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Original Article

Trans-3,4,5,4'-tetramethoxystilbene, a resveratrol analog, potently inhibits angiogenesis *in vitro* and *in vivo*

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Aim: *Trans*-3,4,5,4'-tetramethoxystilbene (DMU-212) has shown strong antiproliferative activities against a variety of cancer cells. The aim of this study was to investigate the anti-angiogenic effects of DMU-212 *in vitro* and *in vivo*.

Methods: Human umbilical vein endothelial cells (HUVECs) were used in this study. Cell viability was studied with MTT assay, and cell apoptosis was evaluated using TUNEL assay and morphological observation. The expression of the related genes and proteins was analyzed with qRT-PCR and Western blot, respectively. Angiogenesis of HUVECs were studied using cell migration and capillary-like tube formation assays *in vitro*, and mouse Matrigel plug assay and chick chorioallantoic membrane (CAM) assay *in vivo*. The tyrosine kinase activities of VEGFR1 and VEGFR2 were measured using commercial kits.

Results: DMU-212 (5–80 μmol/L) significantly inhibited VEGF-stimulated proliferation of HUVECs (IC_{50} value was approximately 20 μmol/L), and induced apoptosis. Furthermore, DMU-212 concentration-dependently inhibited VEGF-induced migration of HUVECs and capillary-like structure formation *in vitro*. DMU-212 also inhibited VEGF-induced generation of new vasculature in Matrigel plugs *in vivo* with significantly decreased area of infiltrating CD31-positive endothelial cells, and inhibited newly formed microvessels in chick CAMs. Moreover, DMU-212 concentration-dependently suppressed VEGF-induced phosphorylation of VEGFR2, and inhibited phosphorylation of multiple downstream signaling components in the VEGFR2 pathway, including c-Src, FAK, Erk1/2, Akt, mTOR, and p70S6K in HUVECs. DMU-212 had no effect on VEGF-induced phosphorylation of VEGFR1.

Conclusion: DMU-212 is a potent inhibitor of angiogenesis that exerts anti-angiogenic activity at least in part through the VEGFR2 signaling pathway.

Introduction

Angiogenesis, a biological process of new blood vessel formation by sprouting from preexisting vessels, is a key component of a variety of physiologic processes, including embryonic vascular development, differentiation, wound healing and organ regeneration[1]. Angiogenesis is tightly regulated by a balance of pro-angiogenic and anti-angiogenic factors[2]. However, many serious diseases, including arthritis, psoriasis, atherosclerosis, diabetic retinopathy, immune disorders, and age-related macular degeneration depend on up-regulated angiogenesis[1, 3, 4]. Moreover, angiogenesis is a fundamental process in the transition of tumors from a dormant state to a malignant state and is considered a hallmark of cancer[5], playing a significant role in tumor growth, invasion, and metastasis[1]. Considering that endothelial cells are genetically stable and less susceptible to drug resistance than many cell types[6], therapies targeting angiogenesis have become increasingly important for tumor chemotherapy and the treatment of other angiogenesis-related diseases.

Resveratrol (*trans*-3,4',5-trihydroxystilbene), a naturally polyphenolic phytoalexin found in grapes, cranberries, peanuts and other dietary constituents, has a wide spectrum of anticancer activities both *in vitro* and *in vivo*[7, 8]. In recent years, resveratrol has been found to possess angiogenesis-regulating properties[9, 10]. Unfortunately, resveratrol can induce either pro- or anti-angiogenic effects depending on the situation, applied dosage and cell type[10]. In addition,

resveratrol has a poor systemic bioavailability and metabolic stability because it has three hydroxyl groups[11]. Structural modifications of resveratrol are needed to increase its bioavailability while preserving its beneficial activities. Attempts to modify resveratrol have predominantly been concerned with the introduction of additional hydroxy moieties into the trihydroxystilbene framework and various degrees of methylation of the phenol groups[12]. An especially auspicious finding concerning these analogs is the fact that *trans*-3,4,5,4'-tetramethoxystilbene (DMU-212; Figure 1) possesses more favorable pharmacokinetic properties than resveratrol and stronger anti-proliferative activities in a variety of cancer cells, including HeLa cervical cancer cells, LnCaP prostate cancer cells, HepG2 hepatoma cells, MCF-7 and MDA-MB-435/LCC6 breast cancer cells, and HCA-7, HCEC, and HT-29 colon cancer cells[13–15]. Despite these widely described anti-cancer properties, DMU-212 has not been investigated for its anti-angiogenic potential. The present study aimed to evaluate the effect of DMU-212 on angiogenesis and to determine the molecular mechanism by which this compound specifically regulates endothelial cells.
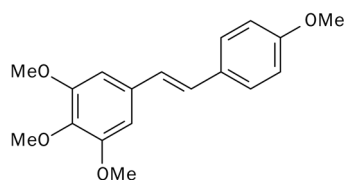
Trans-3,4,5,4'-tetramethoxystilbene (DMU-212)
Chemical formula: $C_{18}H_{20}O_4$
Molecular weight: 300.3490

Figure 1. The chemical structure of *trans*-3,4,5,4'-tetramethoxystilbene (DMU-212). DMU-212 has the molecular formula $C_{18}H_{20}O_4$ and a molecular weight of 300.3490 g/mol. DMU-212 was dissolved in DMSO (0.1 mol/L) as a stock solution.

