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The inhibition of angiogenesis and tumor growth by denbinobin is associated with the blocking of insulin-like growth factor-1 receptor signaling

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Abstract

Denbinobin, which is a phenanthraquinone derivative present in the stems of *Ephemerantha lonchophylla*, has been demonstrated to display antitumor activity. Recent reports suggest that the enhanced activity of insulin-like growth factor-1 receptor (IGF-1R) is closely associated with tumor angiogenesis and growth. This study aims at investigating the roles of denbinobin in suppressing these effects and at further elucidating the underlying molecular mechanisms. In the present study, we used an *in vivo* xenograft model antitumor and the Matrigel implant assays to show that denbinobin suppresses lung adenocarcinoma A549 growth and microvessel formation. Additionally, crystal violet and capillary-like tube formation assays indicated that denbinobin selectively inhibits insulin-like growth factor-1 (IGF-1)–induced proliferation ($GI50=1.3\times10^{-8}$ M) and tube formation of human umbilical vascular endothelial cells (HUVECs) without influencing the effect of epidermal growth factor; vascular endothelial growth factor and basic fibroblast growth factor. Furthermore, denbinobin inhibited the IGF-1-induced migration of HUVECs in a concentration-dependent fashion. Western blotting and immunoprecipitation demonstrated that denbinobin causes more efficient inhibition of IGF-1-induced activation of IGF-1R and its downstream signaling targets, including , extracellular signal-regulated kinase, Akt, mTOR, p70S6K, 4EBP and cyclin D1. All of our results provide evidences that denbinobin suppresses the activation of IGF-1R and its downstream signaling pathway, which leads to the inhibition of angiogenesis. Our findings suggest that denbinobin may be a novel IGF-1R kinase inhibitor and has potential therapeutic abilities for angiogenesis-related diseases such as cancer.

Keywords: Denbinobin; Insulin-like growth factor-1 receptor; Angiogenesis; Endothelial cell; Lung adenocarcinoma A549

1. Introduction

Cancers are diseases characterized by the uncontrolled growth and spread of abnormal cells. Blood vessels provide the oxygen and nutrients that tumors require for growth and progression; therefore, agents that inhibit neovascularization have therapeutic potential for the treatment of angiogenesis-dependent cancers [1]. Endothelial cells play an important role in angiogenic processes, including proliferation, adhesion, migration and tube formation [2].

Denbinobin (5-hydroxy-3,7-dimethoxy-1,4-phenanthraquinone) is a natural product isolated from the stem of the orchid *Ephemerantha lonchophylla* ("Shi-Hu" in traditional Chinese medicine) [3]. It has been reported to exhibit anti-platelet aggregation, antiviral, anti-inflammatory and antitumor properties [3–9]. Previous studies have shown that denbinobin exerts its apoptotic effects by inhibiting Bcr-Abl activity and promoting tubulin polymerization in leukemia cells [6], inducing mitochondrial dysfunction in non-small cell lung cancer (NSCLC) [7], activating apoptosis-inducing factor and DNA damage in colorectal cancer cells [8]. However, it has not yet been established whether denbinobin inhibits angiogenesis, which is a critical step in tumor growth and metastasis.

Insulin-like growth factor-1 (IGF-1) is a 70-amino acid peptide with characteristics of a naturally circulating hormone as well as a potent tissue growth factor. Many recent studies have shown that high concentrations of serum IGF-1 are associated with an increased risk for various types of cancer and may also contribute to resistance towards certain chemotherapeutic agents [10,11]. The actions of IGF-1 are predominantly mediated through the type 1 insulin-like growth factor receptor (IGF-1R), which is frequently overexpressed and constitutively activated in many human malignancies such as pancreatic, colorectal, prostate, breast and lung cancers [12,13]. IGF-1R is a transmembrane receptor tyrosine kinase that comprises of two extracellular α subunits and two membrane-spanning β subunits. When IGF-1 binds to the extracellular α domain of IGF-1R, it activates

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its tyrosine kinase β domain; this results in the autophosphorylation of the β-subunits on specific tyrosine residues, which then act as a docking site for a variety of signaling molecules, resulting in downstream signal transmission [13,14]. Key downstream networks of IGF-1R include the PI3K-Akt-TOR system and the Raf-MAPK-extracellular signal-regulated kinase (ERK) system [15].

Extensive epidemiologic and experimental data have implicated that the IGF-1/IGF-1R system exerts powerful effects on every stage of cancer progression, including promotion of malignant transformation, tumor growth, local invasion, distant metastasis, resistance to apoptosis and eventual tumor recurrence [16–18]. IGF-1 has been acknowledged to be a potent angiogenic factor that can stimulate endothelial cell proliferation, migration and morphological differentiation via binding to IGF-1R [19,20]. It is therefore possible that the disruption of the IGF-1/IGF-1R pathway may be an attractive therapeutic approach for cancer treatment and prevention, in part by blocking angiogenesis.

In this study, we first demonstrated that denbinobin inhibits angiogenesis *in vitro* and *in vivo* as well as in a NSCLC A549 xenograft model. Our results show that denbinobin selectively inhibits the IGF-1-induced signaling pathway and its downstream kinases in human umbilical vein endothelial cells (HUVECs).

2. Materials and methods

2.1. Reagents

Denbinobin was kindly provided by Dr. Chien-Chih Chen (National Research Institute of Chinese Medicine, Taipei, Taiwan). The methods of extraction and isolation of denbinobin were reported previously [4], and the purity, based on high-performance liquid chromatography analysis, was over 98%.

2.2. Cell lines and Cell culture

HUVECs were isolated from freshly obtained human umbilical cords by digestion with collagenase type I (Sigma Chemical, St Louis, MO, USA) using the modified protocol of Jaffe et al. [21]. HUVECs were maintained and propagated in 75-cm^2 plastic flasks and grown in M199 culture medium containing 20% (v/v) heat-inactivated fetal bovine serum (FBS) and 15 μg/ml endothelial cell growth supplements (Upstate Biotechnology, Lake Placid, NY, USA). The lung adenocarcinoma cell line A549 was cultured in RPMI-1640 supplemented with 10% inactivated FBS (v/v), 100 U/ml penicillin and 100 μg/ml streptomycin. These cells were cultured at 37°C in a humidified atmosphere of 95% air-5% CO_2. HUVECs from the second to the fifth passage were utilized in the experiments below.

