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Molecular mechanism of the inhibitory effect of KS-5 on bFGF-induced angiogenesis *in vitro* and *in vivo*

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Abstract

Inhibition of angiogenesis controls the expansion and metastasis of many solid tumors and other related-diseases. KS-5 (1,7-dihydroxy-3-methoxyacridone), is an inactive analogue of the substituted 1-hydroxy acridone antiviral class. This study aimed at studying the effects of KS-5 on bFGF-induced angiogenesis in cultured human umbilical vein endothelial cells (HUVECs) *in vitro* and *in vivo*. KS-5 inhibited bFGF (10 ng/ml)-induced cell proliferation in a concentration-dependent manner, but did not exhibit significant cytotoxic effect examined by LDH release assay. KS-5 inhibited bFGF-induced angiogenesis was associated with decreasing DNA synthesis as evaluated by BrdU incorporation assay, and abrogating endothelial cell ERK1/2 and Akt protein phosphorylation, the major signaling pathways involved in cellular processes of angiogenesis. In addition, KS-5 also inhibited bFGF-induced phosphorylation of mTOR and the major downstream effectors, eIF4E and $p70^{S6K}$. Moreover, bFGF-induced protein synthesis was also inhibited by KS-5. Most importantly, KS-5 treatment in nude mice inhibited *in vivo* angiogenesis as revealed by Matrigel implant assay. In conclusion, the present study suggests that KS-5 has potential anti-angiogenetic effect for cancer therapy and other angiogenesis-dependent diseases.
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Keywords: Angiogenesis; Acridone derivatives; Endothelial cells

1. Introduction

Angiogenesis, the growth of microvessel sprouting the size of capillary blood vessels, is a tightly regulated process and restricted to a few physiological conditions including reproduction, embryonic development, and wound healing [1]. In contrast, uncontrolled angiogenesis is a driving force for a number of pathologies, such as cancer, ocular neovascular disease and chronic inflammatory diseases [2]. Angiogenesis is a multiple-step process involving matrix degradation, endothelial cell proliferation, migration and differentiation. To complete the process, all of the steps must

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occur [3]. One of the most potent stimulators of angiogenesis is basic fibroblast growth factor (bFGF), which regulates endothelial cell proliferation, migration, protease production, integrin and cadherin receptor expression [4]. Inhibition of angiogenesis is becoming one promising, alternative approach to treat cancer and other related-diseases.

The mammalian target of rapamycin (mTOR) is an evolutionarily conserved serine/threonine kinase. This kinase is centrally involved in protein translation, cell cycle progression and cellular proliferation [5,6]. The best-understood roles of mTOR in mammalian cells are related to control of cap-dependent mRNA translation. The eIF4E and $p70^{S6K}$ pathways are the major mTOR-dependent downstream signaling pathways that mediate mTOR-dependent control of cell size and cell growth [6,7]. A key regulatory step in translation is initiation, eIF4E is the limiting initiation factor in most cells. Thus, eIF4E activity plays a principle role in determining global translation rates [8]. The function of eIF4E is inhibited by the eIF4E-binding proteins (4E-BP1, 4E-BP2, 4E-BP3), that bind eIF4E and block its function [9]. The regulation of the 4E-BP1 is much better understood than that of 4E-BP2/3. 4E-BP1 undergoes phosphorylation at seven sites, at least four of which are linked to mTOR signaling [10,11]. Phosphorylation of these sites is required for release of eIF4E from 4E-BP1.

Acridine derivatives are interesting chemotherapeutic agents that were first used as antibacterial and antiparasite agents. The biological activity of acridines is mainly attributed to the planarity of these aromatic structures, which can intercalate within the double-stranded DNA structure, thus interfering with the cellular machinery. More recent understanding reveal that many acridine derivates have biological targets such as topoisomerases I and II, telomerase/telomere and protein kinases and leading to anti-tumor property [12]. KS-5 (1,7-dihydroxy-3-methoxyacridone) was originally synthesized as an analogue of substituted 1-hydroxyacridone antivirals active against HSV-1, HSV-2 and or HCMV replication [13,14]. However, preliminary screening work identified KS-5 as a potential anti-angiogenesis compound and this study reports a detailed study of KS-5 effects on bFGF-induced angiogenesis *in vitro* and *in vivo*.

