Industries) with 10% heat-inactivated fetal bovine serum (Biological Industries) and 1% penicillin-streptomycin, and maintained at 37°C and 5% CO<sub>2</sub>. After DFMG was dissolved in dimethyl sulfoxide, different concentrations of DFMG (synthesized by our research group; patent no. ZL200710104389.4) were added to the culture medium of HL-60 cells and HUVE-12 cells for 48 h. HL-60 cells were pre-incubated with TLR4 activator lipopolysaccharide (LPS; Beyotime Institute of Biotechnology) and TLR4 blocker TAK-242 (MedChemExpress) for 4 h at 37°C and 5% CO<sub>2</sub>, and then treated with 100  $\mu$ M DFMG for 48 h at 37°C and 5% CO<sub>2</sub>. The cells were divided into the following groups: Blank control; solvent control (1% dimethyl sulfoxide); DFMG; LPS; LPS + DFMG; TAK-242; and TAK-242 + DFMG.

**CCK-8 assay.** The CCK-8 assay was performed to detect cell viability in different groups. Cells were seeded into a 96-well culture plate at a density of 5x10<sup>4</sup>/ml, 100  $\mu$ l per well, with three replicate wells per group. Cells were cultured with different concentrations of drugs (0, 25, 50, 100 and 200  $\mu$ mol/l) for 48 h, and then 10  $\mu$ l CCK-8 solution (Nanjing Jiancheng Bioengineering Institute) was added into the well at 37°C for 2 h. The optical density (OD) at 450 nm was measured with a microplate reader (Elx800; BioTek Instruments, Inc.), and the relative cell viability was calculated in terms of OD values.

**Lactate dehydrogenase (LDH) assay.** HL-60 cells were seeded into 24-well culture plates at a density of 5x10<sup>5</sup> cells

per well. Different concentrations of DFMG (0, 25, 50, 100 and 200  $\mu$ mol/l) were used to treat HL-60 cells for 48 h at 37°C. The cell culture supernatant was collected by centrifugation (300 x g for 8 min at room temperature) to evaluate the concentration of LDH released from the cells using an LDH kit (Nanjing Jiancheng Bioengineering Institute), according to the manufacturer's instructions. The OD at 450 nm was measured with a microplate reader (Elx800; BioTek Instruments, Inc.), and the concentration of LDH was calculated according to the manufacturer's protocols.

**Chorioallantoic membrane (CAM) experiment.** Fertilized chicken eggs (Lvjian Ecological Agriculture Institute) were incubated in a hatching incubator (model 150; Weizhen Instruments, Inc.) equipped with an automatic rotator at 37°C and a relative humidity of 60% for 10 days. After incubation the eggshell was disinfected with 75% ethanol (Sinopharm Chemical Reagent Co., Ltd). The eggshell was removed along the fracture line and one part of the CAM was exposed. The HL-60 cells were incubated with DFMG for 48 h at 37°C and 5% CO<sub>2</sub>, following which the cell culture supernatant (collected by centrifugation at 300 x g for 8 min at room temperature) was discarded in order to eliminate the influence of the drugs in the supernatant. The cells were rinsed three times with serum-free medium, and then incubated in 1 ml RPMI-1640 serum-free medium (Biological Industries) for an additional 10 h at 37°C and 5% CO<sub>2</sub>. After the second incubation, the cell culture supernatant was collected by centrifugation (300 x g for 8 min at room temperature). The gelatin sponge was cut into small squares of ~5 mm, and immersed in HL-60 cell culture supernatants treated with different drugs, and then the gelatin sponges soaked with different cell culture supernatants were added to the CAM. Eggs were incubated at 37°C and a relative humidity of 60% for 3 days, and then images of the CAM blood vessels were captured using a microscope (SZX16; Olympus Corporation) at magnification, x1. The angiogenesis area was analyzed using Image-Pro Plus 7.0 software (Media Cybernetics, Inc.). All experimental procedures involving the use of animals were approved by the Animal Use and Care Committee of Hunan Normal University (Changsha, China).

**Matrigel tubule formation assay.** Matrigel (BD Biosciences) was thawed at 4°C overnight. Matrigel was diluted 1:3 with RPMI1640, and then added to a pre-cooled 24-well culture plate at ~300  $\mu$ l per well and placed in an incubator at 37°C and 5% CO<sub>2</sub> for 1 h. To eliminate the influence of the drugs in the cell culture supernatant, the HL-60 cells were incubated with DFMG for 48 h, and the supernatant was collected by centrifugation (300 x g for 8 min at room temperature) and then discarded. The HL-60 cells were rinsed three times with RPMI-1640 serum-free medium (Biological Industries) and then incubated for an additional 10 h in 1 ml serum-free medium at 37°C and 5% CO<sub>2</sub>. After the second incubation, the cell culture supernatant was collected by centrifugation (300 x g for 8 min at room temperature). HUVE-12 cells were resuspended in the culture supernatant of HL-60 cells treated with different drugs. The HUVE-12 cell density was adjusted to 1x10<sup>6</sup>/ml, and 1 ml per well cells was inoculated onto the Matrigel surface and incubated at 37°C and 5% CO<sub>2</sub> for 8 h. Images of tube formation of HUVE-12 cells were captured