2.3. In vivo A549 xenograft model

Male nude mice were implanted with A549 cancer cells (10^7 cells per mice) that were suspended in a volume of 0.1 ml on Day 0. When the tumors reached the average volume of ~100 mm^3, five mice per group were treated. Vehicle (Cremophor EL/ethanol=1:1, 0.2 ml per mouse) or denbinobin was administered intraperitoneally at a dose of 25 mg/kg per day three times a week. Tumor volume was determined every 3–4 days by caliper measurements and by using the formula for an ellipsoid sphere: volume (V)=$lw^2/2$, where l and w are the length and width of the tumor, respectively. Tumor masses were excised and then the histological sections of the xenograft tissue samples were stained with the primary antibody CD31 to permit determination of blood vessels. All protocols of the *in vivo* animal studies were approved by the Animal Care and Use Committee at National Taiwan University.

2.4. Capillary-like tubule formation assay on Matrigel (in vitro angiogenesis)

A 96-well tissue culture plate was coated with 0.06 ml/well of cold unpolymerized liquid Matrigel (BD Biosciences, San Jose, CA, USA) at 4°C, and it was allowed to polymerize at 37°C for a minimum of 1 h. HUVECs were plated onto a layer of polymerized Matrigel at a density of 5×10^4 cells per well with or without denbinobin and 20 ng/ml IGF-1, EGF, VEGF, or bFGF. After incubation at 37°C in a humidified chamber with 5% CO_2 for 6–8 h, changes in cell morphology were visualized and photomicrographs were taken.

2.5. Crystal violet proliferation assay

The growing A549 cells (10^4 cells per well) and HUVECs (5×10^3 cells per well) were seeded into 96-well plates in 10% FBS RPMI-1640 and 20% FBS-M199 culture medium, respectively. After attachment of cells, the medium was removed and the cells were incubated with RPMI-1640 only (A549), 2% heat-inactivated FBS-M199 medium alone (HUVECs) or supplemented with insulin-like growth factor-1 (IGF-1; 20 ng/ml; R&D Systems, Minneapolis, MN, USA), epidermal growth factor (EGF; 20 ng/ml; PeproTech, Rocky Hill, NJ, USA), vascular endothelial growth factor (VEGF; 20 ng/ml; PeproTech) or basic fibroblast growth factor (bFGF; 20 ng/ml; PeproTech) in the absence or presence of various concentrations of denbinobin (0.01–1 μM) for 72 h. Thereafter, the medium was discarded, and the cells were fixed and stained with 50 μl of crystal violet solution in 20% methanol for 10 min at room temperature. After washing with water, the dye was eluted with 0.1 M sodium citrate and 75% ethanol (1:1) and the eluted solution in each well was measured at optical density (OD) 550 nm. The OD_{550} of the basal (treatment without supplement) was considered 100%. The data are represented as the means and standard deviations of quadruplicate data points, and this experiment was repeated eight times.

2.6. DNA synthesis assay

Quantification of DNA synthesis was assessed by the incorporation of the BrdU (5-bromo-2′ deoxyuridine) into newly synthesized DNA using a commercial BrdU labeling and detection enzyme-linked immunosorbent assay (ELISA) kit (Amersham Biosciences, Piscataway, NJ). Quiescent cells in 2% heat-inactivated FBS-M199 medium were stimulated by adding IGF-1 to synthesize DNA. In brief, HUVECs (5×10^3 cells per well) were plated in 96-well tissue culture plates and incubated overnight. The medium was removed, and the cells were washed with phosphate-buffered saline (PBS) once and then starved with 2% heat-inactivated FBS-M199 medium for 24 h. The old medium was replaced with 2% heat-inactivated FBS-M199 medium in the presence or absence various concentration of denbinobin. IGF-1 was added at 20 ng/ml, and the incubation continued for 48 h. BrdU (10 μM) was added for the last 16–18 h of the incubation period. The cells were fixed, and the incorporation of BrdU was quantified using a peroxidase-conjugated monoclonal antibody to BrdU in accordance with the manufacturer's guidelines. HUVECs were seeded in quadruplicate, and independent experiments were repeated six times.

2.7. Cell migration assay

Cell migratory activity was assayed by using a transwell migration apparatus as previously described [22], with modifications. The subconfluent HUVECs were serum-starved overnight and were then harvested with 0.05% trypsin/0.02% EDTA. After trypsinization, HUVECs were suspended in 0.2 ml cultured medium and seeded in the upper chamber of 8-μm pore-sized transwell plates (Costor, Corning, Inc, Corning, NY, USA) for 4 h. After incubation to allow for cell attachment, the medium was replaced with 0.2 ml of 2% heat-inactivated FBS-M199 containing denbinobin, then incubated in the bottom chamber with 0.6 ml 2% heat-inactivated FBS-M199 with IGF-1 (20 ng/ml) alone or in combination with the indicated concentration of denbinobin. The transwell cell culture plate was incubated at 37°C in a humidified atmosphere of 95% air/5% CO_2 for another 8 h. At the end of the incubation, non-migrated cells on the upper face of the filters were mechanically wiped off using a cotton swab and cells that migrated and were attached to the lower face of the filters were fixed and stained with 0.5% crystal violet in 20% methanol for 10 min, rinsed with water and then air dried. After photographed, cut off the filter and placed into a 96-well plate; the stain was eluted with 0.1 M sodium citrate/75% ethanol (1:1 vol/vol), and the absorbance was measured at 550 nm with an ELISA reader.

2.8. In vivo mouse Matrigel plug assay

A mixture of 0.5 ml Matrigel with denbinobin or vehicle and/or 150 ng/ml IGF-1 was injected subcutaneously near the abdominal midline of the C57BL/6 mice. Mice were sacrificed on Day 7. The Matrigel plugs were then removed and photographed, and the extent of blood vessel formation was assessed by measuring the hemoglobin content using the Drabkin's reagent kit (Sigma-Aldrich, St. Louis, MO, USA). Some of the excised Matrigel plugs were fixed with 4% formalin and embedded in paraffin. Histological sections were then stained with Masson's Trichrome.

2.9. Immunoprecipitation

A total of 300 μg of cellular protein from HUVECs was incubated overnight at 4°C with 3 μg anti-IGF-1Rβ antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and 30 μg of protein A/G agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The beads were spun down after centrifugation (5,000 rpm, 5 min, 4°C) and washed three times with lysis buffer RIPA-B (1% Triton X-100, 5 M NaCl, 0.5 M Na_2PO_4, pH 7.4) at 4°C. Sodium dodecyl sulfate (SDS) sample buffer (5×) was added and boiled for 10 min. After brief centrifugation at 13,000 rpm, the supernatant was loaded onto 7% SDS–polyacrylamide electrophoresis (SDS-PAGE) gels. The phosphorylated IGF-1R was detected by using an anti-phosphotyrosine 4G10 antibody (Upstate, Temecula, CA, USA) against phospho-tyrosine kinase.