2. Materials and methods

2.1. Cell culture

Human umbilical vein endothelial cells (HUVECs) were obtained from human umbilical cord veins with collagenase, isolated according to protocols from Jaffe et al. [15], and cultured in 75-cm^2 plastic flasks in M199 medium containing 20% inactivated fetal bovine serum (FBS) and 15 μg/ml endothelial cell growth supplements. Confirmation of cell identity as endothelial cells was provided by detection of CD31 (PECAM-1) by immunostaining. The human nasopharyngeal cancer cell line, KB cell, was cultured in RPMI1640 medium containing 10% FBS. Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. Medium was changed every 2 days, and cells were passaged after treatment with a solution of 0.05% trypsin/0.02% EDTA. Experiments were conducted on HUVECs that had gone through two to five passages.

2.2. Growth inhibition assay

Method is according to the recommendation of the NCI DTP Angiogenesis Resource Center used. HUVECs (5×10^3 cells/well) are plated in a 96-well plate in 100 μl of EGM-2 (Clonetic #CC3162). After 24 h (day 0), the test compound (100 μl) is added to each well at 2× the desired concentration in EGM-2 medium. On day 0, one plate is stained with 0.5% crystal violet in 20% methanol for 10 min, rinsed with water, and air-dried. The remaining plates are incubated for 72 h at 37 °C. After 72 h, plates are stained with 0.5% crystal violet in 20% methanol, rinsed with water and air-dried. The stain is eluted with 1:1 solution of ethanol: 0.1 M sodium citrate (including day 0 plate), and absorbance is measured at 540 nm with an ELISA reader. Day 0 absorbance is plotted as basal.

2.3. Cytotoxicity assay

The lactate dehydrogenase (LDH) released into cell cultures is an index of cytotoxicity and evaluates the permeability of the cell membrane. After an incubation of 24 h with various drug concentration in EBM2, the culture supernatants were collected. The LDH assay was performed using the CytoTox96 Nonradioactive Cytotoxicity Assay kit (Promega, Madison, WI) according to the manufacturer's instructions. The percentage of LDH released was expressed as a proportion of the LDH released into the medium compared to the total amount of LDH present in cells treated with 2% Triton X-100.

2.4. DNA synthesis assay

Based on the measurement of 5-bromo-2-deoxyuridine (BrdU) incorporation during DNA synthesis, the

colorimetric ELISA kit (Amersham Biosciences, Piscataway, NJ) was used for the quantification of DNA synthesis. HUVECs were seeded at 5.0×10^3 cells per well into 96-well plates in M199 medium supplemented with 20% FBS, and starved with 1% FBS-M199 medium for 24 h. The cells were then incubated with or without indicated reagents and bFGF (10 ng/ml) (Biovision, Palo Alto, CA) for 48 h. BrdU (10 μM) was added during last 16 h of incubation period. The assay was performed according to the manufacturer's instructions.

2.5. Western blot analysis

After exposure of cells to indicated agents over specified time courses, cells were washed twice with ice-cold PBS, and reaction was terminated by addition of 100 μl of ice-cold lysis buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 0.5 mM phenylmethyl sulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1% Triton X-100). Protein (30 μg/lane) was separated by electrophoresis on 7.5–12% SDS–polyacrylamide gels. Proteins were electrophoretically transferred to polyvinylidene difluoride membranes and blots were blocked with 5% nonfat milk for 1 h. The membrane was immunoreacted with the primary antibody to phospho-ERK1/2 (Thr202/Tyr204), phospho-Akt (Ser473), phospho-4E-BP (Thr37/46), phospho-eIF4E (Ser209), phospho-$p70^{S6K}$ (Thr389) (Cell Signaling Technologies, Boston, MA), phospho-mTOR (Ser2448) (Novus Biologicals, Littleton, CO) and GAPDH (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for overnight incubation at 4 °C. After four washings with PBS/0.1% Tween 20, the secondary antibody (diluted 1:1000) was applied to the membranes for 1 h at room temperature. Antibody-reactive bands were detected with an enhanced chemiluminescence kit (Amersham, Buckinghamshire, UK).