Materials and methods
Materials
DMU-212 was synthesized in our laboratory (purity was established as at least 99% by HPLC analysis) and dissolved in dimethyl sulfoxide (DMSO) to form a 0.1 mol/L stock solution, which was stored at -20 °C in small aliquots until needed and then diluted to various concentrations as needed. Fetal bovine serum (FBS) was purchased from HyClone Laboratory (Logan, UT, USA). M199 medium and recombinant human vascular endothelial growth factor (VEGF) were purchased from Gibco (Carlsbad, CA, USA). DMSO, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium (MTT), Tween 20, Tris, sodium dodecyl sulfate (SDS), glycerol, β-mercaptoethanol, bromophenol blue, Coomassie brilliant blue, bovine serum albumin (BSA) and phenylmethanesulfonyl fluoride (PMSF) were obtained from Shanghai Sangon Biotech (Shanghai, China). Matrigel was purchased from BD Biosciences (San Diego, CA, USA). A ter-

minal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) kit was obtained from Promega (Madison, WI, USA). Protease inhibitor cocktail was purchased from Sigma (St Louis, MO, USA). Polyvinylidene difluoride (PVDF) membranes were purchased from Millipore (Billerica, MA, USA). TRIzol reagent was acquired from Invitrogen (Carlsbad, CA, USA). PrimeScript™ RT reagent kit and SYBRH Premix Ex Taq™ were purchased from TaKaRa (Kyoto, Japan). The antibodies anti-cleaved PARP (Asp[214]), anti-cleaved caspase-3 (Asp[175]), anti-cleaved caspase-9 (Asp[175]), anti-phospho (p)-VEGFR2 (Tyr[1175]), anti-p-VEGFR1 (Tyr[1213]), anti-p-c-Src (Tyr[416]), anti-p-focal adhesion kinase (FAK) (Tyr[576/577]), anti-p-extracellular signal-regulated kinase 1/2 (Erk1/2) (Thr[202]/Tyr[204]), anti-p-Akt (Ser[473]), anti-p-mammalian target of rapamycin (mTOR) (Ser[2448]), anti-ribosomal protein S6 kinase (p70S6K) (Thr[389]), anti-c-Src, anti-FAK, anti-Erk1/2, anti-Akt, anti-mTOR, and anti-p70S6K were purchased from Cell Signaling Technology (Beverly, MA, USA). The antibodies anti-CD-31, anti-Bcl-2, anti-survivin, anti-α-tubulin, anti-VEGFR2, anti-VEGFR1, anti-β-actin, anti-GAPDH, and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

Cell culture
Human umbilical vein vascular endothelial cells (HUVECs) were obtained in our laboratory as described[16]. Cells were cultured on gelatin-coated plastic dishes in M199 medium supplemented with 20% FBS and 70 ng/mL fibroblast growth factor 2 in a humidified incubator at 37 °C with 5% CO_2. Cells were used at or before passage 8. The identity of HUVECs was confirmed by their cobblestone morphology and strong positive immunoreactivity to CD31.

Exposure of HUVECs to DMU-212
HUVECs were plated in 24-well cell culture plates. When cells reached 80% confluency, they were treated with VEGF (10 ng/mL) and various concentrations (0, 5, 10, 20, 40, or 80 µmol/L) of DMU-212. The morphological changes of cells were observed under a phase contrast microscope (Nikon, Japan) at 24 or 48 h.

Cell viability assay
HUVECs were plated in 96-well cell culture plates. When cells reached 80% confluency, they were incubated with VEGF (10 ng/mL) and various doses of DMU-212 (0, 5, 10, 20, 40, or 80 µmol/L) for 24 or 48 h. Cell viability was determined by MTT assay as described[17]. The viability (%) was expressed as=[optical density (*OD*) of treated group/*OD* of control group]×100%. The viability of the control group was set to 100%.

TUNEL assay
HUVECs were plated in 24-well cell culture plates. When cells reached 80% confluency, they were incubated with VEGF (10 ng/mL) and various doses of DMU-212 (0, 5, 10, 20, 40, or 80 µmol/L) for 24 or 48 h. DNA fragmentation was detected by

the DeadEnd™ Fluorometric TUNEL System according to the manufacturer's protocol. Samples were evaluated under fluorescence microscopy (Nikon, Japan). The apoptosis rate was quantified according to the TUNEL-positive rate.

Western blot analysis

After treatment, HUVECs were lysed in lysis buffer containing 25 mmol/L Tris-HCl (pH 6.8), 2% SDS, 6% glycerol, 2 mmol/L PMSF, 1% β-mercaptoethanol, 0.2% bromphenol blue and a protease inhibitor cocktail for 10 min on ice and then boiled in a digital dry bath incubator for 10 min. The protein concentration was determined by Coomassie brilliant blue protein assay. Total endothelial protein extracts (30 μg) were separated by 12% SDS-polyacrylamide gel electrophoresis and transferred to a PVDF membrane. Blots were incubated with the corresponding primary antibody (1:1000) and then with an HRP-conjugated secondary antibody (1:5000). The immunoreactive bands were developed with an ECL Western blotting system. Band intensity was quantified by Quantity One software.

Cell migration assay

HUVECs were plated in 24-well cell culture plates. Cells were wounded by scraping with pipette tips and then washed twice with medium to remove detached cells. Then, cells were treated with VEGF (10 ng/mL) and various doses of DMU-212 (0, 5, 10, 20, 40, or 80 μmol/L). Migration was documented by photos taken immediately after scraping, as well as 24 or 48 h later. Initial and final wound sizes were measured using Axio-Vision Rel.4.7 software, and the difference between the two was used to determine migration distance using the following formula: Migration distance=(Initial wound size–Final wound size)/2.

Capillary-like tube formation assay

Growth factor-reduced Matrigel was pipetted into pre-chilled 96-well plates (40 μL Matrigel per well) and polymerized for 1 h at 37°C. HUVECs (4×10^4 per well) were simultaneously seeded with VEGF (10 ng/mL) and various doses of DMU-212 (0, 5, 10, 20, 40, or 80 μmol/L) on Matrigel-coated plates. After 24 h of incubation, tubular structures were photographed under inverted phase-contrast microscopy (Nikon, Japan) at a magnification of ×100. The degree of tube formation was quantified by measuring the length of tubes in random fields from each well using the National Institutes of Health (NIH) Image Program.