2.10. Western blot analysis

HUVECs were seeded and allowed to attach overnight. They were starved for 24 h and then pretreated with denbinobin for 1 h. After exposure of cells to IGF-1 (20 ng/

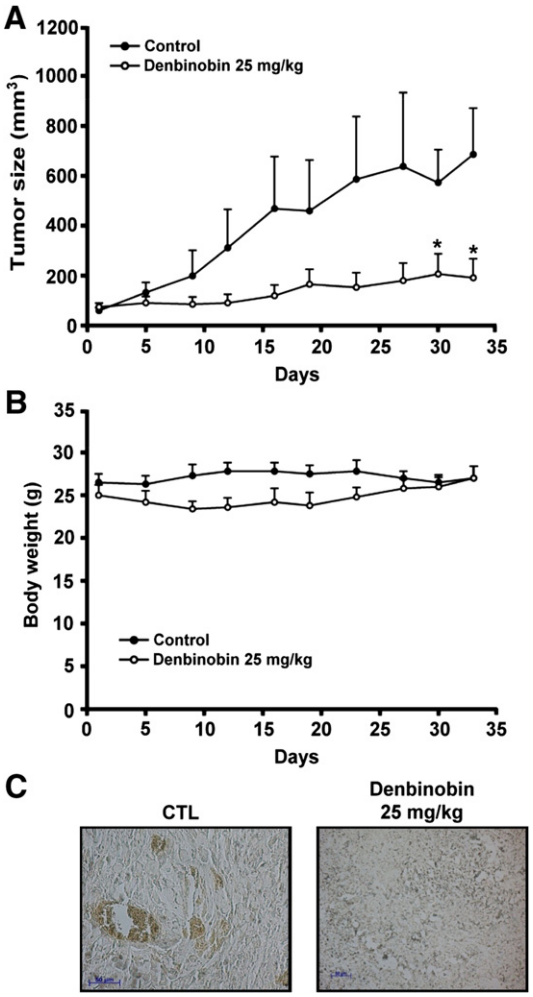


Fig. 1. Denbinobin suppressed A549 human lung adenocarcinoma growth and vascularization *in vivo*. A549 xenograft nude mice were administered denbinobin (25 mg/kg ip) or a vehicle after tumor cell implantation. *P<.05 compared with control group. (A) Tumor size. (B) Body weight. (C) Histology. Formalin-fixed, paraffin-embedded sections from xenograft tumors were stained with CD31 antibody to detect endothelial cells and tumor vascularization. Brown color: CD31-positive area.

ml) over a specified time, the cell culture dishes were washed once with ice-cold PBS before adding ice-cold modified RIPA buffer [150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40 (NP-40), 0.5% Na deoxycholate, 0.1% SDS, 20 mM Tris, pH 8.0]. After scraping, cell lysates were kept on ice for 30 min and then centrifuged (13,000 rpm, 30 min, 4°C), after which the supernatants were obtained. Cell lysates in the 5× sample buffer were denaturalized for 10 min at 100°C and kept at −20°C until Western blot analysis. Equivalent aliquots of protein were separated by SDS-PAGE and transferred onto a polyvinylidene fluoride membrane. After blocking with PBS plus 5% nonfat milk for 1 h and washing three times with PBS/0.1% Tween 20, the membrane was immunoreacted with a primary antibody to phospho-IGF-1R (Tyr1131)/InsR (Tyr1158) (Upstate Biotechnology), phospho-ERK1/2 (Thr202/Tyr204), ERK1/2, phospho-Akt (Ser473), Akt, phospho-4E-BP (Thr37/46), phospho-4E-BP (Thr70), phospho-p70S6K (Thr389) (Cell Signaling Technologies, Boston, MA, USA), phospho-mTOR (Ser2448) (Novus Biologicals, Littleton, CO, USA), IGF-1Rβ (Santa Cruz Biotechnology), Cyclin D1 (Calbiochem, San Diego, CA, USA), or actin (Millipore, Temecula, CA, USA) and incubated overnight at 4°C. After three washings with PBS/0.1% Tween 20, the secondary antibody (1:1000 dilution) was applied to the membranes at room temperature for 1 h. Antibody-reactive bands were detected with an enhanced chemiluminescence kit (ECL; Amersham Biosciences, Buckinghamshire, UK). The density of each band was quantified by ImageQuant software.

Table 1
The effect of denbinobin on growth factor-induced proliferation in HUVECs

Growth Factors (20 ng/ml)	GI_{50}(M)
IGF-1	1.3×10^{-8}
EGF	3.6×10^{-7}
VEGF	7.5×10^{-4}
bFGF	$>1.0\times10^{-6}$

2.11. Data analysis and statistics

The significance of differences in *in vivo* xenograft data was analyzed by the Mann–Whitney *U* test; others represent mean±S.E.M. from at least three independent experiments. Statistical analysis was performed by the *t* test, and *P* values less than .05 (*P<.05, **P<.01, ***P<.001) were considered significant.

3. Results

3.1. Denbinobin suppressed A549 xenograft tumor growth and angiogenesis in nude mice

Firstly, we explored the effects of denbinobin on A549 human NSCLC growth *in vivo* by using a nude mice xenograft model. Our results showed that intraperitoneal administration of denbinobin (25 mg/kg) significantly inhibited tumor growth (Fig. 1A) without the loss of body weight (Fig. 1B). To examine the effect of denbinobin on the vasculature of biopsy specimens of lung cancers, we performed immunohistochemistry analysis on paraffin-embedded tissue sections that were derived from tumor-bearing mice. Our results indicate that denbinobin treatment reduced tumor vasculature, as indicated by the lack of CD31 staining (Fig. 1C). These results suggest that denbinobin may inhibit tumor growth via an anti-angiogenic effect.

3.2. Denbinobin selectively inhibited IGF-1-induced cell proliferation and tube formation

As angiogenesis critically depends on the ability of endothelial cells to proliferate, we investigated the *in vitro* anti-angiogenic activity of denbinobin by studying its effect on endothelial cell proliferation. We used a crystal violet proliferation assay to evaluate the effect of denbinobin on the growth of HUVECs. Denbinobin (0.01–1 μM) significantly inhibited IGF-1-induced HUVECs growth ($GI_{50}=1.3\times10^{-8}$ M), but EGF ($GI_{50}=3.6\times10^{-7}$ M), VEGF ($GI_{50}=7.5\times10^{-4}$ M) and bFGF ($GI_{50}>1.0\times10^{-6}$ M) did not (Table 1). We further examined the effect of denbinobin on IGF-1-induced A549 cells viability. As shown in Fig. 2, denbinobin did not significantly inhibit lung cancer A549 cell proliferation ($GI_{50}>1.0\times10^{-6}$ M). These results demonstrated that HUVECs were more sensitive to denbinobin compared to A549 cells. Additionally, we evaluated how denbinobin affects tube formation induced by various angiogenic factors in

