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Antiangiogenic effect of ZSTK474, a novel phosphatidylinositol 3-kinase inhibitor

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ABSTRACT

Angiogenesis is known to be required for tumour growth and metastasis. Recent reports indicated that phosphatidylinositol 3-kinase (PI3K) promoted angiogenesis by inducing expressions of HIF-1α and vascular endothelial growth factor (VEGF). The present study aims to investigate the antiangiogenic effect of ZSTK474, a novel pan-PI3K inhibitor. ZSTK474 significantly inhibited tumour growth in the RXF-631L xenograft model. Immunohistochemical staining of the tumour tissue with anti-von Willebrand Factor antibody showed a significantly reduced number of microvessels in the ZSTK474-treated mice, suggesting the highly promising antiangiogenic activity *in vivo*. In human umbilical vein endothelial cells (HUVECs), submicromolar concentrations of ZSTK474 inhibited cell growth, blocked VEGF-induced cell migration and the tube formation, and thus revealed potent *in vitro* antiangiogenic activity. Furthermore, ZSTK474 inhibited phosphorylation of Akt at submicromolar concentrations. In RXF-631L cancer cells, on the other hand, ZSTK474 treatment inhibited the expression of HIF-1α and secretion of VEGF. Together, these results suggest that ZSTK474 has potent antiangiogenic activity, which could be attributed to dual-target inhibitory properties: inhibition of VEGF secretion by cancer cells and inhibition of PI3K in endothelial cells.

1. Introduction

Angiogenesis, the process of generating new blood vessels from a primitive vascular network, is required for various physiologic events such as embryonic development, tissue modelling and wound healing.[1] However, angiogenesis is also involved in various human pathologies, including tumour growth and metastasis. Indeed, it was shown that a tumour can not continue to grow without angiogenesis after its size reaches 1 mm^3.[2] Angiogenesis is a multi-step process involving basement membrane dissolution, endothelial cell proliferation and migration, vessel lumen and branch formation, vessel maturation and new basement membrane formation.[3]

The understanding that angiogenesis is required for tumour growth has raised significant interests among researchers to develop inhibitors against angiogenesis for cancer therapy. Especially, since the FDA (USA) approval of Avastin, a recombinant human monoclonal antibody against VEGF, for colorectal cancer therapy in 2004, more than 30 inhibitors of angiogenesis have been either approved or are in clinical trials for the treatment of cancer.[2,4]

Phosphatidylinositol 3-kinases (PI3Ks) are a family of lipid kinases that phosphorylate phosphatidylinositol 4,5-bisphosphate (PIP2) at the 3-hydroxyl group of the inositol ring[5,6] to generate phosphatidylinositol 3,4,5-trisphosphate (PIP3), which serves as a key second messenger and plays funda-

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mental roles in cellular responses such as proliferation, survival, motility and metabolism.[7,8] Frequent mutations found in the PIK3CA gene, which encodes PI3Kα in human tumours, and subsequent amplification suggest that PI3K is a potential target for cancer therapy.[9,10] ZSTK474, 2-(2-difluoromethylbenzimidazol-1-yl)-4,6-dimorpholino-1,3,5-triazine, is a novel PI3K inhibitor, which was identified in our laboratory by COMPARE analysis using a panel of 39 human cancer cell lines (termed JFCR39) coupled to a drug activity database.[11–15] Most notably, ZSTK474 inhibited the four PI3K isoforms with high specificity over 141 protein kinases including mTOR and DNA-PK.[11,16,17] *In vivo* it showed highly promising antitumour activity against A549, PC-3 and WiDr xenografts without any obvious toxicity.[11]

An increasing number of studies demonstrate the involvement of PI3K in angiogenesis. Thus, knockdown of PI3Kα using siRNA (small interfering RNA) inhibited angiogenesis both *in vivo* and *in vitro*.[18,19] It was also shown that over expression of the wild type PTEN, which dephosphorylates PIP3 to PIP2, decreased the capillary-like tube formation in human umbilical vein endothelial cells (HUVECs).[20] VEGF and HIF-1α were demonstrated to mediate PI3K-induced tumour angiogenesis signals.[18,21]As an endothelial cell-specific mitogen, as well as a vascular permeability factor, VEGF is well known to be involved in angiogenesis.[22] HIF-1α transactivates VEGF by binding to the hypoxia-responsive element (HRE) of the VEGF promoter.[23,24] Although the detailed mechanism remains unclear, HIF-1α seems to act downstream of p70S6K.[21]