In vivo Matrigel plug assay

Eight-week-old C57BL/6 mice were obtained from Department of Laboratory Animal Science, Peking University Health Science Center (PUHSC), China. They (each group, *n*=10) were subcutaneously injected with 500 μL of Matrigel containing 100 ng/mL VEGF, 100 units of heparin and 0 or 20 μmol/L of DMU-212. The injected Matrigel rapidly formed a single, solid gel plug. After 2 weeks, mice were sacrificed, and the skin of each mouse was pulled back to expose the Matrigel plug. Pictures of Matrigel plugs were taken using a Canon EOS 550D

Digital SLR camera. Matrigel plugs were then snap-frozen in optimal cutting temperature (OCT) embedding medium (Tissue-Tek). Frozen sections were subjected to immunofluorescent staining with anti-CD31 antibody. Microvessel density was defined as the percentage of vessel area, which was calculated as (CD31-positive)/Matrigel plug area. All procedures were in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No 85–23, revised 1996).

Chick chorioallantoic membrane (CAM) assay

Fertilized chick eggs were kept in a humidified incubator at 37°C for 5 d. After incubation, eggs were opened on the air sac side, and chick embryos were prepared by separating the CAM from the shell membrane. Filter disks with various doses of DMU-212 (0, 5, 10, 20, 40, or 80 μmol/L) were placed on the CAM. The cavity was covered with parafilm, eggs were incubated for additional 2 d, and CAMs were removed for analysis. Angiogenesis was quantified by counting the number of branching blood vessels. Assays for each test sample were carried out using 15 eggs.

Quantitative real-time PCR (qRT-PCR)

Total RNA was prepared from HUVECs using TRIzol reagent. RNA was reverse-transcribed to single-stranded cDNA using the PrimeScript™ RT Reagent Kit, followed by qRT-PCR using the SYBRH Premix Ex Taq™ in Real-Time PCR System (Eppendorf, Germany). The reverse-transcribed RNA was primed with oligonucleotides specific for VEGFR1 (forward: 5′-GGGCAGACTCTTGTCCTCAACT-3′; reverse: 5′-CAGCT-CATTTGCACCCTCGT-3′), VEGFR2 (forward: 5′-GACTGTG-GCGAAGTGTTTTTGA-3′; reverse: 5′-GTGCAGGGGAGGGT-TGGCGTAG-3′), and β-actin (forward: 5′-GTGCGGGACAT-CAAGGAGAA-3′; reverse: 5′-AGGAAGGAGGGCTGGAA-GAG-3′) (Applied Biosystems, USA).

In vitro VEGFR1 and VEGFR2 kinases activity assays

After treatment, the tyrosine kinase activities of VEGFR1 and VEGFR2 were assayed with HTScan VEGFR1 and VEGFR2 kinase assay kits following the manufacturer's protocol (Cell Signaling Technology, USA). The results are expressed as percent kinase activity of the control (100%).

Statistical analysis

All experiments were performed in duplicate and repeated independently at least 3 times. Data are expressed as the mean±SEM. Treatment groups were compared by *t*-test with SPSS 17.0 (SPSS Inc, Chicago, USA). Differences were considered statistically significant at $P<0.05$.

Results

DMU-212 inhibits cell viability and potentiates apoptosis in HUVECs

Because angiogenesis is primarily initiated by growth factors, we first investigated the effect of DMU-212 (Figure 1) on VEGF-stimulated HUVEC viability by MTT assay. We found
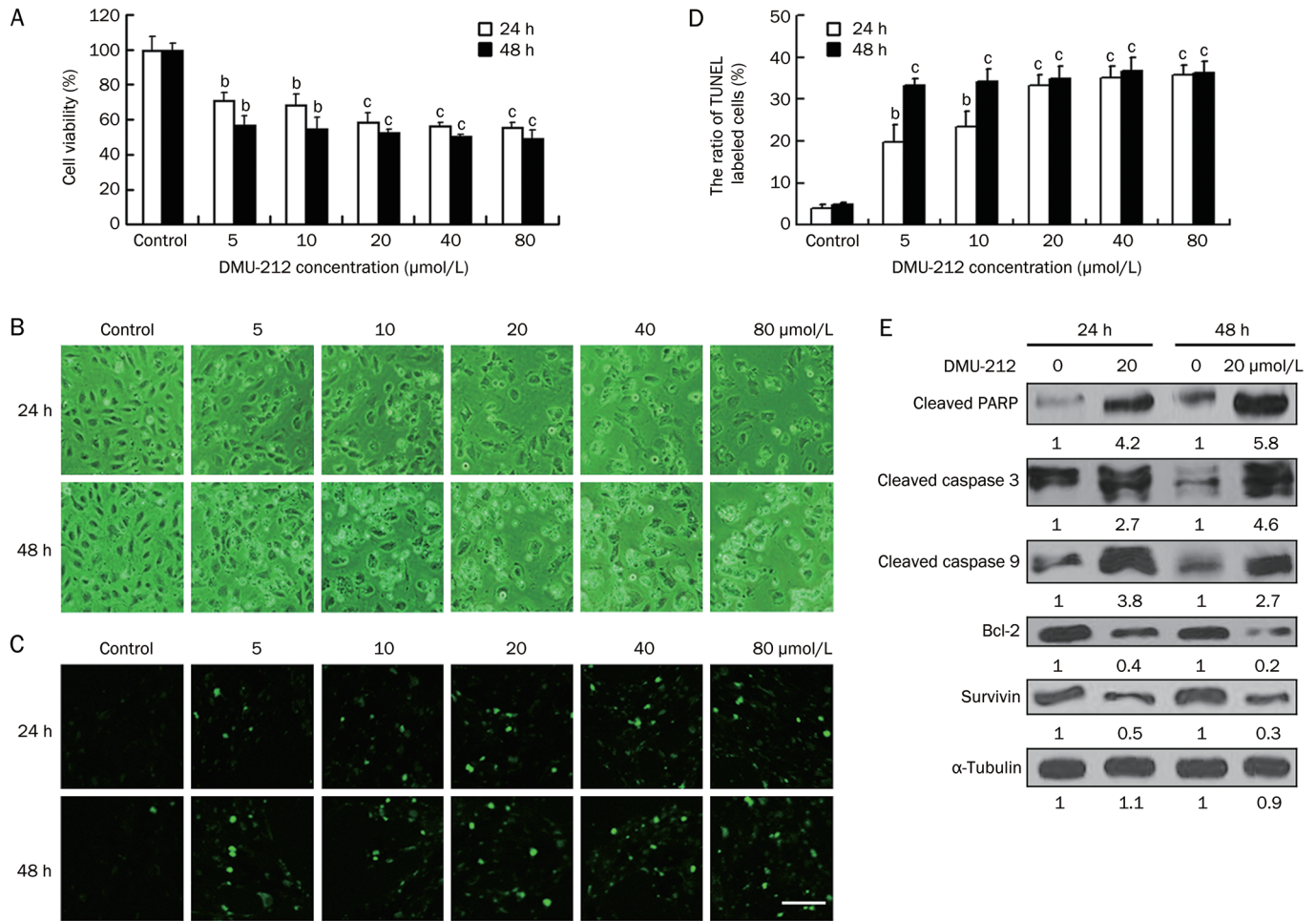
Figure 2. DMU-212 inhibits cell viability and induces apoptosis in HUVECs. (A) Cells were treated with various doses of DMU-212 (0, 5, 10, 20, 40, or 80 µmol/L) in the presence of VEGF (10 ng/mL) for 24 or 48 h. Cell viability was determined by MTT assay. (B) Morphological micrographs were obtained under a phase-contrast microscope (×100). (C) Cell apoptosis was detected by TUNEL assay at 24 or 48 h. Bar=40 µm. (D) Histogram shows the ratio of TUNEL-positive cells. (E) Cells were treated with (20 µmol/L) or without DMU-212 in the presence of VEGF (10 ng/mL) for 24 or 48 h. After treatment, total cell lysates were prepared and analyzed for cleaved PARP, cleaved caspase 3, cleaved caspase 9, Bcl-2, and survivin by Western blot. In each case, the membrane was stripped and reprobed with anti-α-tubulin antibody to confirm equal protein loading. Data are expressed as the mean ±SEM of three independent experiments. [b]$P<0.05$, [c]$P<0.01$ *vs* control.