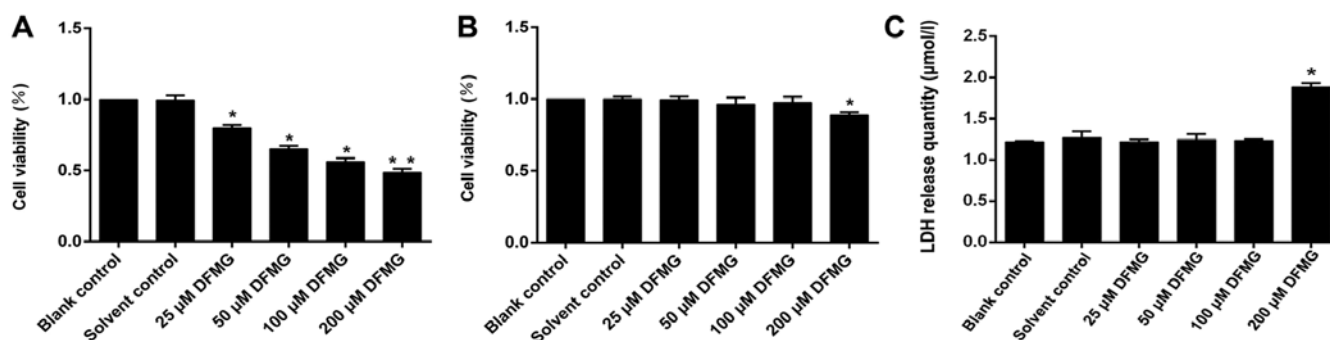


Figure 1. Effect of DFMG on HL-60 cells and HUVE-12 cells at different concentrations. (A) HL-60 cell viability. (B) HUVE-12 cell viability. (C) LDH release from HL-60 cells. Compared with the blank control, the solvent control was not statistically different. \* $P < 0.05$ , \*\* $P < 0.001$  vs. solvent control group. DFMG, difluoromethyl-5,4'-dimethoxygenistein; LDH, lactate dehydrogenase.

using a microscope (IX51; Olympus Corporation) at magnification, x200, and tube numbers were analyzed with ImageJ 1.52t software (National Institutes of Health).

**Western blot (WB) analysis.** Protein was extracted from HL-60 cells lysed in RIPA buffer (Beijing ComWin Biotech Co., Ltd.). The total protein concentration was determined with a bicinchoninic acid protein assay kit (Beijing Solario Science Technology Co., Ltd.). Proteins were then mixed with protein loading buffer and denatured in boiling water at 100°C for 10 min. Equal amounts of protein (30 μg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes at 4°C. After blocking the membranes with 5% skimmed milk at room temperature for 2 h, they were incubated with primary antibodies at 4°C overnight and probed with the appropriate secondary antibodies for 1 h at room temperature the following day. The primary antibodies included VEGF (1:10,000; Abcam; cat. no. ab52917), TLR4 (1:1,000; ABclonal Biotech Co., Ltd.; cat. no. A11226), NF-κB p65 (1:1,000; Abbkine Scientific Co., Ltd.; cat. no. Abp57495), phosphorylated (P)-NF-κB p65 (1:2,000; ABclonal Biotech Co., Ltd.; cat. no. AP0123), IκB-α (1:2,000; ABclonal Biotech Co., Ltd.; cat. no. A1187), and β-actin (1:10,000; Abbkine Scientific Co., Ltd.; cat. no. Abp50151). The secondary antibodies included horseradish peroxidase-conjugated anti-rabbit (1:10,000; Abbkine Scientific Co., Ltd.; cat. no. A21020) and anti-mouse (1:10,000; Abbkine Scientific Co., Ltd.; cat. no. A25012). Immunoreactive bands were visualized using electrochemiluminescence reagent (New Cell & Molecular Biotech Co., Ltd) and were scanned using a chemiluminescence imaging analysis system (Tanon 5200 Chemiluminescent Imaging System). The relative quantitative analysis of proteins was performed using ImageJ software version 1.52t (National Institutes of Health).