2.6. Protein synthesis measurements

Incorporation of [^{3}H]leucine was used as a sensitive parameter to measure de novo protein synthesis. HUVECs were seeded at a density 5.0×10^3 cells/well on 24-well plates. Cells were starved with DME/F12 medium containing 1% FBS for 24 h. Cells incubated with leucine-free DME/F12 medium for 1 h and pretreat drug for 30 min, and then treated with or without growth factor followed by incubation with 2.5 μCi/ml [^{3}H]leucine for 6 h. Plates were washed twice with cold phosphate-buffered saline, and 10% trichloroacetic acid was added at 4 °C and incubated for 60 min. The precipitates were washed twice and dissolved in 1 N NaOH. The radioactivity of the incorporated [^{3}H]leucine was measured using liquid scintillation counting.

2.7. In vivo Matrigel plug assay

Nude mice (6 weeks of age) were given subcutaneous injections of 500 μl of Matrigel (BD Biosciences) at 4 °C containing growth factor (150 ng/ml bFGF) and drug. After injection, the Matrigel rapidly formed a plug. After 7 days, the skin of the mouse was easily pulled back to expose the Matrigel plug, which remained intact. For histological examination, the plug were fixed, embedded, and stained with Hematoxylin & Eosin.

2.8. Data analysis and statistics

Data are presented as mean ± SEM or as percentage of control. Statistical comparisons between groups were performed using the Student's t test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Anti-proliferative effect of KS-5 on endothelial and tumor cells

The anti-proliferative activity of KS-5 was determined on HUVECs and the human nasopharyngeal cancer cell line, KB cells. KS-5 inhibited cellular proliferation in a concentration-dependent manner with IC_{50} of 0.6 μM (Fig. 1A) and 8.23 μM (Fig. 1B), against HUVECs and KB cells, respectively. Therefore, the results shown that KS-5 is about 10 times more selective against endothelial cells as opposed to cancer cells. Subsequently, we used LDH assay to test whether the inhibition of cell proliferation was the consequence of KS-5-mediated cellular toxicity. For cells treated with KS-5 in EBM-2 medium for 24 h, there was no significant difference in LDH release between basal and treated cells even with a KS-5 concentration as high as 40 μM (about 70-fold higher than IC_{50}, Fig. 1C).

3.2. Effect of KS-5 on bFGF-induced DNA synthesis of HUVECs

Cell proliferation involves both cell growth and cell division [6]. Cell growth (increase cell mass) needs protein synthesis, and cell division (increase cell number) needs DNA synthesis. To determine that the effect of KS-5 on bFGF-induced DNA synthesis the BrdU incorporation assay was used. As shown in Fig. 2, KS-5 (0.1–20 μM) reduced bFGF-stimulated DNA synthesis in a concentration-dependent manner with IC_{50} value of 0.98 μM and significant inhibition was observed from 1 μM upwards ($P < 0.05$).

3.3. KS-5 inhibits MAPK and Akt protein phosphorylation

The mitogenic signaling pathways are activated in response to a variety of extracellular stimuli such as growth factors, cytokines and cell stress [16]. We exam-