that when cells were cultured in M199 medium containing FBS and 10 ng/mL VEGF, 5–80 µmol/L of DMU-212 significantly inhibited VEGF-stimulated HUVEC proliferation and survival. Approximately 50% inhibition (IC_{50}) was observed at a DMU-212 concentration of 20 µmol/L after 48 h of treatment. We found that DMU-212 was effective even at low concentrations (5 and 10 µmol/L, Figure 2A).

To clarify whether the reduction of cell viability induced by DMU-212 was related to apoptosis, we detected the effect of DMU-212 on HUVEC apoptosis. Phase-contrast microscopy was used to evaluate the effect of DMU-212 on cell morphological features. Exposure of HUVECs to DMU-212 (5–80 µmol/L) for 24 or 48 h increased the number of shrinking cells and cells detached from the culture dish (Figure 2B). The percentage of TUNEL-positive cells in DMU-212 (5–80 µmol/L)-treated groups was markedly increased compared with the control (Figure 2C and 2D). DMU-212 induced HUVEC

apoptosis in a dose-dependent manner, with a plateau at 20 µmol/L. Accordingly, we used 20 µmol/L DMU-212 to further analyze its effect on apoptosis-related signaling molecules in HUVECs.

As shown in Figure 2E, DMU-212 treatment for 24 or 48 h resulted in poly(ADP-ribose) polymerase (PARP) cleavage, which is a central event in the execution of the apoptotic program in a variety of cells[18]. DMU-212 also increased the levels of cleaved caspase-3 and -9, two well established biomarkers for apoptotic death in HUVECs, after 24 or 48 h of treatment. Furthermore, DMU-212 treatment for 24 or 48 h decreased the levels of anti-apoptotic proteins Bcl-2 and survivin (Figure 2E). These data clearly suggest that DMU-212 inhibited cell viability and potentiated apoptosis in HUVECs, at least partly by modulating the levels of pro-apoptotic and anti-apoptotic molecules.
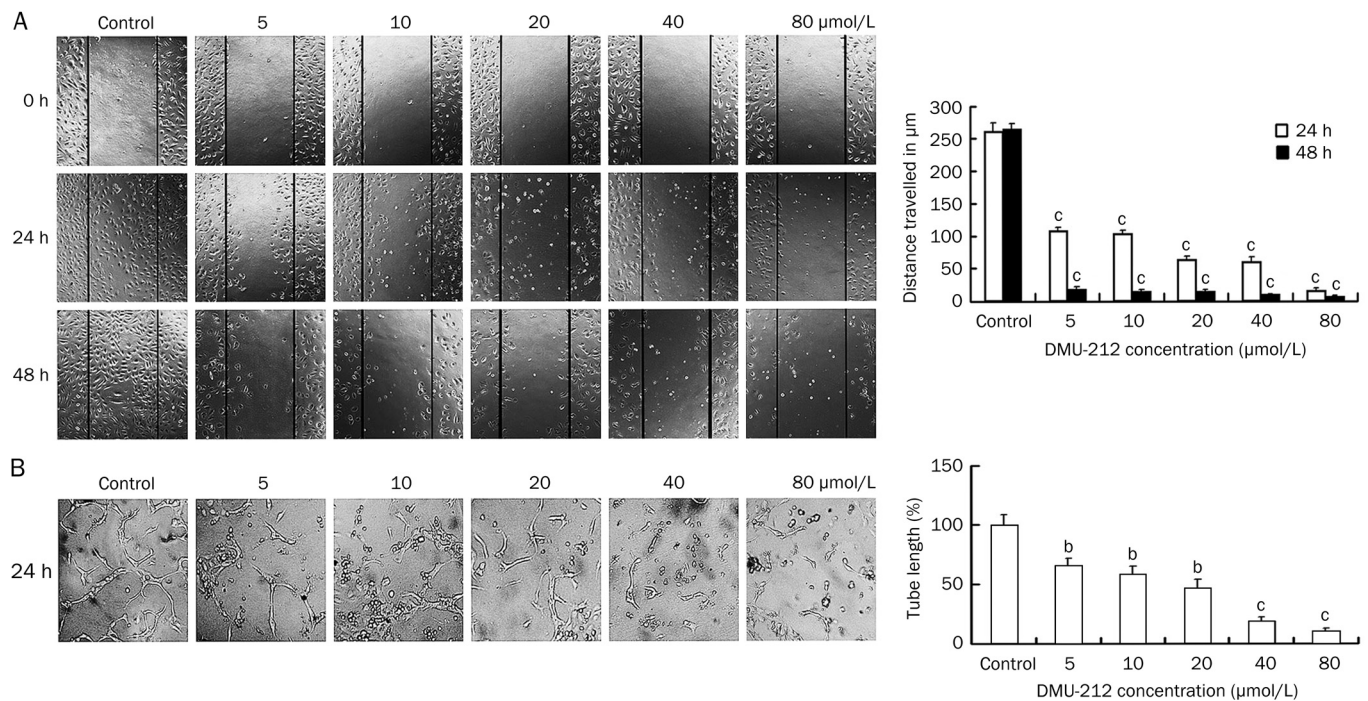
Figure 3. DMU-212 inhibits VEGF-induced migration and capillary structure formation of HUVECs. (A) The effect of DMU-212 (0, 5, 10, 20, 40, or 80 μmol/L) on the migratory potential of VEGF-treated HUVECs was analyzed by wound healing assay. Top photos were taken immediately after scraping. Middle photos were taken 24 h after scraping. Bottom photos were taken 48 h after scraping. These photos were obtained under a phase-contrast microscope (×40). Histogram shows the cell migration distance data. (B) The effect of DMU-212 (0, 5, 10, 20, 40, or 80 μmol/L) on VEGF-treated tube formation of HUVECs was examined by plating cells on Matrigel. Histogram shows the total length of tubes after 24 h of treatment. These photos were obtained under a phase-contrast microscope (×100). Data are expressed as the mean±SEM of three independent experiments. [b]P<0.05, [c]P<0.01 *vs* control.