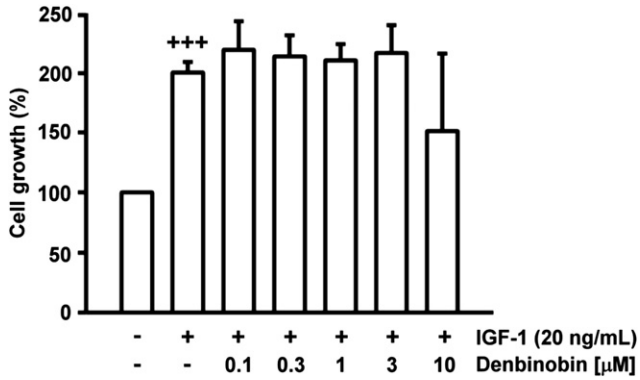


Fig. 2. Effect of denbinobin on IGF-1-induced proliferation of A549 cells. A549 cells (10^4 cells per well) were stimulated with or without 20 ng/ml IGF-1 in the absence or presence of various concentration of denbinobin (0.1–10 μM). Following incubation for 72 h, crystal violet 50 μM was added to stain the cells, which were then solubilized with 75% enthanol:0.1 M sodium citrate, and absorbance of the solution at 550 nm was determined. Data represent the mean+S.E.M. of four independent experiments (each performed in triplicate). +++, P<.001 versus basal group.

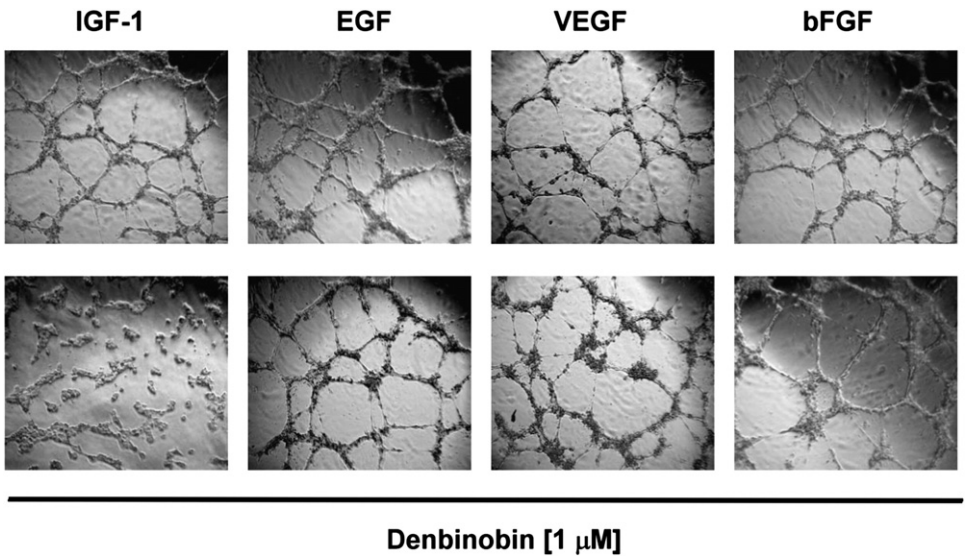


Fig. 3. Denbinobin selectively inhibited IGF-1-induced tube formation. HUVECs (5×10^4 cells per well) were stimulated with or without 20 ng/ml IGF-1, EGF, VEGF or bFGF in the absence or presence of 1 μM denbinobin. Following incubation for 6–8 h, representative photomicrographs (original magnification ×100) showing tube formation by HUVECs were taken. Representative results from one of three independent experiments.

HUVECs. Denbinobin is selective for IGF-1 and does not inhibit EGF-, VEGF- and bFGF-induced tube formation (Fig. 3).

3.3. Denbinobin inhibited IGF-1-induced angiogenesis in vitro

To further verify the role of denbinobin in IGF-1-induced angiogenesis, we performed an angiogenic cellular functional assay using HUVECs. We first determined the effect of denbinobin on IGF-1-induced DNA synthesis using a BrdU incorporation assay. Addition of denbinobin (0.1–1 μM) significantly inhibited IGF-1-stimulated DNA synthesis in HUVECs in a concentration-dependent manner (GI50=0.27 μM) (Fig. 4A). Because migration of endothelial cells is one of the critical features in the formation of new blood vessels, we proceeded to examine the effect of denbinobin on IGF-1-induced migration of endothelial cells *in vitro*. Denbinobin (0.1–1 μM) attenuated IGF-1-induced migratory activity of HUVECs in a concentration-dependent manner (19.18–50.91% decrease) ($P<.001$) (Fig. 4B). We proceeded to perform an *in vitro* Matrigel tube formation assay to determine the effect of denbinobin on IGF-1-mediated differentiation of HUVECs. As shown in Fig. 4C, HUVECs rapidly aligned and formed networks of tubes when incubated with IGF-1 alone. However, in the presence of denbinobin, HUVECs on Matrigel failed to form tubule-like structures (21.87–97.15% decrease) ($P<.001$). Taken together, these results indicated that denbinobin can block IGF-1-stimulated angiogenesis by inhibiting all three of the major steps of angiogenesis.