**Reverse transcription-quantitative (RT-q)PCR analysis.** Total RNA of HL-60 cells was extracted with TRIzol™ reagent (Vazyme Biotech Co., Ltd.), and then a reverse transcription kit (Vazyme Biotech Co., Ltd.) was used to produce cDNA from RNA under the following conditions: 50°C for 15 min, followed by 85°C for 5 sec. RT-qPCR was carried out using SYBR Premix Extaq™ (Vazyme Biotech Co., Ltd.) and a 7500 fast qPCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) under the following conditions: 95°C for 5 min,

then 40 cycles of 10 sec at 95°C and 30 sec at 60°C, with a final extension for 15 sec at 95°C and 1 min at 60°C. The sequences of the RT-qPCR primers were as follows: VEGF, forward 5'-GCACATAGAGAGAATGAGCTTCC-3', reverse 5'-CTCCGCTCTGAACAAGGCT-3'; TLR4, forward 5'-CCG AGGCCATTATGCTATGT-3', reverse 5'-TCCCTTCTCCT TTTCCCTA-3'; and GAPDH, forward 5'-CAGGAGGCATTG CTGATGAT-3', reverse 5'-GAAGGCTGGGGCTCATTT-3'. The relative quantitative analysis of mRNA expression was normalized to the reference gene GAPDH and calculated using the  $2^{-\Delta\Delta C_q}$  method (24).

**Statistical analysis.** Data from three independent repeats were analyzed with SPSS 20.0 software (IBM Corp.), and the results are shown as the mean ± standard deviation (SD). One-way ANOVA followed by Tukey's post hoc test was used for the multiple-group comparison analysis.  $P < 0.05$  was considered to indicate statistically significant difference.

## Results

**DFMG decreases viability of HL-60 cells but not HUVE-12 cells.** Cells were treated with different concentrations of DFMG (blank control, solvent control, 25, 50, 100 and 200 μM DFMG) for 48 h, and cell viability was observed. It was found that when the concentration of DFMG reached 50 μM, the viability of HL-60 cells was significantly decreased compared with the solvent control (Fig. 1A). However, when DFMG was <100 μM, the viability of HUVE-12 cells was unaffected (Fig. 1B).

**High concentration of DFMG promotes release of LDH from HL-60 cells.** HL-60 cells were treated with different concentrations of DFMG (blank control, solvent control, 25, 50, 100, and 200 μM DFMG) for 48 h, and LDH release was detected. It was shown that 200 μM DFMG promoted the release of LDH from HL-60 cells, indicating that 200 μM DFMG is toxic towards HL-60 cells (Fig. 1C).

**DFMG inhibits angiogenesis induced by HL-60 cells.** HL-60 cell viability was decreased by a >50 μM dosage of DFMG and treatment with 200 μM DFMG appeared to be toxic towards HL-60 cells, additionally <100 μM DFMG did not affect HUVE-12 viability. Therefore, 100 μM DFMG was selected to investigate its effects on angiogenesis induced by