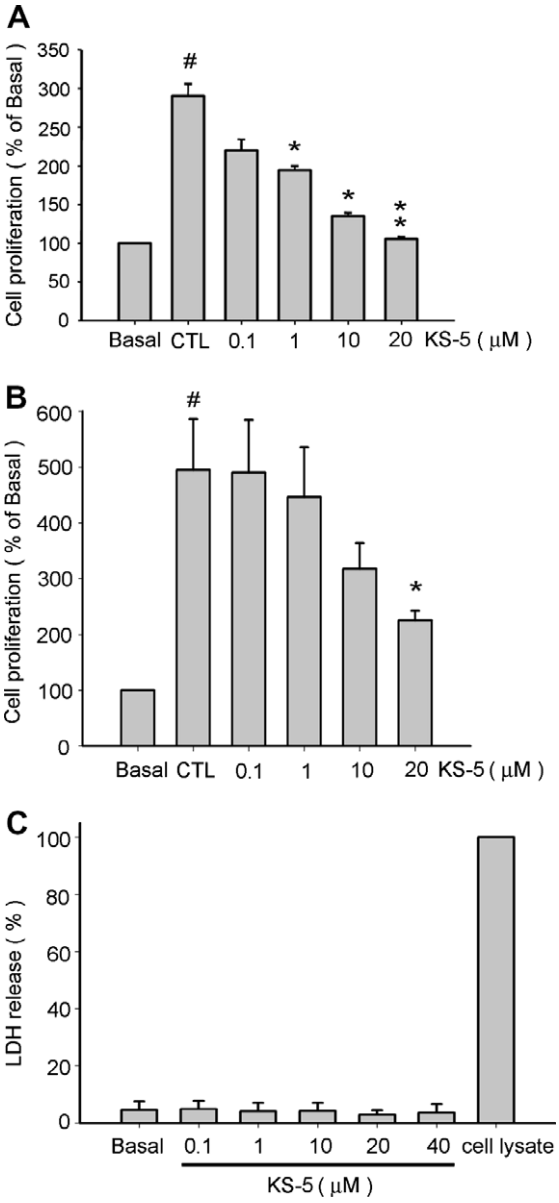


Fig. 1. Effects of KS-5 on the proliferation of HUVECs and KB cells. HUVEC(A) and KB (B) cells were seeded in a 96-well plate at 5000 cells per well, after 72 h (A) or 48 h (B) incubation with varying concentrations of KS-5 (0.1–20 μM), crystal violet 50 μl were added to stain the cells, then wash twice and air dry. Bound dye was subsequently solubilized with enthanol: 0.1 M sodium citrate, and absorbance of the solution at 540 nm was determined. The IC_{50} value of KS-5 is 0.61 (A) and 8.23 (B) μM, respectively. Data represent the mean ± SEM of three independent experiments (each performed in triplicate). $^{\#}P < 0.001$ versus basal group; $^{*}P < 0.05$; $^{**}P < 0.01$ versus control group. (C) Effect of KS-5 on the LDH release in HUVECs. HUVECs were exposed to 0.1, 1, 10, 20 and 40 μM of KS-5. At 24 h of incubation after exposure, 0.05 ml of culture medium were collected for determination of LDH activity according to the manufacturer's instructions.

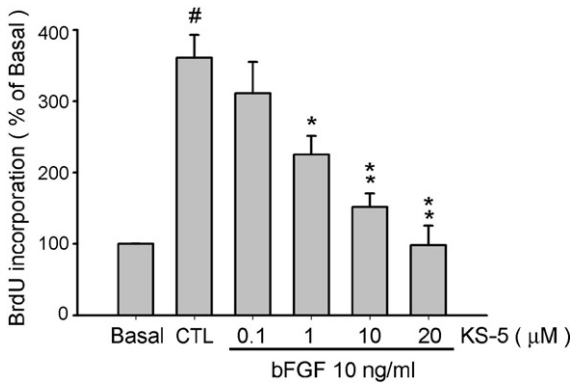


Fig. 2. Effects of KS-5 on bFGF-induced HUVEC DNA synthesis. HUVECs were stimulated with bFGF 10 ng/ml containing varying concentrations of KS-5 for 48 h, and labeled with BrdU 10 μM during the last 16 h of stimulation. Data represent the mean ± SEM of three independent experiments (each performed in triplicate). $^{\#}P < 0.001$ versus basal group; $^{*}P < 0.05$; $^{**}P < 0.01$ versus control group.