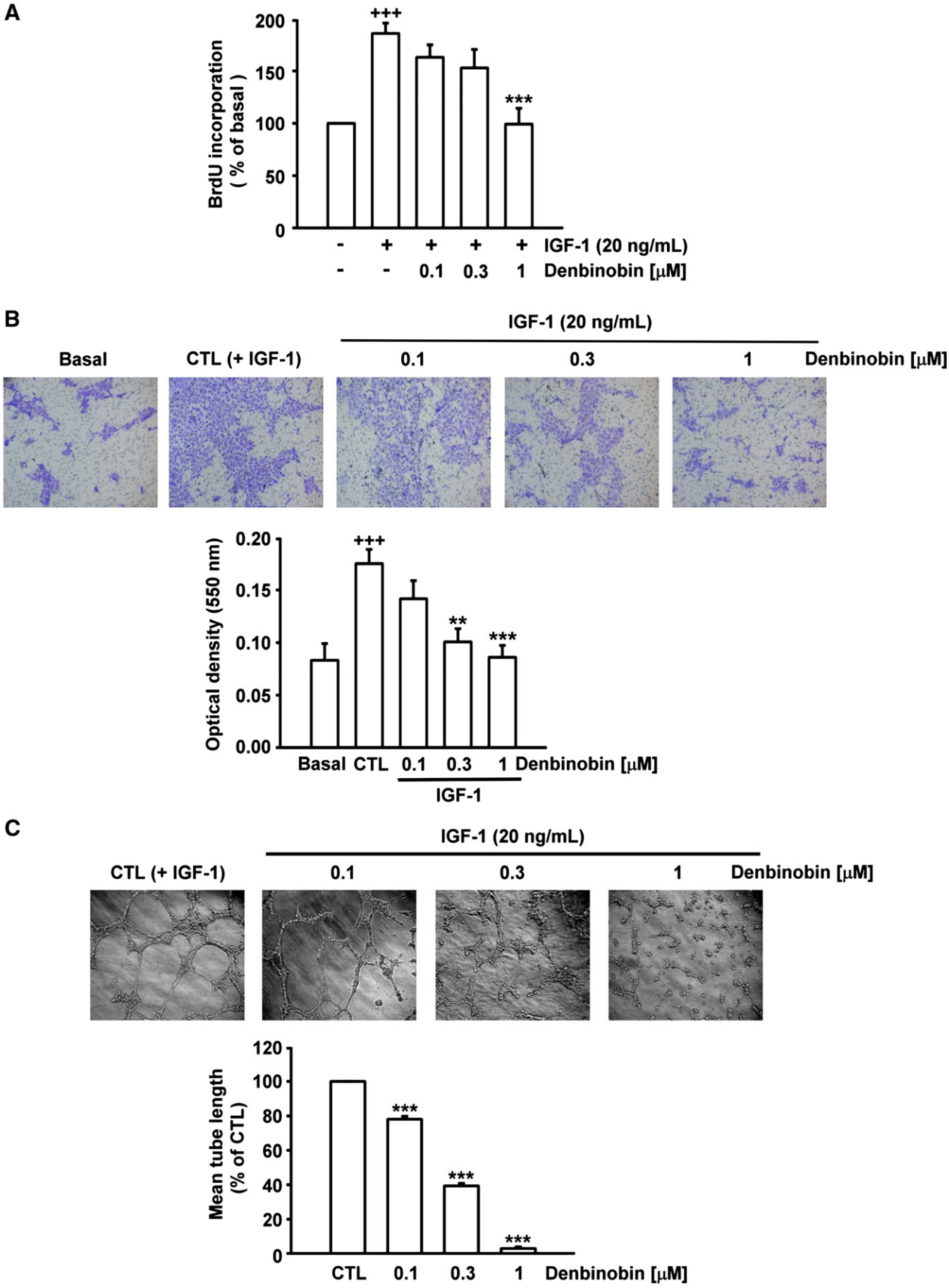
3.4. Denbinobin inhibited IGF-1-induced angiogenesis in vivo

To confirm the hypothesis that denbinobin is capable of blocking IGF-1-induced blood vessel formation, an *in vivo* mouse Matrigel plug assay was carried out. The Matrigel containing IGF-1 with or without denbinobin was injected subcutaneously into C57BL/6 mice for 7 days, and the Matrigel plug in mice was excised, photographed and histologically examined. Plugs with IGF-1 only (controls) significantly appeared dark-red in color. In contrast, plugs mixed with denbinobin (1 and 10 μM) were visually pale in their color, indicating no or less blood vessel formation (Fig. 5A, upper panel). Microscopic examination showed that the new vessels in the controls were abundantly filled with erythrocytes, indicating the formation of functional vasculature within the Matrigel and blood circulation in the newly formed vessels (Fig. 5A, lower panel). We also measured the hemoglobin content within the Matrigel plugs to quantify the extent of angiogenesis. Denbinobin (1 and 10 μM) dramatically reduced the amount of hemoglobin in comparison to the control (75.35–94.18% decrease) ($P<.001$) (Fig. 5B). These results clearly demonstrate that denbinobin strongly suppresses IGF-1-induced angiogenic activity *in vivo*.

3.5. Denbinobin inhibited IGF-induced tyrosine phosphorylation of IGF-1R

The IGF-1/IGF-1R system is well known to play an important role in many cancer cells through promoting angiogenesis and metastasis. Furthermore, recent reports have demonstrated that over-expression of IGF-1R was observed in many human cancers, including small and non-small cell lung cancers [12]. Thus, IGF-1R has become a target of several new anti-cancer drugs [13]. We first used western immunoblot analysis to determine whether denbinobin could inhibit the activation of IGF-1R by IGF-1 in HUVECs. As illustrated in Fig. 6A, IGF-1 enhanced tyrosine phosphorylation of IGF-1R in HUVECs. Pretreatment of the cells with denbinobin (0.3–3 μM) suppressed this effect in a concentration-dependent manner. Moreover, we found that denbinobin (1 μM) dramatically inhibited IGF-1R tyrosine kinase by using immunoprecipitation (Fig. 6B). These results suggest that denbinobin is a potential inhibitor of IGF-1R.