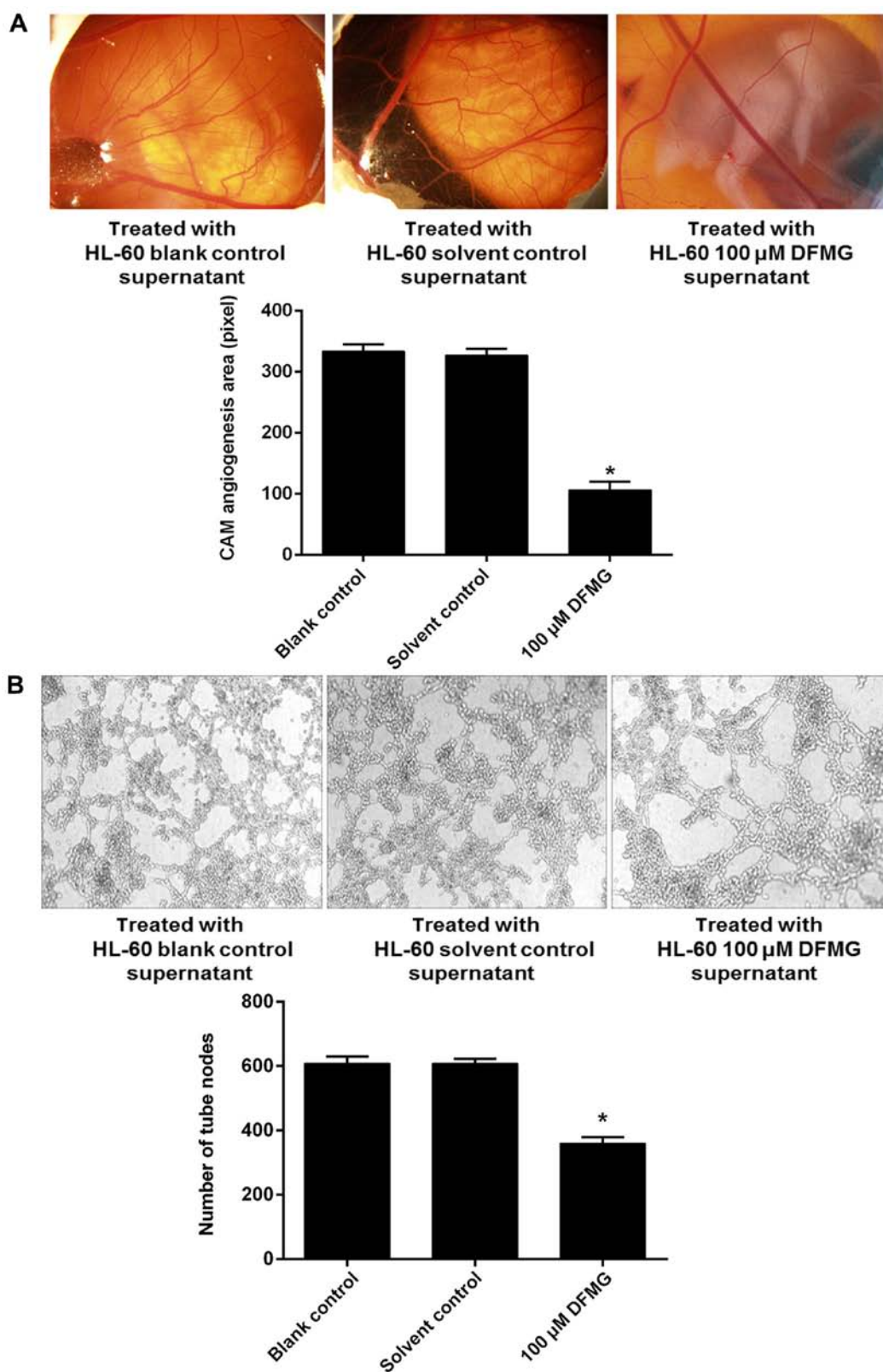


Figure 2. Effect of the culture supernatant in HL-60 cells treated with 100  $\mu$ M DFMG on the angiogenesis area. (A) CAM angiogenesis and (B) Matrigel tubule formation. There was no statistical difference between the blank control and the solvent control. \* $P < 0.05$  vs. solvent control group. DFMG, difluoromethyl-5,4'-dimethoxygenistein; CAM, chorioallantoic membrane.

APL HL-60 cells in the following experiments. CAM assays showed that the area of angiogenesis in the membrane was reduced by the culture supernatant of HL-60 cells treated with 100  $\mu$ M DFMG (Fig. 2A). Matrigel tubule formation

assays showed that the tube formation ability of HUVE-12 cells was significantly reduced when they were incubated in the culture supernatant of HL-60 cells treated with 100  $\mu$ M DFMG (Fig. 2B).