Consistent with the idea that PI3K is involved in angiogenesis, several PI3K inhibitors were also shown to have antiangiogenic activity. Thus, the widely used PI3K inhibitor LY294002 effectively reduced the expression of VEGF in ovarian cancer cells,[21] blocked PC-3 cell-induced angiogenesis in chicken chorioallantoic membrane (CAM),[25] and showed antiangiogenic activity *in vivo* by reducing the microvessels in the tumour tissue of a U87 xenograft model.[26] Similarly, another PI3K inhibitor, PI-103, inhibited the migration of HUVEC and decreased the microvessels in the tumour tissue of MDA-MB-435 xenograft.[27] Additionally, PI3K inhibitor NVP-BEZ235 potently inhibited the VEGF-induced HUVEC proliferation *in vitro* and chamber implant angiogenesis *in vivo*.[28] Together, these results suggested that the PI3K inhibitors, at least in part, are potentially antiangiogenic.

In the present study, we have examined the antitumour effect of ZSTK474 on a renal cancer RXF-631L xenograft model, with special interest in its antiangiogenic activity.

2. Materials and methods

2.1. Materials

ZSTK474 was provided by Zenyaku Kogyo Co. Ltd (Tokyo, Japan). SU5416 was purchased from Sigma-Aldrich (St. Louis, MO, USA). Recombinant human VEGF 165, recombinant human FGF-basic (fibroblast growth factor-basic, bFGF), and Quantikine® human VEGF, bFGF and PDGF (platelet-derived growth factor) kits were purchased from R&D Systems (Minneapolis, USA); mouse anti-human HIF-1α antibody was obtained from BD Biosciences (San Jose, CA, USA); anti-phospho-Akt (Ser473) and anti-Akt antibody were from Cell Signaling Technology (Danvers, MA, USA); anti-β-actin antibody was from Sigma; anti-von Willebrand factor was from Dako Cytomation (Glostrup, Denmark). Benchmark Plus™ microplate spectrophotometer was from BIO RAD (Hercules, CA, USA); Odyssey infrared imaging system was from LI-COR® Biosciences (Lincoln, Nebraska, USA).

2.2. Cell lines and cell culture

HUVECs were purchased from Clonetics and maintained in endothelial cell growth medium-2 (EGM2 BulletKit, Conetics) at 37 °C in a humidified atmosphere containing 5% CO_2. The human renal cancer cell line RXF-631L was cultured in RPMI 1640 medium supplemented with 5% foetal bovine serum and kanamycin (100 U/ml) at 37 °C in a humidified atmosphere containing 5% CO_2.

2.3. In vivo antitumour effect of ZSTK474

The antitumour effect of ZSTK474 was tested *in vivo* in RXF-631L xenograft mouse models. Animals were handled and treated in accordance with the guidelines of the animal use and care committee of the Japanese Foundation for Cancer Research and conformed to the NIH guide for the *Care and Use of Laboratory Animals*. Female nude mice with BALB/c genetic backgrounds were purchased from Charles River Japan, Inc. (Yokohama, Japan), maintained under specific pathogen-free conditions and provided with sterile food and water *ad libitum*. RXF-631L xenografts were generated by subcutaneous injection with RXF-631L cells. Each nude mouse was inoculated with the generated tumour fragment of size 3 mm × 3 mm × 3 mm. When the tumours reached a volume of 100–300 mm^3, animals were randomly divided into control and ZSTK474 groups (each group containing six mice) (day 0). The experimental group of mice was orally administered daily with a given dose of ZSTK474 (100, 200, or 400 mg/kg of body weight) from day 0 to 13, except for days 3 and 10. The control group of mice was orally administered with 5% hydroxypropyl methyl cellulose (HPMC) instead of ZSTK474. After being photographed on day 14, the length (*L*) and width (*W*) of the subcutaneous tumour mass were measured by calipers in live mice, and the tumour weight (TW) was calculated as $TW = (L \times W^2)/2$. The antitumour efficacy was evaluated by *T/C*, which was calculated based on the following formula: *T/C* (%) = 100 × average tumour weight of ZSTK474-treated group/average tumour weight of control group.