Fig. 4. Denbinobin inhibited IGF-1-induced angiogenesis *in vitro*. (A) BrdU incorporation assay of cell proliferation. After overnight seeding of HUVECs (5.0×10^3 cells/well) into 96-well plates, cells were starved with 2% FBS-M199 medium for another 24 h. Cells were stimulated with IGF-1 (20 ng/ml) containing the indicated concentration of denbinobin (0.1–1 μM) for 48 h and were then labeled with BrdU (10 μM) for the last 18 h of treatment. These experiments were performed six times, and each time cell cultures were examined in quadruplicate. (B) Effect of denbinobin on the migratory behavior of endothelial cells, as measured by the transwell migration assay. Upper panel, The characteristic feature of migratory cells on the transwell membrane. Lower panel, The quantification of cell migration. (C) The *in vitro* Matrigel capillary-like tubule formation assay. HUVECs were plated on Matrigel-coated plates in the presence of IGF-1 with or without varying concentration of denbinobin, and the changes in cell morphology were recorded by phase contrast microscopy. (Upper panel) Representative photographs of tube formation in HUVECs on polymerized Matrigel layers are shown. (Lower panel) The quantification of the average tubular length was determined by the image analysis software. Data are the mean of three independent experiments. $+++P<.001$ versus basal group; $^{**}P<.01$ and $^{***}P<.001$ versus control group.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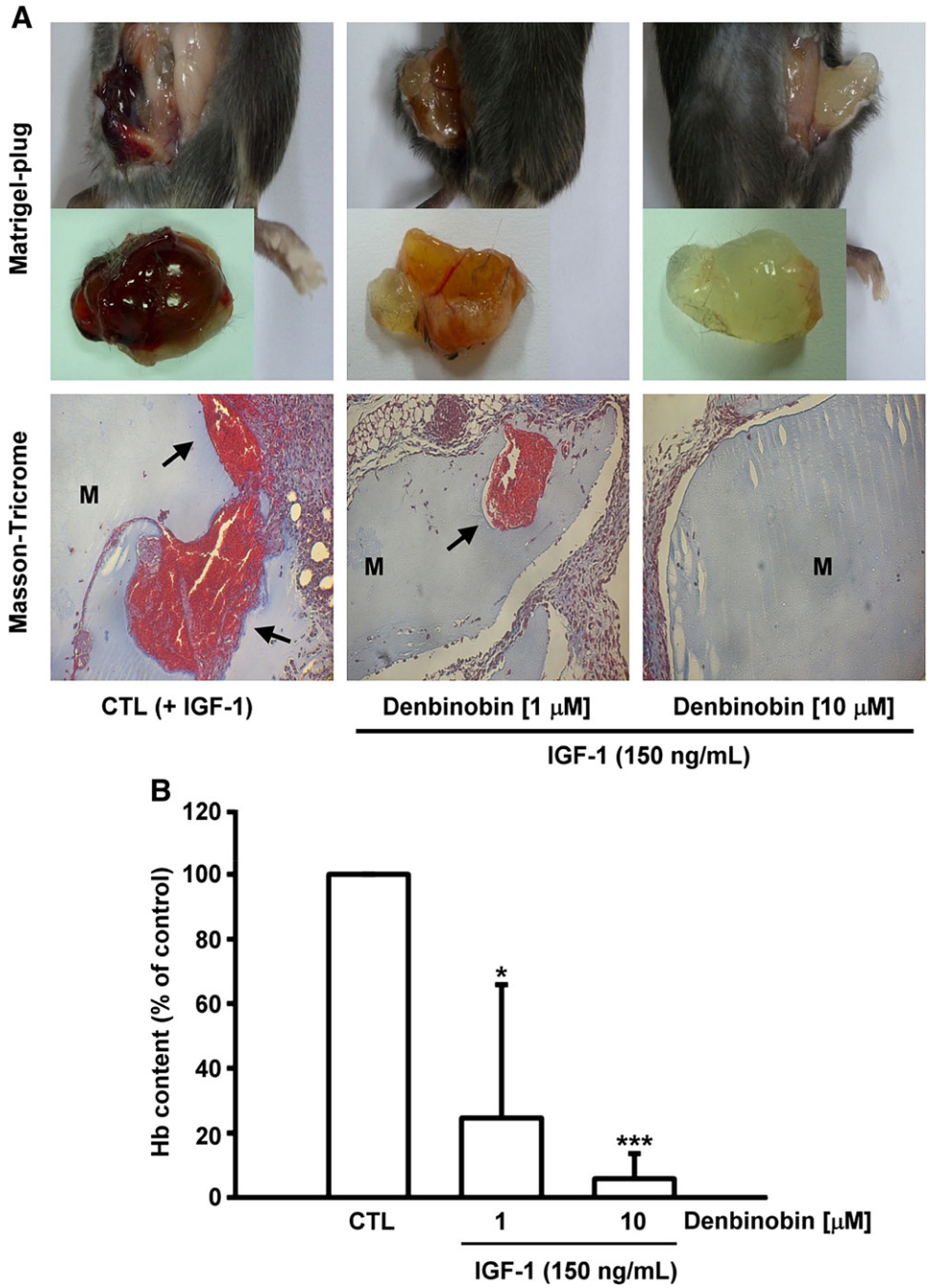

Fig. 5. Denbinobin suppressed IGF-1-induced angiogenesis in an *in vivo* mouse Matrigel-plug assay. The experimental procedures are described under "Materials and Methods." (A) Upper panel, Photographs of the representative Matrigel plug from each group. Lower panel, Photomicrographs of the histological sections from Matrigel plugs stained with Masson's trichrome. Arrows indicate regions with functional neovessels that contained red blood cells within the Matrigel (M, Matrigel plug). (B) Quantification of active vasculature inside the Matrigel by measurement of hemoglobin content using the Drabkin's reagent kit. Each value represents the mean of at least three animals. *P<.05; ***P<.001 versus control group.

3.6. Denbinobin inhibited downstream signals of IGF-1/IGF-1R in HUVECs

Next, we investigated whether denbinobin interferes with the activation of two major downstream molecules involved in the IGF-1/IGF-1R signaling cascade. As indicated in Fig. 7A and 7B, IGF-1 significantly induced phosphorylation of ERK and Akt. In contrast, pretreatment with denbinobin (0.3–3 μM) dramatically reduced the levels of phospho-ERK1/2 and phospho-Akt in a concentration-dependent manner. In addition, Western immunoblot analysis showed that pretreatment of HUVECs with denbinobin caused significant reduction in the activation of their downstream targets including mTOR, p70S6K, 4EBP phosphorylation and cyclin D1 expression, which play an important role in cell protein synthesis and cell growth (Fig. 7C).

4. Discussion

The important current commentary by Magwere [23] has illustrated that denbinobin exhibits the anti-cancer activity via suppressing escape immune surveillance, and suggests it may be the first of a futuristic new class of anticancer "dendrobicides." In this

study, we report a novel molecular mechanism by which denbinobin inhibits tumor angiogenesis. First, in addition to studying its *in vitro* antiangiogenic activity, we have demonstrated the effect of denbinobin on tumor growth and angiogenesis using a NSCLC A549 xenograph model. Our results show that denbinobin inhibits tumor growth *in vivo*, concomitant with a reduction in CD31-stained tumor vessel. Second, the anti-angiogenic effect of denbinobin on HUVECs is significantly more potent than its anti-proliferative effect on cancer cells. In particular, our previous studies showed that denbinobin inhibits the proliferation of HT-29, A549, PC-3, Hep3B and HCT-116 cells with GI_{50} values of 18.0, 8.5, 7.9, 7.4 and 3.4 μM, respectively [8]; however, the present study shows that denbinobin inhibits the angiogenesis of endothelial cells with a GI_{50} value of 0.27 μM (BrdU assay).

Epidermal growth factor receptor tyrosine kinase inhibitors have been found to be effective against lung cancer, but clinical resistance to these agents has developed with an increase in their usage [24]. IGFR-1 is a novel molecular target for the treatment of NSCLC, and recent studies have identified a critical role for IGF-1 in pathological neovascularization, a process that is characteristic of proliferative cancers [17,25,26]. Abundant evidence supports the theory that angiogenesis is critically important for the progression and survival of tumor cells and tumor metastasis [27]. Therefore, development of agents that effectively block the IGF-1/IGF-1R axis-mediated angiogenesis is a promising anti-cancer strategy. This is the first study to demonstrate that denbinobin inhibits tumor angiogenesis by suppressing tyrosine phosphorylation of IGF-1R and the related signaling pathway.