DMU-212 inhibits VEGF-induced migration and capillary structure formation of HUVECs

Endothelial cell migration is essential for new blood vessel formation during neo-angiogenesis[19]. Accordingly, we investigated the effect of DMU-212 on the migratory ability of endothelial cells using a monolayer-culture wound-healing assay. As shown in Figure 3A, DMU-212 treatment for 24 h decreased the distance migrated by HUVECs approximately by 58.4%, 59.9%, 75.4%, 76.6%, and 93.8% at 5, 10, 20, 40, and 80 μmol/L, respectively. DMU-212 treatment for 48 h almost completely abolished VEGF-induced HUVEC migration, even at lower concentrations (5 and 10 μmol/L).

Another important step during angiogenesis is the formation and merging of tubes by endothelial cells, forming a complex network of vessels and capillaries[20]. The Matrigel tube formation assay serves as an ideal *in vitro* model system to study the formation of the vascular loop during angiogenesis[21]. Upon seeding on Matrigel, HUVECs rapidly aligned with one another and formed a tube-like network in the presence of VEGF for 24 h. Consistent with its effect on migration, DMU-212 significantly reduced the numbers of branch points and ends and the total tube length in VEGF-stimulated HUVECs (Figure 3B).

DMU-212 inhibits VEGF-stimulated angiogenesis *in vivo*

Considering that the effect of DMU-212 on HUVECs *in vitro* cannot be directly translated into its *in vivo* anti-angiogenic activity, we further assessed the *in vivo* anti-angiogenic activity of DMU-212 using mouse Matrigel plug and chick CAM angiogenesis assays. As shown in Figure 4A, the presence of VEGF in the Matrigel plug induced strong vascularity and led the cells to appear dark red with blood. The addition of DMU-212 (20 μmol/L) to the Matrigel plugs markedly blocked the VEGF-induced vascularity. We next stained the Matrigel plugs with anti-CD31 antibody to analyze vessel density. The CD31-positive staining indicated a lower density of functional vasculature in the DMU-212-incorporated plugs (Figure 4B). In the chick CAM model, newly formed microvessels regressed in the areas near the DMU-212-implanted disks in a dose-dependent manner (Figure 4C). DMU-212 induced avascular zone formation in the developing embryos, supporting the strong anti-angiogenic effect of DMU-212 *in vivo*.