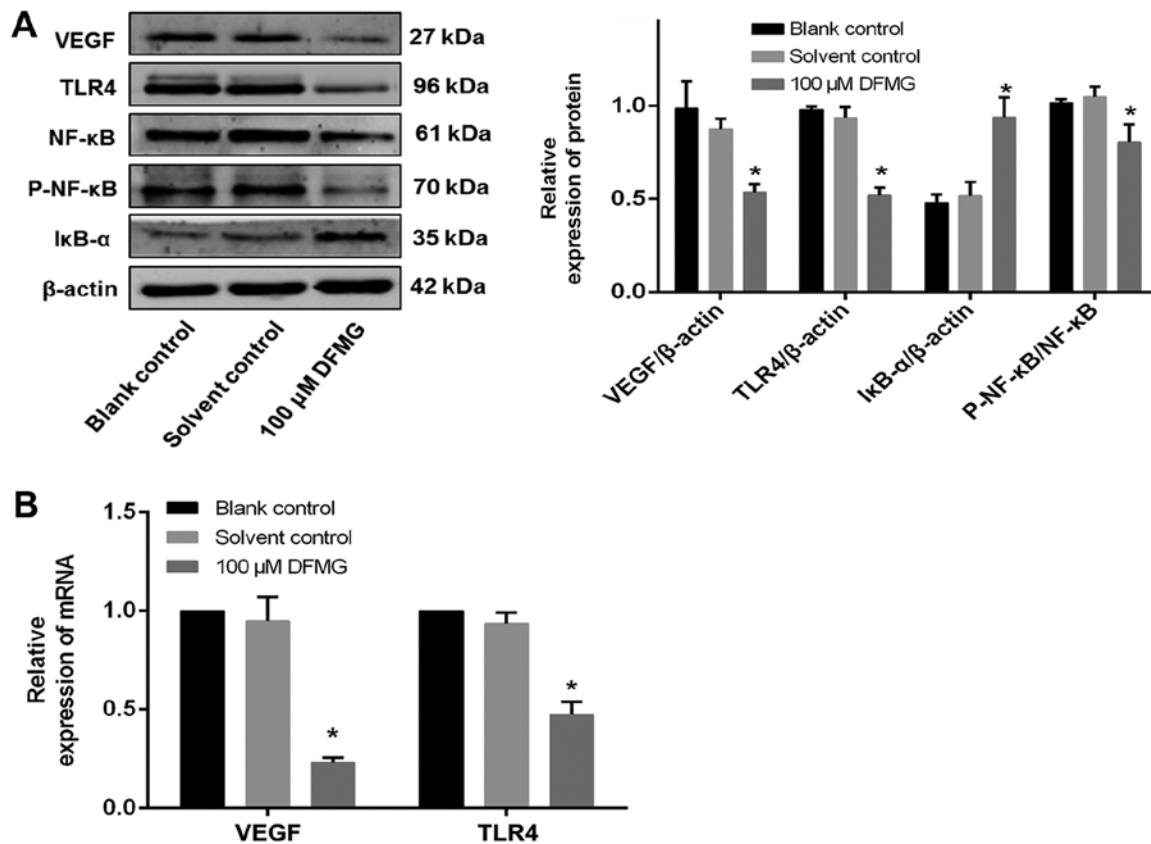


Figure 3. Effect of DFMG on the TLR4/NF- $\kappa$ B signaling pathway and the protein and mRNA expression of VEGF in HL-60 cells. (A) Protein expression of TLR4, P-NF- $\kappa$ B, I $\kappa$ B- $\alpha$  and VEGF in HL-60 cells. (B) mRNA expression of VEGF and TLR4 in HL-60 cells. There was no statistical difference between the blank control and the solvent control. \* $P$ <0.05 vs. solvent control group. DFMG, difluoromethyl-5,4'-dimethoxygenistein; TLR4, toll-like receptor 4; VEGF, vascular endothelial growth factor; P, phosphorylated; NF, nuclear factor.

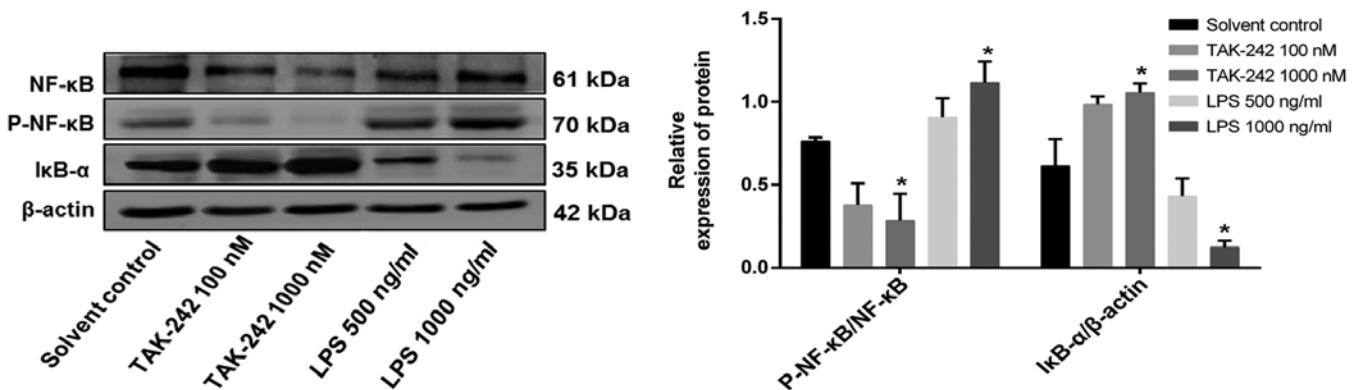


Figure 4. Effect of toll-like receptor 4 blocker, TAK-242, and activator, LPS, on the protein expression of P-NF- $\kappa$ B and I $\kappa$ B- $\alpha$  in HL-60 cells. \* $P$ <0.05 vs. solvent control group. LPS, lipopolysaccharide; P, phosphorylated; NF, nuclear factor.

DFMG inhibits the TLR4/NF- $\kappa$ B signaling pathway and reduces the protein and mRNA expression of VEGF in HL-60 cells. VEGF is an important pro-angiogenic regulator of APL (25). Numerous studies have shown that the TLR4/NF- $\kappa$ B signaling pathway plays an important role in regulating the activity of VEGF (26-28). Therefore, the effects of DFMG on the TLR4/NF- $\kappa$ B signaling pathway and VEGF in HL-60 cells were examined by WB and RT-qPCR. This demonstrated that 100  $\mu$ M DFMG could reduce the protein and mRNA expression of VEGF and TLR4, as well as reduce the protein

expression of P-NF- $\kappa$ B and increase the protein expression of I $\kappa$ B- $\alpha$  (Fig. 3). Together, this suggested that DFMG could inhibit the TLR4/NF- $\kappa$ B signaling pathway in HL-60 cells.