2.4. Immunohistochemical (IHC) staining

Tumours were removed from the control and ZSTK474-treated mice, fixed with 10% neutral formalin and embedded in paraffin. Tumour sections (4-μm-thick) were cut and dewaxed with xylene, rehydrated by dipping in a series of ethanol solutions (100% to 50%), treated with 3% H_2O_2 in PBS, and blocked with normal goat serum. Then the tissue sections were exposed to anti-von Willebrand Factor (vWF) antibody to detect microvessels. The bound antibodies were stained using the DAKO Envision™ kit (DAKO cytomation, Glostrup, Denmark), and the sections were then counterstained with haematoxy-

lin. The number of microvessels was quantified as reported previously.[29]

2.5. Cell growth inhibition assay

Cell viability was determined using the WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfohenyl)-2H-tetrazolium, monosodium salt) assay kit (Kishida Chemicals, Osaka, Japan). To investigate the effect of ZSTK474 on the growth of HUVEC, 0.1 ml of cells (5×10^3 cells/well) was seeded in a 96-well plate and incubated in EGM2 medium at 37 °C in a humidified atmosphere containing 5% CO_2. Twenty four hours later, 0.5 μl of various stock solutions of ZSTK474 was added to achieve different final concentrations. After further incubation for 72 h at 37 °C, 10 μl of WST-8 was added to each well and the cells were further incubated at 37 °C. Three hours later, the absorbances at 450 nm and 650 nm (background) were measured with a microplate spectrophotometer (Benchmark Plus™, Bio Rad, Hercules, CA). The number of viable cells remaining after the treatment was calculated using the following formula: Cell number (% control) = 100 × (absorbance of a given sample – absorbance of blank well)/(absorbance of control well – absorbance of blank well), where the blank well contained medium but no cells and the control well contained cells but no inhibitor. The IC_{50} value was calculated by fitting the data points to a logistic curve using GraphPad Prism 4 software (GraphPad Software, San Diego, CA).

To examine the effect of ZSTK474 on VEGF- or bFGF-induced growth of HUVEC, these cells were initially grown in a 96-well plate (1×10^4 cells/well) in EGM2. Twenty four hours later, the medium was replaced with endothelial cell basic medium 2 (EBM2) supplemented with either 60 ng/ml VEGF or 30 ng/ml bFGF, and the cells were grown in the absence or presence of various concentrations of ZSTK474 or SU5416, a well known angiogenesis inhibitor. After further incubation for 48 h, WST-8 was added and the number of viable cells remaining was determined as described above.

2.6. In vitro assay for cell migration

The effect of ZSTK474 on migration of HUVECs *in vitro* was investigated by using a Transwell Boyden Chamber (Corning, MA) containing a polycarbonate filter with pore size of 8 μm as described previously[30] with a little modification. Briefly, 0.1 ml of HUVEC suspension (2×10^4 cells) in EGM2 with various concentrations of ZSTK474 was placed in the upper compartment of the Transwell Boyden Chamber. The lower compartment of each chamber contained 0.65 ml of EGM2 supplemented with 30 ng/ml of VEGF and the same concentration of ZSTK474 as in the upper compartment. Following incubation at 37 °C for 18 h, the culture medium from the upper compartment was discarded, and the non-migrant cells on the upper face of the Transwell membrane were removed using a cotton swab. Then the membrane was washed with 37 °C PBS. Cells that migrated to the bottom face of the membrane were fixed with 90% ethanol, stained with 0.5% eosin and observed under the Olympus DP50 microscope. A representative field of each membrane was photographed and used for the quantification of migrated cells. The number of migrated cells was counted, and the percentage of HUVEC migrated after the ZSTK474 treatment relative to that after the DMSO (vehicle solvent) treatment (control) was calculated. SU5416, a well known angiogenesis inhibitor, was used as a positive control for this experiment. Representative data from two independent experiments, each carried out in triplicate, were used for plotting.

2.7. In vitro assay for capillary-like tube formation

The effect of ZSTK474 treatment on tube formation *in vitro* was determined following the method reported previously[30] after a little modification. Briefly, matrigel (BD Biosciences, San Jose, CA) was thawed overnight at 4 °C. For coating, 50 μl of matrigel was pipetted into each well of a pre-chilled 96-well plate, and then the plate was incubated at 37 °C for 45 min. 0.1 ml of HUVECs (1×10^5 cells/ml) was seeded in each well of the coated plate. 0.5 μl of various stock solutions of ZSTK474 was added to achieve desired final concentrations and the cells were then incubated for 18 h at 37 °C. The capillary-like tubes formed were visualised under the Olympus DP50 microscope. A representative network of tube structures formed in each well was photographed. For quantification, the length of the tubes was measured using the MetaMorph 6.3 software, and from this data, the percentage of tubes formed in each ZSTK474-treated well relative to the control well (DMSO-treated cells) was calculated. SU5416 was used as a positive control in this experiment. Representative data from three independent experiments, each performed in triplicate, were used for analysis.