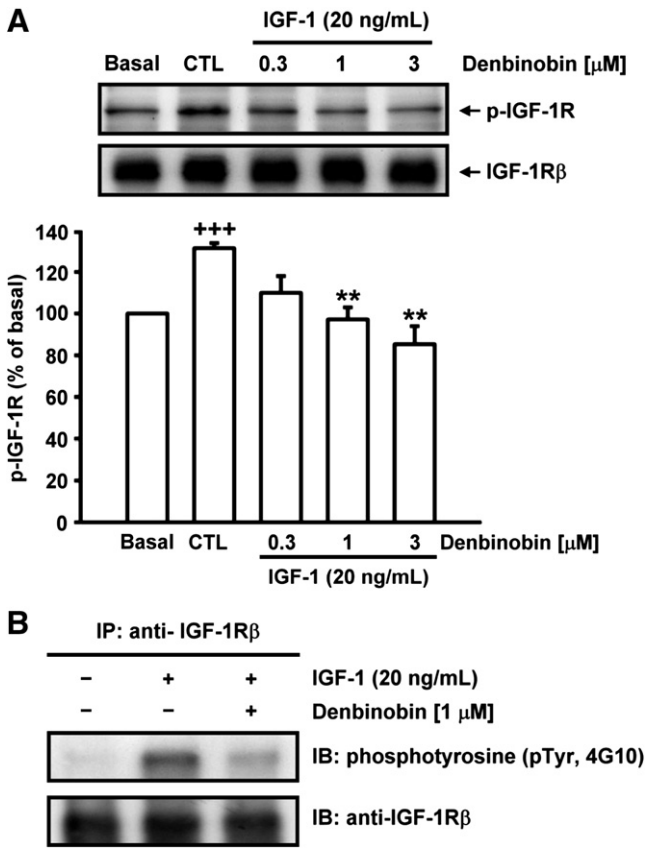

Fig. 6. Denbinobin abrogated IGF-1-induced activation of IGF-1R. HUVECs were grown to 80-90% confluence in dishes. The exchange of culture medium for low-serum medium (for another 24 h) was followed by pretreatment with denbinobin for 1 h. Vehicle (basal) or IGF-1 (20 ng/ml) was added to the cells for another 10 min. After treatment, cells were harvested and cellular proteins were isolated. The inhibitory effect of denbinobin on tyrosine phosphorylation of IGF-1R by IGF-1 was determined by immunoblotting (A) and immunoprecipitation (B). +++P<.001 versus basal group; **P<.01 versus control group.

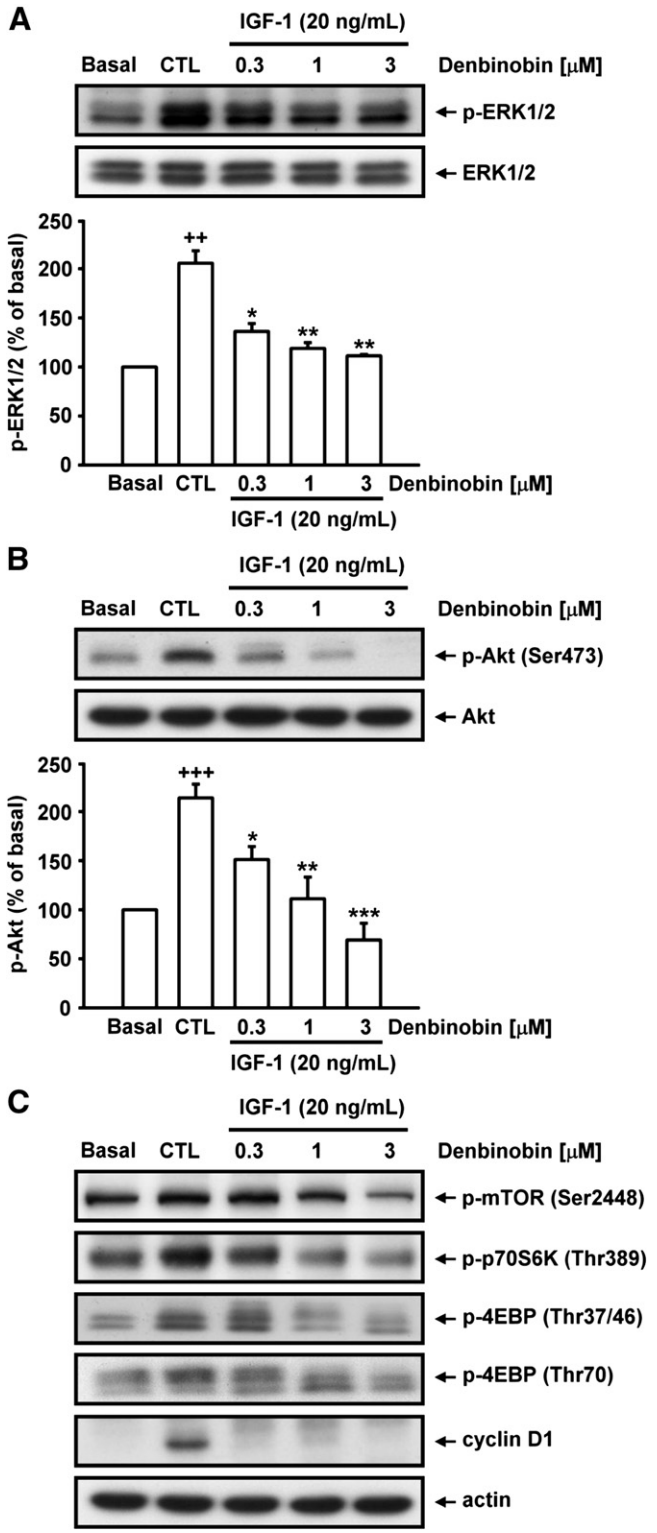

Fig. 7. Denbinobin inhibited IGF-1-induced signaling pathway activation in HUVECs. HUVECs were preincubated in the absence or presence of denbinobin for 1 h and subsequently stimulated by IGF-1 (20 ng/ml). Next, cells were harvested and total cell lysates were subjected to western blotting analysis to determine the impact on IGF-1R associated pathways. ++P<.01; +++P<.001 versus basal group; *P<.05; **P<.01, ***P<.001 versus control group.