*Inhibition of the TLR4/NF- $\kappa$ B signaling pathway enhances the anti-angiogenic effect of DFMG and activation of the TLR4/NF- $\kappa$ B signaling pathway attenuates its anti-angiogenic effect.* HL-60 cells were treated with different concentrations of TAK-242, a selective TLR4 inhibitor (100 or 1,000 nM), or LPS (500 or 1,000 ng/ml) for 4 h. WB

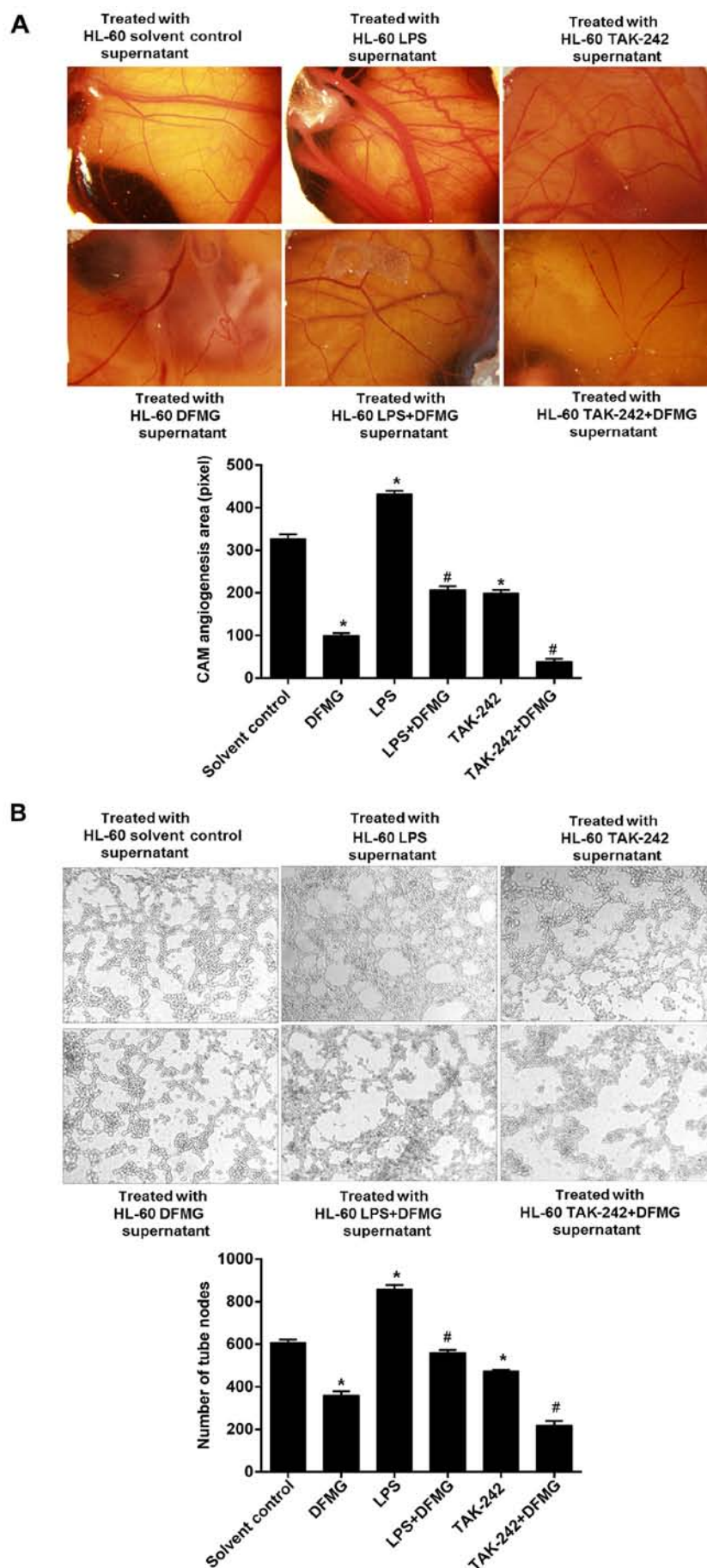


Figure 5. Effect of the culture supernatant in HL-60 cells treated with 100  $\mu$ M DFMG on the angiogenesis area. (A) CAM angiogenesis and (B) Matrigel tube formation after activation or inhibition of the Toll-like receptor 4/nuclear factor  $\kappa$ B signaling pathway. \* $P < 0.05$  vs. solvent control group; # $P < 0.05$  vs. DFMG-only group. DFMG, difluoromethyl-5,4'-dimethoxygenistein; CAM, chorioallantoic membrane.