DMU-212 is a potent VEGFR2 but not VEGFR-1 tyrosine kinase inhibitor

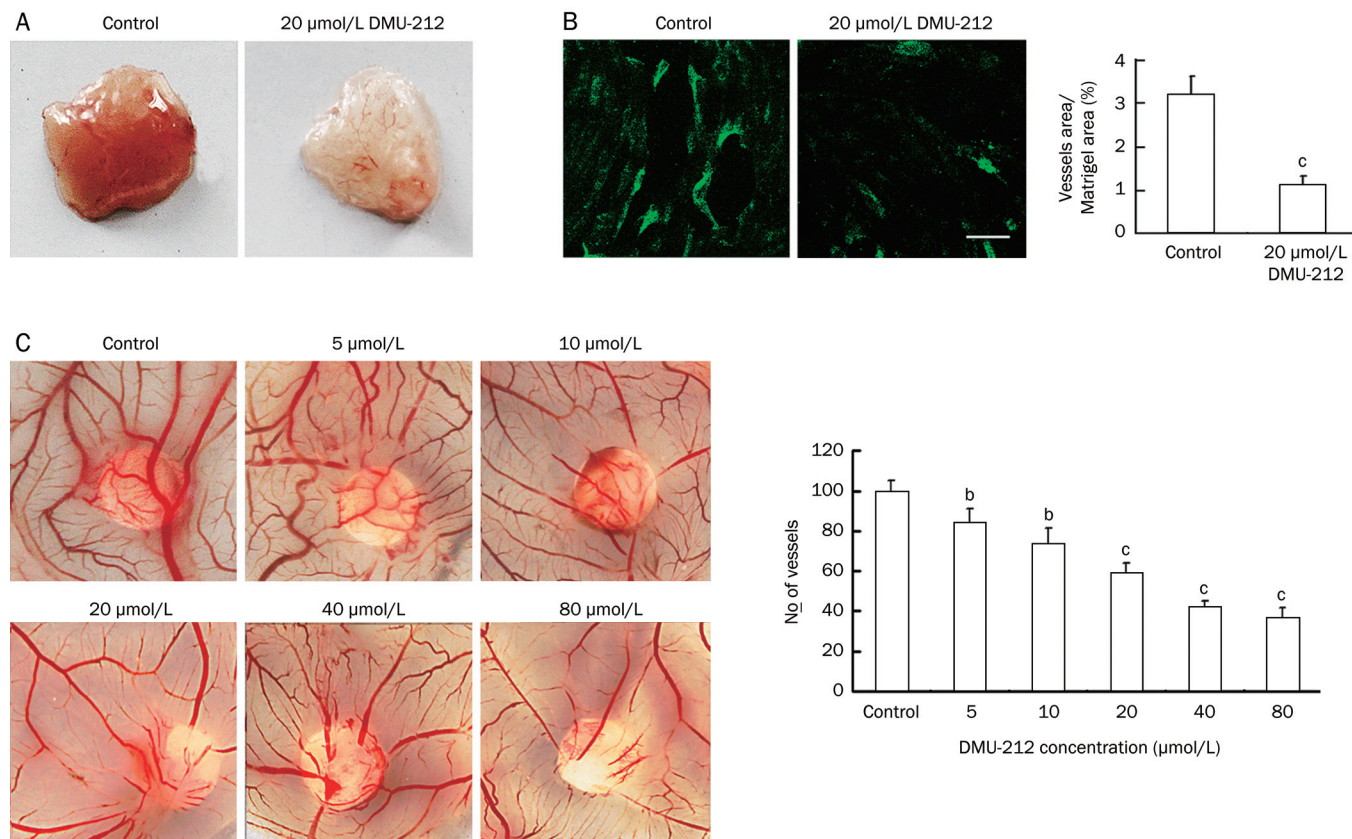VEGF is a master regulator of both physiologic and pathologic angiogenesis[22], which acts mainly through two recep-

Figure 4. DMU-212 inhibits VEGF-induced angiogenesis *in vivo*. (A) Representative photographs of Matrigel plugs containing 100 ng/mL VEGF with or without 20 μmol/L DMU-212. Plugs were removed from C57BL/6 mice at 2 weeks post-implantation. (B) Immunofluorescent labeling of microvessels with anti-CD31 antibody. Bar=40 μm. Histogram shows quantitative analysis of vessel density within Matrigel plugs. Microvessel density was defined as the ratio of vessel area CD31-positive/Matrigel plug area in 200× fields. (C) The effect of DMU-212 (0, 5, 10, 20, 40, or 80 μmol/L) on the newly formed blood vessels was examined by chick CAM assay. Histogram shows the relative number of newly formed blood vessels on disks. Assays for each test sample were carried out using 15 eggs. Data are expressed as the mean±SEM of three independent experiments. [b]$P<0.05$, [c]$P<0.01$ *vs* control.

tor tyrosine kinases: VEGFR2 and VEGFR1. To identify the molecular targets of the anti-angiogenic activity of DMU-212 *in vitro*, we examined whether DMU-212 affects VEGF-induced VEGFR2 and VEGFR1 gene expression in HUVECs using qRT-PCR. As shown in Figure 5A, there were no significant differences in the expression of either VEGFR1 or VEGFR2 mRNA between DMU-212-treated groups and the VEGF-treated group, indicating that the DMU-212 anti-angiogenic activity did not involve down-regulating VEGF-stimulated VEGFR1 and VEGFR2 gene expression in HUVECs.

Thus, we investigated whether DMU-212 affects VEGFR1 and VEGFR2 protein expression in HUVECs using Western blot analysis. The results showed that DMU-212 dramatically inhibited VEGF-induced VEGFR2 phosphorylation at Tyr[1175] but did not inhibit phosphorylation of VEGFR1 Tyr[1213] (Figure 5B). Moreover, we found that DMU-212 did not exert obvious influences on total VEGFR2 or VEGFR1 protein expression (Figure 5B). These findings suggest that inhibition of VEGFR2 by DMU-212 was not due to down-regulation of VEGF-induced VEGFR2 expression but rather to direct blockage of VEGF-induced phosphorylation of VEGFR2.

To verify whether DMU-212 affects the VEGFR1 and VEGFR2 tyrosine kinase activities, we performed ELISA-based *in vitro* VEGFR1 and VEGFR2 tyrosine kinase assays. Our results showed that DMU-212 directly inhibited VEGFR2 tyrosine kinase activity in a dose-dependent manner but did not suppress VEGFR1 tyrosine kinase activity (Figure 5C and D), indicating that DMU-212 is a potent VEGFR2 but not VEGFR-1 tyrosine kinase inhibitor.