Angiogenesis has an important role in numerous physiological functions and pathological states. It is well established that endothelial cells play a pivotal role in each step of angiogenesis, so they should be an attractive target for antitumor therapies. In fact, most antiangiogenesis agents block one or more steps of angiogenesis by targeting endothelial cells [28]. Here, we demonstrate that denbinobin effectively inhibits *in vitro* angiogenesis processes, including endothelial cell proliferation, migration and capillary-like tubule formation (Fig. 4) and that it also suppresses angiogenesis *in vivo* (Fig. 5). A previous study reported that treatment with 20 μM denbinobin inhibits A549 lung cancer cell viability by 56.1±11.0% [7]; this concentration is nearly 20-fold higher than that used in HUVECs. In the present study, we found that denbinobin prevents tumor growth and tumor-angiogenesis without discernible toxicity in an A549 NSCLC xenograft model (Fig. 1). Furthermore, the results of crystal violet assay showed that the inhibition of denbinobin on IGF-1-induced proliferation of A549 cells with GI_{50} value of $>1.0\times10^{-6}$ M (Fig. 2) and HUVECs with GI_{50} value of 1.3×10^{-8} M (Table 1). Taken together, our results suggest that denbinobin is a potent anti-angiogenesis agent, and it inhibits tumor growth by suppressing the formation of new blood vessels.

The IGF-1 axis, particularly IGF-1R, is recognized as an important target in cancer therapy because it is crucial in the regulation of tumorigenicity [29,30]. In response to IGF-1 stimulation, IGF-1R undergoes autophosphorylation of the β-subunit, which activates its substrate tyrosine kinase activity and then initiates numerous intracellular signaling cascades [31]. In this study, we used Western blotting and an immunoprecipitation assay to demonstrate that denbinobin dramatically decreases the IGF-1-mediated phosphorylation of IGF-1R (Fig. 6). There are two major signaling pathways downstream of the IGF-1/IGF-1R axis, the PI3K-Akt pathway and the Raf-MEK-ERK pathway, which are tightly associated with the signal transduction reactions that regulate cell proliferation, growth, survival, migration and capillary-like tubule formation [15,31]. Akt (a serine/threonine-specific protein kinase) and ERK regulate gene expression, protein synthesis and translation and, thus, play pivotal roles in essential cellular functions, such as cell cycle regulation, proliferation, growth, migration, survival and apoptosis. Activation of both pathways is needed for essential cellular processes of endothelial cells and angiogenesis.

Earlier studies have shown that the PI3K-Akt pathway regulates protein translation through its downstream mammalian target of rapamycin (mTOR), which is a central serine/threonine kinase. mTOR conveys much of its cell growth and proliferation through phosphorylation of two downstream effectors, the 70 kDa ribosomal protein S6 kinase (p70S6K) and eukaryotic initiation factor 4E-binding protein (4EBP). The serine/threonine kinase p70S6K is a highly conserved element in a wide array of cellular processes, which are activated through phosphorylation at a number of sites and the primary target of the activated kinase is the 40S ribosomal protein S6. Downstream signaling from PI3K-Akt generally leads to the phosphorylation of mTOR at serine 2448, which then phosphorylates its substrate p70S6K at threonine 389 (Thr389). Unphosphorylated 4EBP is a translational inhibitor that binds to eukaryotic translation initiation factor 4E (eIF4E) to repress cap-dependent translation initiation [32]. Stimulation of the PI3K-Akt-mTOR pathway leads to hierarchical phosphorylation of 4EBP1 (first Thr 37/46, then Thr 70 and last Ser65), dislodging 4EBP from eIF4E and subsequently increasing cap-dependent translation. eIF4E promotes translation of proteins like c-Myc, VEGF and cyclin D [32,33]. Presently, we demonstrated that denbinobin inhibits the activation of IGF-1-induced IGF-1R and attenuates the phosphorylation of its major downstream effectors, ERK1/2 (Thr202/Tyr204), mTOR (S2448), p70S6K (Thr389) and 4EBP (Thr 37/46 and Thr 70), resulting in a markedly decreased expression of proteins like cyclin D1. However, to examine whether denbinobin affects other proteins involved in IGF-1/IGF-1R-medated PI3K-Akt-mTOR or Raf-MEK-ERK signaling pathways and whether these effects also occur in tumor cells, further experiments are required.

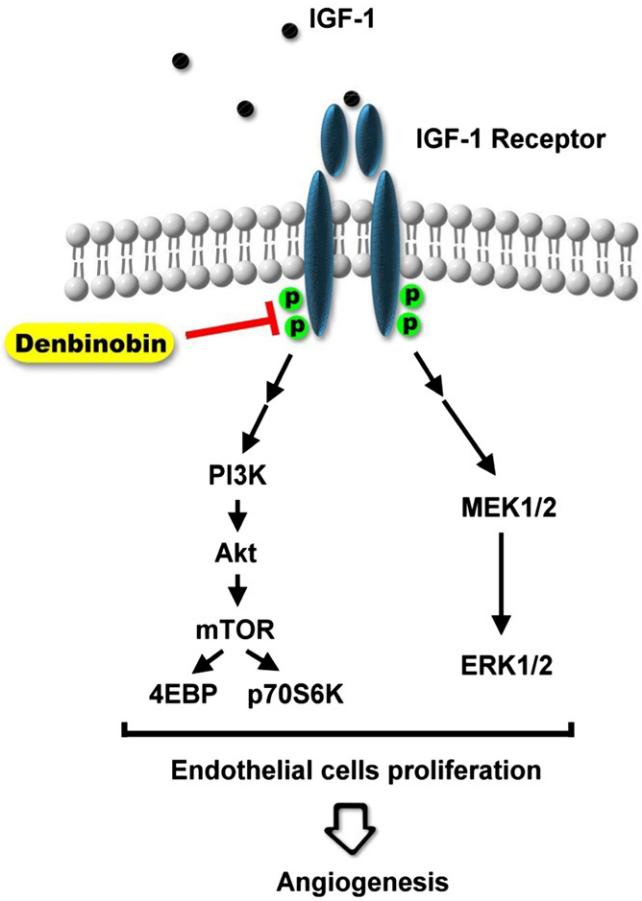

Fig. 8. Schematic diagram representing the mechanism of angiogenesis inhibition by denbinobin. Binding of IGF-1 to IGF-1R leads to the autophosphorylation of tyrosine residues in the β subunits of the receptor, and activates a cascade of downstream transduction processes, the MEK-ERK pathway and the PI3K-Akt-mTOR pathway. These two pathways further trigger the cell proliferation, which is tightly associated with the angiogenesis. Denbinobin strongly reduced IGF-1-induced angiogenesis through inhibiting IGF-1R phosphorylation.

Based on our systemic demonstration, we conclude that denbinobin effectively inhibits endothelial cell proliferation, migration and capillary-like tubule formation; simultaneously, the suppression of angiogenesis and tumor growth is observed *in vivo*. At the molecular level, our results indicate that denbinobin dramatically suppresses IGF-1-induced IGF-1R phosphorylation and thus interferes with downstream signaling pathways (Fig. 8). Taken together, our results suggest that denbinobin is a promising candidate for treatment of cancer and other angiogenesis-related diseases.

Acknowledgments

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