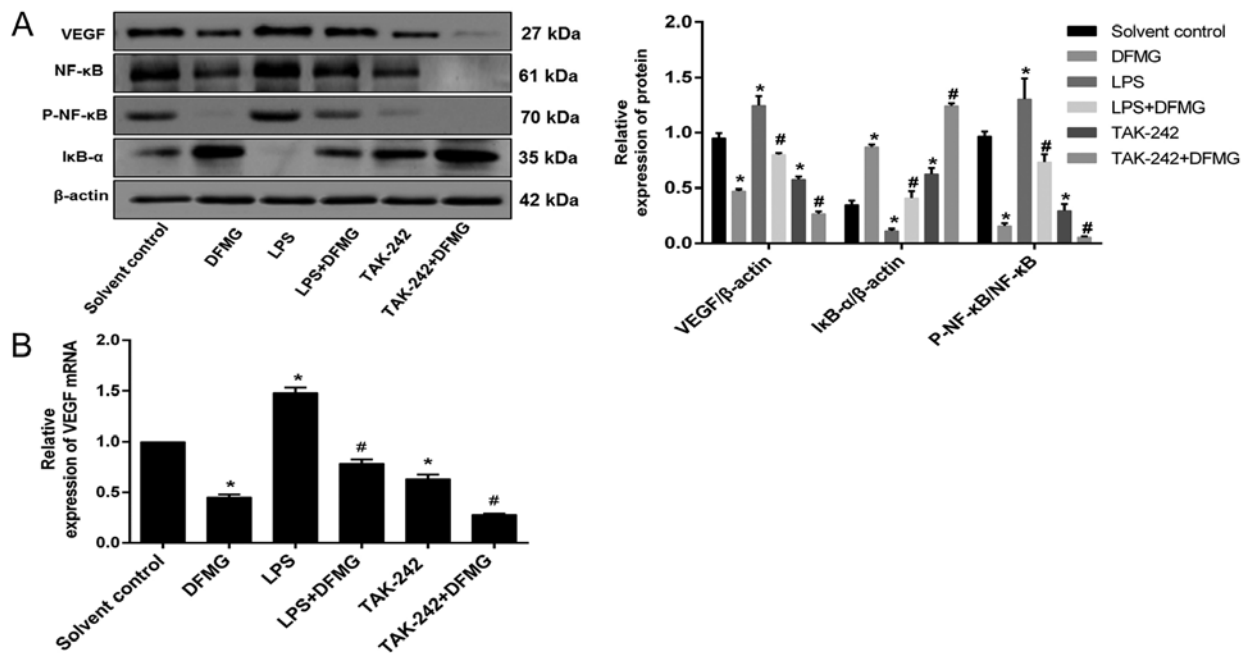


Figure 6. Protein and mRNA expression of VEGF in HL-60 cells treated with DFMG after activation or inhibition of the TLR4/NF- $\kappa$ B signaling pathway. (A) Protein expression of P-NF- $\kappa$ B, I $\kappa$ B- $\alpha$  and VEGF in HL-60 cells. (B) mRNA expression of VEGF in HL-60 cells. \* $P < 0.05$  vs. solvent control group; # $P < 0.05$  vs. DFMG-only group. VEGF, vascular endothelial growth factor; DFMG, difluoromethyl-5,4'-dimethoxygenistein; TLR4, toll-like receptor 4; NF, nuclear factor; P, phosphorylated.

was then performed to investigate the protein expression of I $\kappa$ B- $\alpha$  and P-NF- $\kappa$ B p65. It was observed that TAK-242 treatment increased the protein expression of I $\kappa$ B- $\alpha$  and decreased the protein expression of P-NF- $\kappa$ B in HL-60 cells. However, LPS had the opposite effect. Overall, these results suggested that 1  $\mu$ M TAK-242 and 1  $\mu$ g/ml LPS significantly inhibited or activated the TLR4/NF- $\kappa$ B signaling pathway, respectively (Fig. 4).

After activation or inhibition of the TLR4/NF- $\kappa$ B signaling pathway, HL-60 cells were treated with DFMG for 48 h respectively. The angiogenesis area of the CAM was analyzed after HL-60 cells culture supernatant was collected and added to the CAM. Compared with the solvent control group, in the DFMG group, the area of angiogenesis on the CAM was significantly reduced. However, compared with the DFMG-only group, the area of angiogenesis on the CAM decreased significantly in the TAK-242 + DFMG group, while it increased significantly in the LPS + DFMG group (Fig. 5A).

Similar to the CAM experiment, the data from tubule formation assay showed that compared with the solvent control group, in the DFMG group, the numbers of tubules were significantly reduced. However, compared with the DFMG-only group, the number of tubules decreased significantly in the TAK-242 + DFMG group, whereas numbers increased significantly in the LPS + DFMG group (Fig. 5B).

The protein and mRNA expression of VEGF in HL-60 cells was assessed by WB and RT-qPCR. The data demonstrated that compared with the solvent control group, in the DFMG group, the protein and mRNA expression of VEGF was significantly reduced. However, compared with the DFMG-only group, protein and mRNA expression of VEGF decreased significantly in the TAK-242 + DFMG group and increased significantly in the LPS + DFMG group (Fig. 6).