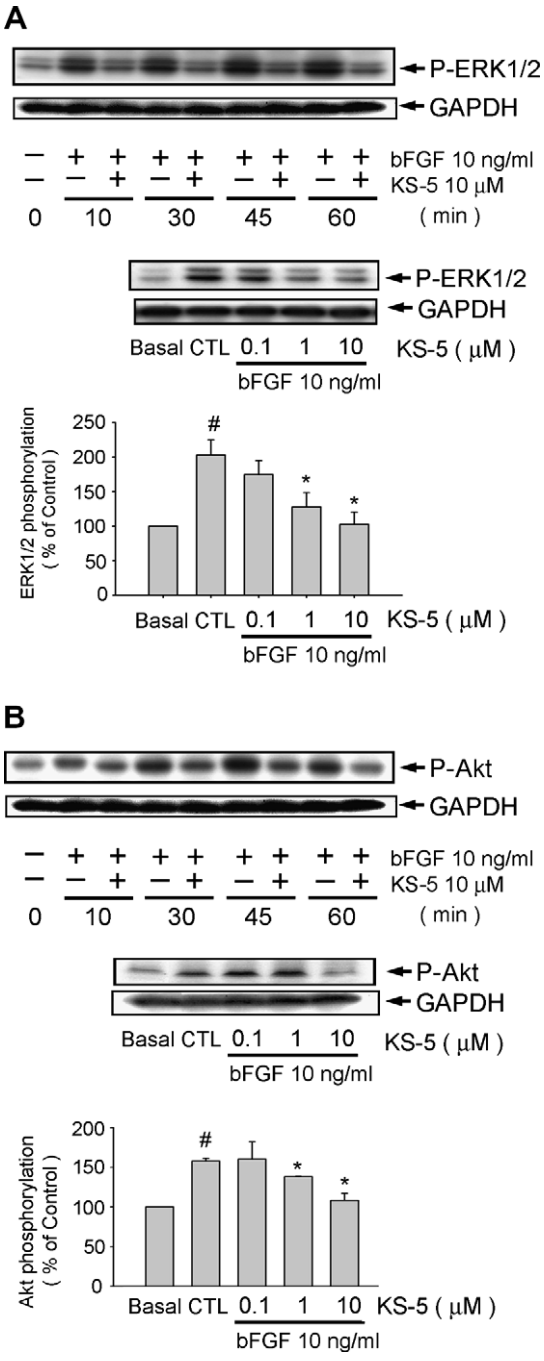
ined whether KS-5 caused alteration of mitogenic signaling pathways activated by bFGF. First, we examined its effect on the MAP kinase pathway under bFGF-induction conditions. We utilized an antibody to detect phospho-ERK1/2, commonly used indirect assay to assess MAP kinase activation. As shown in Fig. 3A, treatment with KS-5 significantly decreased phospho-ERK1/2 levels after bFGF-stimulated for the indicated times. However, the PI3 kinase/Akt signalling pathway is now recognized as being at least an important as the ras-MAP kinase pathway in cell survival and proliferation [17]. As shown in Fig. 3B, treatment with KS-5 also inhibited bFGF-induced Akt phosphorylation. These data suggest that KS-5 is able to block bFGF-induced ERK1/2 and Akt signaling pathways in endothelial cells.

3.4. Effect of KS-5 on bFGF-induced mTOR pathway activation in HUVECs

Cell proliferation requires enhanced rates of protein synthesis. The mTOR signaling pathway is important for protein synthesis. To determine whether the anti-proliferative effects of KS-5 result from inhibition of mTOR signaling, we first examined the phosphorylation status of mTOR, $p70^{S6K}$, 4E-BP1, and eIF-4E on endothelial cells. As shown in Fig. 4A and B, elevated phosphorylation of mTOR, $p70^{S6K}$, 4E-BP1, and eIF-4E were detected following bFGF treatment in a time-dependent fashion. As expected, basal levels of phosphorylated mTOR, $p70^{S6K}$, 4E-BP1, and eIF-4E were much lower in untreated endothelial cells. However, when we examined the effect of KS-5 on phosphorylation of mTOR, and phosphorylation of $p70^{S6K}$, 4E-BP1 and eIF-4E, these downstream targets of mTOR, were significantly decreased in endothelial cells by KS-5 treatment.

3.5. Effect of KS-5 on bFGF-induced protein synthesis in HUVECs

The present data suggested that KS-5 inhibited bFGF-induced mTOR signaling pathway to modulate endothelial cells growth. Signaling by mTOR regulates protein synthesis by modulating the rate of translation of mRNAs. Therefore, we investigated the effect of KS-5 on bFGF-induced [^{3}H]leucine incorporation into the cells.