DMU-212 inhibits the phosphorylation of VEGFR2-induced downstream signaling molecules

To further clarify the mechanism that underlies the anti-angiogenic activity of DMU-212, we assessed some key molecules involved in VEGFR2-mediated angiogenesis signaling. We found that DMU-212 significantly inhibited the phosphorylation of multiple downstream signaling components of the VEGFR2 pathway, including c-Src, FAK, Erk1/2, Akt, mTOR, and p70S6K (Figure 6). Taken together, our results indicate that DMU-212 reduces angiogenic activity by suppressing the activation of the VEGFR2 signaling cascade in endothelial cells.
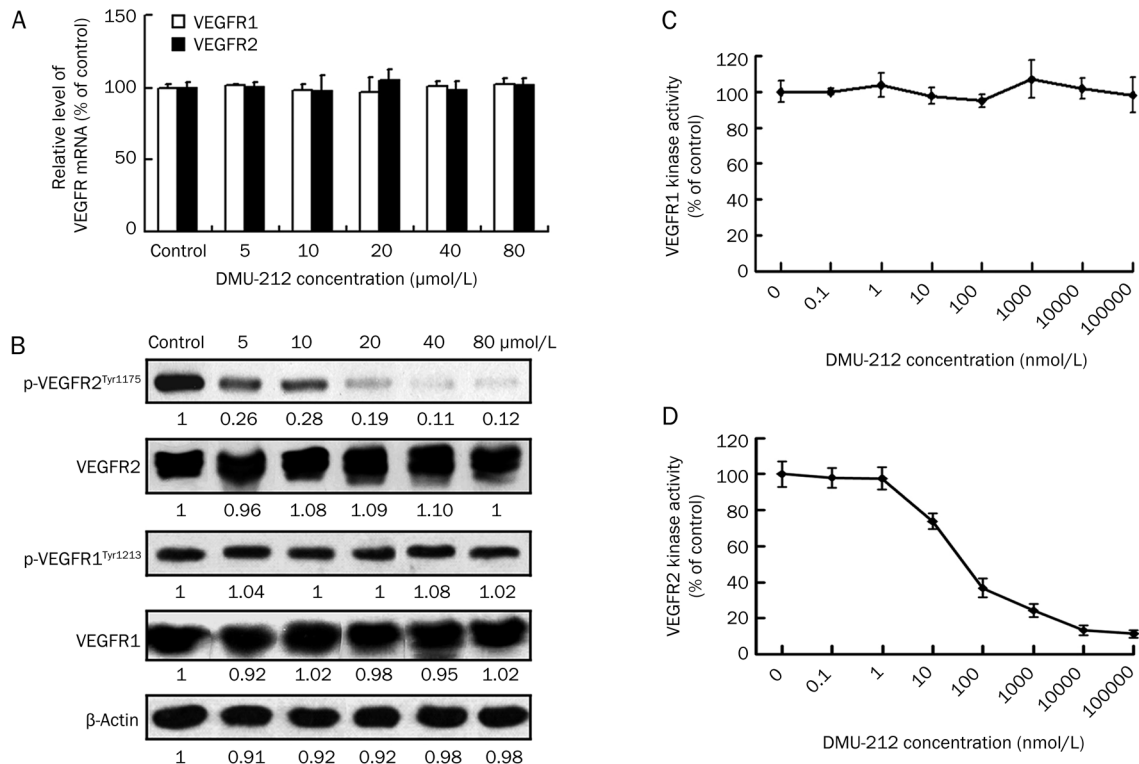
Figure 5. DMU-212 inhibits VEGF-induced phosphorylation of VEGFR2 tyrosine kinase in HUVECs. (A) Cells were treated with various doses of DMU-212 (0, 5, 10, 20, 40, or 80 μmol/L) in the presence of VEGF (10 ng/mL) for 24. The levels of VEGFR1 and VEGFR2 mRNAs were normalized to β-actin and expressed as percentages of the control (100%). (B) Cells were treated with various doses of DMU-212 (0, 5, 10, 20, 40, or 80 μmol/L) in the presence of VEGF (10 ng/mL) for 24 h. After treatment, total cell lysates were prepared and analyzed for p-VEGFR1, p-VEGFR2, VEGFR1, and VEGFR2. In each case, the membrane was stripped and reprobed with anti-β-actin antibody to confirm equal protein loading. (C) The effect of DMU-212 (0–100 000 nmol/L) on the VEGFR1 kinase activity was analyzed using an *in vitro* HTScan VEGF Receptor 1 Kinase Kit according to the manufacturer's instructions. (D) The effect of DMU-212 (0–100 000 nmol/L) on the VEGFR2 kinase activity was analyzed using an *in vitro* HTScan VEGF Receptor 2 Kinase Kit according to the manufacturer's instructions. Data are expressed as the mean±SEM of three independent experiments.
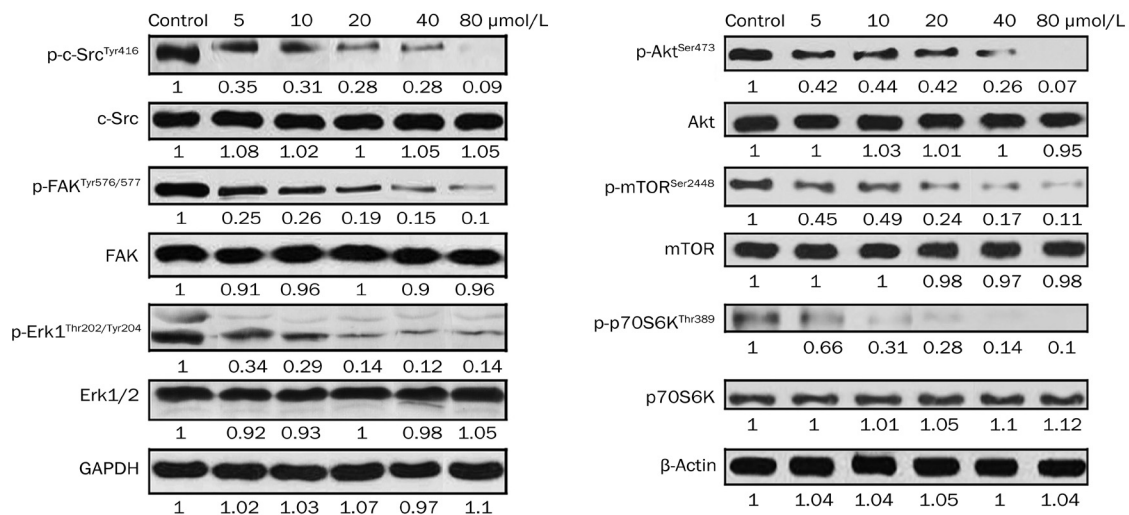

Figure 6. DMU-212 inhibits VEGF-induced phosphorylation of VEGFR2 pathway signaling molecules in HUVECs. Cells were treated with various doses of DMU-212 (0, 5, 10, 20, 40, or 80 μmol/L) in the presence of VEGF (10 ng/mL) for 24 h. After treatment, total cell lysates were prepared and analyzed for p-c-Src, p-FAK, p-Erk1/2, p-Akt, p-mTOR, p-p70S6K, c-Src, FAK, Erk1/2, Akt, mTOR, and p70S6K. In each case, the membrane was stripped and reprobed with anti-GADPH or anti-β-actin antibody to confirm equal protein loading.