## Discussion

In 1971, Folkman suggested that tumor growth and metastasis depend on neovascularization (29). In tumor development, numerous new blood vessels are formed to provide nutrition and moisture for tumor growth while spreading tumor cells to distant locations, forming new metastases in other parts of the body (30,31). Physiological angiogenesis is a highly dynamic process involving multiple pro-angiogenic and anti-angiogenic factors in the body; during tumor development, various stimuli cause the levels of pro-angiogenic factors to increase (30,32), leading to an imbalance in this dynamic process (6,33). Previous studies have shown that angiogenesis occurs in acute myeloid leukemia, an invasive hematological malignancy characterized by malignant proliferation of leukemia cells in the bone marrow leading to inhibition of normal bone marrow hematopoiesis (12,34,35). Bone marrow angiogenesis is an important pathological process in the changing hematopoietic microenvironment, and increased bone marrow microvessel density can aggravate the proliferation of acute leukemia cells (36). In studies of APL, VEGF was found to be the most potent specific pro-angiogenic factor (25). Clinical studies have confirmed high expression of VEGF in the bone marrow of patients with APL (37), and that arsenic trioxide reduces the expression of VEGF in APL and exerted anti-angiogenic effects (18). Therefore, treatment inhibiting bone marrow angiogenesis and targeting pro-angiogenic factors has gained attention in the treatment of acute leukemia (17).

DFMG is a synthesized derivative of genistein. In our previous study, DFMG was found to inhibit angiogenesis in atherosclerotic plaques (23). In the present study, HL-60 cells were treated with different concentrations of DFMG,

and subsequently the viability of HL-60 cells and degree of cell damage caused by the toxicological effects of DFMG was detected. With increasing DFMG concentrations, HL-60 cell viability was inhibited at  $>50 \mu\text{M}$  DFMG and release of LDH from HL-60 cells treated with  $200 \mu\text{M}$  DFMG was significantly increased, indicating that  $200 \mu\text{M}$  DFMG is toxic towards HL-60 cells. In addition, DFMG at the dosage of  $<100 \mu\text{M}$  did not affect HUVE-12 viability. Therefore,  $100 \mu\text{M}$  DFMG was selected to investigate its effects on angiogenesis induced by APL HL-60 cells. The reduction of the angiogenesis area in the CAM, as well as the reduction in Matrigel tubule numbers, revealed that DFMG inhibited angiogenesis induced by APL HL-60 cells compared with the solvent control supernatant treatment. DFMG also reduced the protein and mRNA expression of VEGF in HL-60 cells. Thus, the release of VEGF and ability to promote angiogenesis were significantly attenuated in HL-60 cells treated with DFMG.

Numerous studies have shown that the TLR4/NF- $\kappa$ B signaling pathway plays an important role in regulating the activity of VEGF (25,28,38). The proteasomal degradation of I $\kappa$ B- $\alpha$  is an important event in the canonical pathway of NF- $\kappa$ B activation. Subsequently, phosphorylated NF- $\kappa$ B dimers enter the nucleus from the cytoplasm to promote transcription of the gene of interest (39,40). In our previous study, DFMG was shown to reduce the protein and mRNA expression of TLR4 in the angiogenesis of atherosclerotic plaques (23). In the present study, it was shown that DFMG inhibited the TLR4/NF- $\kappa$ B signaling pathway and downregulated the protein and mRNA expression of VEGF in HL-60 cells. Next, it was demonstrated that TAK-242 and LPS inhibited or activated the TLR4/NF- $\kappa$ B signaling pathway in HL-60 cells, respectively. When the TLR4/NF- $\kappa$ B signaling pathway was activated, the CAM angiogenesis area, Matrigel tubule numbers and the mRNA and protein expression of VEGF were all increased in the HL-60 cells. The opposite results were observed in the HL-60 cells when the TLR4/NF- $\kappa$ B signaling pathway was inhibited. Therefore, when the TLR4/NF- $\kappa$ B signaling pathway was activated, downregulation of the protein and mRNA expression of VEGF and inhibition of angiogenesis induced by DFMG were attenuated. On the other hand, when the TLR4/NF- $\kappa$ B signaling pathway was inhibited, downregulation of the protein and mRNA expression of VEGF and inhibition of angiogenesis induced by DFMG were further enhanced.

In summary, DFMG downregulates the protein and mRNA expression of VEGF, and inhibits angiogenesis induced by APL HL-60 cells by inhibiting the TLR4/NF- $\kappa$ B signaling pathway. However, as the present study used only *in vitro* HL-60 cells, it will be important to verify these findings in other leukemic cell lines and *in vivo*. APL is a common malignant disease in the hematopoietic system with an increasing incidence rate. Thus, improved treatment strategies are needed (41). The findings from the present study extend the implications of DFMG and provide a potential new drug candidate for the treatment of patients with APL.

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## Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

## Authors' contributions

YZ, XF, LL and XuX designed the study. XuX, PB, TK, SL, XiX and SG performed the experiments. XuX, LL, XF and YZ analyzed the data. XuX, PB, TK and YZ prepared the manuscript. YZ, LL, XF and XuX revised the manuscript. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

All experimental procedures involving the use of animals were approved by the Animal Use and Care Committee of Hunan Normal University School of Medicine (approval no. 2013-289; Changsha, China).

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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