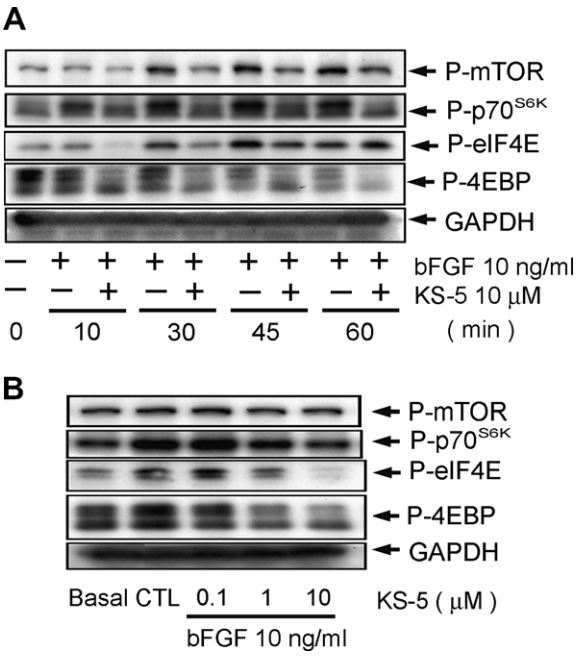


Fig. 4. Effects of KS-5 on bFGF-induced translational regulatory proteins. (A) Quiescent HUVECs were untreated (0) or treated for 10–60 min with bFGF (10 ng/ml) following a 30 min pretreatment with DMSO or KS-5 (10 μM). (B) Quiescent HUVECs were untreated (Basal) or treated with bFGF (10 ng/ml) for 30 min following a 30 min pretreatment with DMSO (CTL) or KS-5 (0.1, 1, 10 μM). Cell extracts were prepared and equal amounts of protein were analysed by SDS–PAGE and immunoblotting with antibodies specific for phospho-mTOR, phospho-p70^{S6K}, phospho-eIF4E, and phospho-4EBP. Similar results were obtained in three independent experiments.

As shown in Fig. 5, bFGF treatment for 6 h caused a significant increase in [^{3}H]leucine incorporation almost 2-fold. KS-5 significantly abolished, in a concentration-dependent manner, the bFGF-induced increase in [^{3}H]leucine incorporation into HUVECs. These results suggest that KS-5 may inhibit angiogenesis probably through the inhibition of mTOR-dependent pathway by bFGF.

Fig. 3. Effects of KS-5 on bFGF-induced ERK1/2 and Akt protein phosphorylation. Top, Quiescent HUVECs were untreated (0) or treated for 10–60 min with bFGF (10 ng/ml) following a 30 min pretreatment with DMSO or KS-5 (10 μM). Bottom, Quiescent HUVECs were untreated (Basal) or treated with bFGF (10 ng/ml) for 30 min following a 30 min pretreatment with DMSO (CTL) or KS-5 (0.1, 1, 10 μM). Cell extracts were prepared and equal amounts of protein were analysed by SDS–PAGE and immunoblotting with antibodies specific for phosphorylated-ERK 1/2 (A) or phosphorylated-Akt, (B) The quantitative data is shown under each protein, respectively. $^{\#}P < 0.001$ versus basal group; $^{*}P < 0.05$ versus control group.

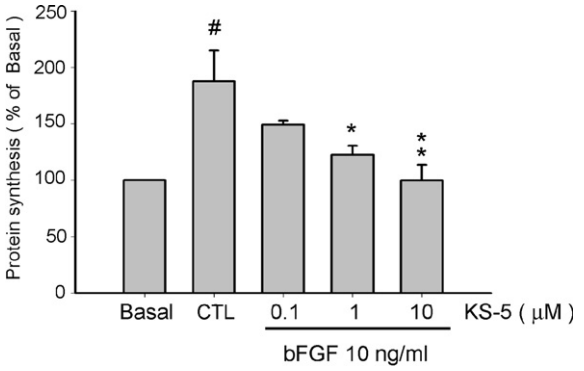


Fig. 5. Effects of KS-5 on bFGF-induced HUVEC protein synthesis. HUVECs were pre-treated with different concentrations of KS-5 for 30 min and added [^{3}H]leucine (2.5 μCi/ml) then stimulated with bFGF (10 ng/ml) for 6 h. After treatment, the cells were washed, and the cellular proteins were precipitated with 10% TCA for 1 h at 4 °C. The precipitates were washed twice, dissolved in 1 M NaOH. The radioactivity of incorporated [^{3}H]leucine was measured and expressed as counts/min. Data represent the mean ± SEM of three independent experiments. $^{\#}P < 0.001$ versus basal group; $^{*}P < 0.05$; $^{**}P < 0.01$ versus control group.