2.8. Western blot analysis

Cell lysates from treated and control cells were prepared as described previously.[11] Proteins in the cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then they were electrophoretically transferred onto Immobilon-FL membranes (Millipore, Bedford, MA). After blocking the membranes with 1:5 diluted (in distilled water) Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE), the membranes were incubated with either anti-HIF1α or anti-p-Akt antibody. The membranes were washed and then incubated with the respective Alexa Fluor 680 goat anti-rabbit IgG or anti-mouse IgG secondary antibodies. Signals from the bound labelled-antibodies were detected using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE). To confirm equal protein loading, the bound antibodies were stripped off from the blots by the stripping buffer (25 mM glycine pH2.0 and 2% SDS) and then they were re-probed with either anti-β-actin or anti-Akt antibody.

2.9. Enzyme-linked immunosorbent assay (ELISA)

RXF-631L cells were grown to a subconfluent stage in a 6-well plate. The culture medium in each well was replaced with fresh medium and then the cells were treated with various concentrations of ZSTK474 for 24 h. The culture medium from each well was collected and centrifuged at 800 rpm for 4 min. The supernatants were stored at –20 °C until used for ELISA.

The assay was performed using the respective Quantikine® kit (for human VEGF, bFGF or PDGF) from R&D Systems (Minneapolis, MN) according to the manufacturer's instructions. The relative amount of each growth factor level in the