Discussion

Angiogenesis is one of the most important hallmarks of tumor development[1, 23]. Endothelial cells represent one of the essential cellular elements in the tumor microenvironment that play a vital role in the growth and progression of cancer through controlling angiogenesis[24]. An anti-tumor strategy of targeting endothelial cells could be efficient because endothelial cells are generally non-transformed and are considered less prone to acquiring drug resistance[25–27]. Therefore, the use of small molecules that target endothelial cells as well as the resultant angiogenesis could be an effective strategy to prevent and treat tumors.

The methylated analog of resveratrol, DMU-212, has improved bioavailability in mouse liver and plasma compared to resveratrol[14]. DMU-212 also possesses superior anti-tumor activity over resveratrol because DMU-212 induces apoptosis and anti-proliferation effects via different mechanisms from those of resveratrol. In addition to the introduction of G_2/M arrest, this compound reduces the expression of anti-apoptotic proteins, including cyclin D1, Bcl-xL, and Bcl-2, in cancer cells[13]. Its strong anti-tumor activity against various cancer cell types prompted our interest in investigating the role of DMU-212 in angiogenesis. In the present study, we report novel biological functions of DMU-212 as an inhibitor of angiogenesis. Our research focused on the inhibitory effects of DMU-212 on endothelial cell migration and capillary structure formation. We showed that DMU-212 strongly inhibited newly formed microvessels in chick CAMs and Matrigel plugs *in vivo*.

The induction of endothelial cell apoptosis is an important anti-angiogenic mechanism[28]. However, some agents, such as ceramide, do not appear to induce endothelial cell apoptosis but do inhibit angiogenic events[29]. To clarify whether the anti-angiogenic activity of DMU-212 is related to apoptosis, we investigated the effect of DMU-212 on endothelial cells. Our data suggest that DMU-212 inhibited cell viability and induced apoptosis in HUVECs in a dose-dependent manner. Cell survival is maintained by a delicate balance between anti-apoptotic and pro-apoptotic stimuli. The present study shows that DMU-212 treatment not only up-regulated caspase-3 and -9 activation as well as cleavage of PARP but also decreased Bcl-2 and survivin levels in HUVECs. More definitive studies are still required to understand the mechanisms underlying DMU-212-induced apoptosis in endothelial cells.

Angiogenesis is tightly regulated by an intricate balance between angiogenic and anti-angiogenic factors[3]. VEGF, a glycoprotein that plays a critical role in endothelial cell proliferation, migration and tube formation, is one of the most important pro-angiogenic regulators of physiological and pathological neo-vascularization, such as tumor angiogenesis[30]. VEGF exerts its biological actions by binding to its two receptor tyrosine kinases expressed on endothelial cells, VEGFR1 and VEGFR2[3]. In the present study, we found that DMU-212 did not suppress VEGFR1 tyrosine kinase activity but significantly blocked VEGFR2 tyrosine kinase activity, making DMU-212 a potent VEGFR2 inhibitor. Our study also demonstrates that the suppression of VEGFR2 by DMU-212 was not due to decreased VEGF-induced VEGFR2 expression but rather to direct inhibition of VEGF-induced phosphorylation of VEGFR2.

VEGFR2 mediates the majority of the downstream effects of VEGF in angiogenesis. Successful anti-angiogenic therapy in clinical trials requires simultaneous blockade of multiple downstream signaling components of VEGFR[31]. Activation of VEGFR2 stimulates Src phosphorylation in endothelial cells[32]. Activated Src family kinases phosphorylate FAK, leading to the formation of a FAK-Src kinase complex, which is involved in VEGF-induced angiogenesis *in vitro* and *in vivo*, particularly its effects on cell survival and migration[33]. In the VEGF signaling pathway, Erk1/2 is a critical regulator of endothelial cell proliferation, growth, migration, and apoptosis[34]. In addition, Akt/mTOR/p70S6K signaling is a novel, functional mediator of VEGF/VEGFR2-mediated angiogenesis[35]. In the present study, we found that DMU-212 inhibited the phosphorylation of multiple downstream signaling components of VEGFR2, including c-Src, FAK, Erk1/2, Akt, mTOR, and p70S6K, suggesting that DMU-212 exerted its anti-angiogenic activity by directly inhibiting VEGFR2 activation and VEGFR2-mediated signaling cascades in endothelial cells.

In conclusion, we have presented the evidence that DMU-212 potently inhibits angiogenesis by blocking the phosphorylation of VEGFR2 and thereby suppressing VEGFR2-mediated signaling pathways, which play multiple roles in regulating neovascularization, as well as by inducing apoptosis in endothelial cells. Sale *et al*[14] indicated that DMU-212 is devoid of any toxicity in rats when administered at single doses of up to 40 mg/kg via the intravenous route or up to 400 mg/kg when administered perorally. These data, coupled with our present findings, suggest that this small molecule may be a promising agent for the treatment of angiogenesis-related diseases.

Author contribution

Lu ZHANG and Fang DAI designed the research; Liang-ke CHEN, Peng-fei QIANG, Qi-ping XU, Yi-hua ZHAO, and Lu ZHANG performed the research; Liang-ke CHEN and Lu ZHANG analyzed the data; and Lu ZHANG wrote the paper.

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