3.6. Effect of KS-5 on neovascular formation in vivo

To determine whether KS-5 is capable of blocking bFGF-induced angiogenesis *in vivo*, we used an experimental model in which angiogenesis is induced by bFGF embedded in a pellet of Matrigel, which was injected subcutaneously in mice; the degree of vascularization was evaluated after 7 days. bFGF (150 ng/ml) significantly induced angiogenic responses compared with Matrigel alone (Fig. 6), and KS-5 significantly inhibited the angiogenic response in a dose-dependent manner. Microscopic examination showed that the addition of bFGF to the Matrigel induced cellularity and the formation of cords, tubules, and several blood-filled channels containing red blood cells. In contrast, Matrigel pellets without angiogenic stimuli had only a few infiltrating, single, elongated cells. bFGF-induced angiogenesis was significantly reduced in mice treated with bFGF plus KS-5. These results clearly indicate that KS-5 is a potent anti-angiogenic molecule *in vivo*.

4. Discussion

Angiogenesis, the process of blood-vessel growth, is important during both normal development and tumor growth and metastasis. The inhibition of angiogenesis controls the expansion and metastasis of solid tumors and other related-diseases [18]. In the present study, we demonstrated that KS-5, a 1-hydroxy acridone derivative, has anti-angiogenic properties *in vitro* and *in vivo*. Using an *in vitro* model system, we demonstrated that KS-5 markedly and concentration dependently inhibited bFGF-induced endothelial cell proliferation. As noted above, the activation of ERK1/2, PI3-K and mTOR

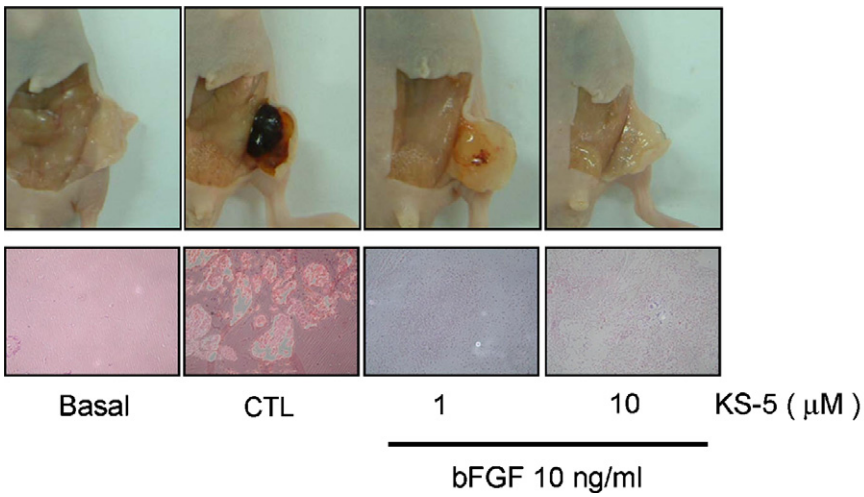


Fig. 6. Effects of KS-5 on bFGF-induced angiogenesis *in vivo*. Top: anti-angiogenesis effect of KS-5 in *in vivo* mouse Matrigel-plug assay. The experimental procedures are described in Materials and methods. Matrigel without growth factor did not show any migration or invasion of endothelial cells. However, Matrigel containing growth factor (bFGF 150 ng/ml), many blood vessels appeared in the gel on mice subcutaneous. Treatment of KS-5 simultaneously significant dose-dependent inhibition of the formation of blood vessels in the gel after 7 days. Bottom: histological analysis (hematoxylin and eosin staining) of the effect of KS-5 in *in vivo* angiogenesis. Matrigel containing bFGF in vehicle-treated mice demonstrated a high degree of cellularity and the presence of blood-containing vessels (magnification 100×).

signaling pathways are required for the growth and proliferation effects of bFGF on endothelial cells [17,19,20]. However, KS-5 significantly inhibits bFGF-induced ERK1/2 and Akt protein phosphorylation in a concentration-dependent manner.

Abnormal activation of Raf/MEK/ERK and PI3K/Akt pathways often occurs in human cancer due to mutation or aberrant expression [21]. In certain cancer types, the Raf/MEK/ERK pathway may induce the phosphorylation of Bcl-2 and other key molecules involved in the regulation of apoptosis [22]. Certain phosphorylation events on Bcl-2 have been associated with prolonged activation [23] and enhanced anti-apoptotic activity [24]. The abnormal activation or mutation of these pathways may result in the selectivity of KS-5 between HUVECs and KB cells.

In eukaryotes, 95–97% of total cellular mRNA translation is via cap-dependent pathway, the others are through cap-independent pathway [25]. mTOR is located at the intersection of several major signaling pathways (including PI3-kinase, ERK and AMPK) and is able to integrate a large and diverse panel of cell signals. mTOR can thereby modulate numerous cellular functions [26]. In this study, we show for the first time that KS-5 can inhibit protein synthesis and pathways involved in mTOR-dependency, revealing a possible new mechanism of endothelial cell growth regulation by KS-5. Overexpression of eIF4E leads to cell transformation and/or enhanced cell growth [27]. It also enhances the translation of certain transcripts by recruiting other translation factors to the mRNA 5′ cap. KS-5 inhibited bFGF-induced eIF4E phosphorylation on Ser209. The effects of phosphorylation on eIF4E activity are not completely understood, but eIF4E phosphorylation has been reported to increase its affinity for mRNA caps [28]. Although phosphorylation of Thr^{37}/Thr^{46} does not regulate the binding of 4E-BP1 to eIF4E directly, phosphorylation at the Ser^{65} and Thr^{70} sites do [11,29]. Phosphoryation of Thr^{37}/Thr^{46} is required for modification of Thr^{70}, following which Ser^{65} undergoes phosphorylation [30]. KS-5 decreased 4E-BP phosphorylation at Thr^{37}/Thr^{46} could be stand for decreasing eIF4E activity indirectly. Treatment of KS-5 also inhibited the phosphorylation of $p70^{S6K}$, which modulates translation of 5′TOP-containing mRNAs, suggesting that a potentially large number of endothelial cell proteins may have their synthesis regulated by KS-5 at the translational level.

Several types of experimental evidence suggest that bFGF induces angiogenesis indirectly by activation of the VEGF/VEGFR system, e.g. (i) both endogenous and exogenous bFGF induces VEGF expression in endothelial cells [31]; (ii) systemic administration of VEGF-A neutralizing antibodies dramatically reduces bFGF-induced angiogenesis [31]; (iii) bFGF induces VEGF receptor expression in endothelial cells [32]. However, in this study, we determinated that KS-5 did not affect bFGF-induced VEGF expression at the mRNA level (data not shown). These data shown that KS-5 directly suppressed bFGF-induced cell proliferation signaling pathway to abolish the angiogenic function.

In conclusion, we evaluated the anti-proliferation activity of a series of acridone-derivatives on HUVECs induced by bFGF and KS-5 was identified as the most effective one; structure-activity relationships are interesting and will be reported elsewhere (Peng, Teng and Bastow, unpublished results). KS-5 inhibited bFGF (10 ng/ml)-induced cell proliferation in a concentration-dependent manner. It is very significant that KS-5 did not cause HUVEC cytotoxicity as revealed by LDH release assay results. The anti-proliferative mechanism involved decreasing phosphorylation of ERK1/2 and Akt proteins using Western blotting analysis. In addition, we have also identified that KS-5 inhibited bFGF-induced protein synthesis. This effect correlated with the phosphorylation state of mTOR and downstream factors, $p70^{S6K}$ and eIF4E, both being decreased when cells were pretreated with KS-5. Finally, in nude mice Matrigel implant model, KS-5 significantly inhibited *in vivo* angiogenesis induced by bFGF. Taken together, our results suggest that KS-5 is a potent angiogenesis inhibitor with the potential to become a useful agent in the treatment of human cancer and other angiogenesis-dependent diseases.

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