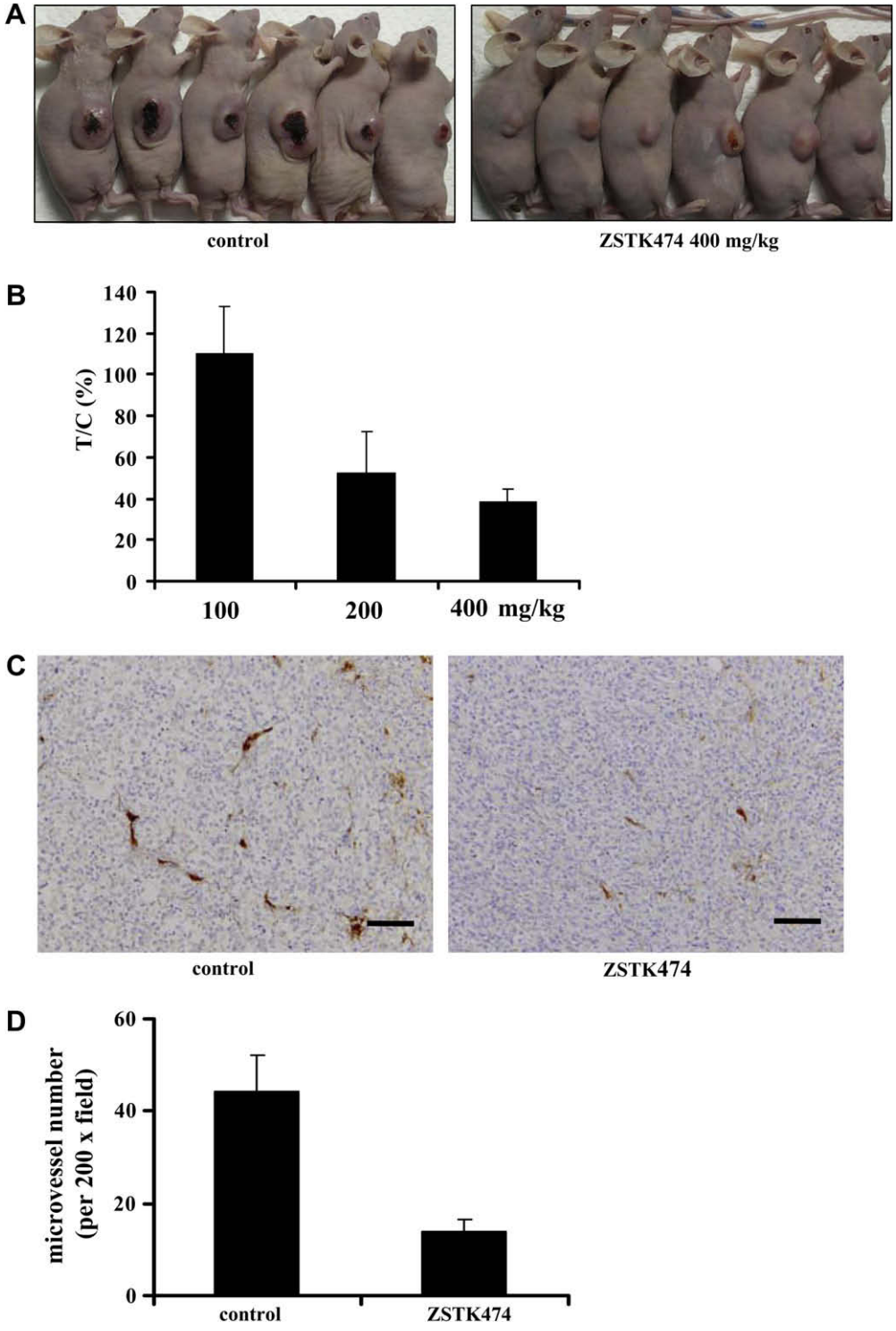

Fig. 1 – Inhibitory effect of ZSTK474 on tumour growth and tumour angiogenesis in a RXF-631L xenograft model. After the xenograft model formed, ZSTK474 (100, 200, or 400 mg/kg of body weight) was administered orally to the mice daily from day 0 to 13, except for days 3 and 10. (A) Photographs of control (5% HPMC administered) and ZSTK474-treated mice (400 mg/kg of body weight) on day 14. (B) Antitumour efficacy of ZSTK474 evaluated by T/C ratio. Data are mean ± SD (n = 6). (C) Immnuno-histochemical staining (IHC) of tumours. The 4-μm-thick tumour sections were exposed to anti-von Willebrand Factor (vWF) antibody to detect microvessels. Scale bar: 100 μm. (D) Quantification of the microvessels identified by IHC staining. The number of microvessels per 200 × field was quantified. Data are mean ± SD (n = 4).

ZSTK474-treated cells was expressed as the percentage of that in the DMSO-treated control cells.

3. Results

3.1. Antitumour efficacy of ZSTK474 in vivo

We used the RXF-631L xenograft mouse model to investigate the antitumour efficacy of ZSTK474 *in vivo*. After the tumours formed (100–300 mm^3), the mice in groups of six were orally administered respectively with 0 (control), 100, 200, and 400 mg/kg of ZSTK474. ZSTK474 showed significant antitumour activity in a dose-dependent manner after 12 days of treatment. (Fig. 1A and B) In addition, administration of ZSTK474 did not reduce the body weights of mice significantly (data not shown), suggesting that ZSTK474 treatment did not cause any severe side effects.

3.2. ZSTK474 inhibited tumour angiogenesis in vivo

To examine the antiangiogenic activity of ZSTK474 *in vivo*, 4-μm-thick tumour sections were prepared from the mice treated with either 400 mg/kg ZSTK474 or 5% HPMC (control), and the sections were then immunohistochemically stained with anti-vWF antibody for detecting microvessels. As shown in Fig. 1C and D, oral administration with 400 mg/kg of ZSTK474 for 12 days clearly reduced the number of microvessels, suggesting that ZSTK474 inhibited tumour angiogenesis *in vivo*.

3.3. ZSTK474 inhibited growth of HUVEC

To further investigate the antiangiogenic activity of ZSTK474, we next examined the effect of ZSTK474 on the growth of HUVEC, a well used endothelial cell for angiogenesis study. In the cell viability assay using WST-8, ZSTK474 treatment reduced the number of viable HUVECs in dose-dependent manner. (Fig. 2A) The IC_{50} of ZSTK474 for inhibiting the growth of HUVECs was calculated to be 0.146 μM, suggesting potent antiangiogenic activity of ZSTK474.

Both VEGF and bFGF are known to play important roles in angiogenesis.[22,31] Thus, we next evaluated the inhibitory effect of ZSTK474 on VEGF- or bFGF-induced HUVEC growth. HUVECs were initially grown in a 96-well plate in EGM2. Twenty four hours later, the medium was replaced with EBM2 supplemented with either 60 ng/ml VEGF or 30 ng/ml bFGF, and then incubated in the absence or presence of ZSTK474 or SU5416, a well known angiogenesis inhibitor that targets the VEGF receptor 2. After further incubation at 37 °C for 48 h, the number of viable cells was determined using the WST-8 assay. As shown in Fig. 2B, ZSTK474 potently inhibited the VEGF-induced HUVEC growth, and its potency of inhibition was higher than that of SU-5416 ($p < 0.05$ at 0.5 and 1 μM). ZSTK474 also inhibited the bFGF-induced HUVEC growth (Fig. 2C). In contrast, SU5416 inhibited the VEGF-induced HUVEC growth (Fig. 2B), but not the bFGF-induced HUVEC growth (Fig. 2C).

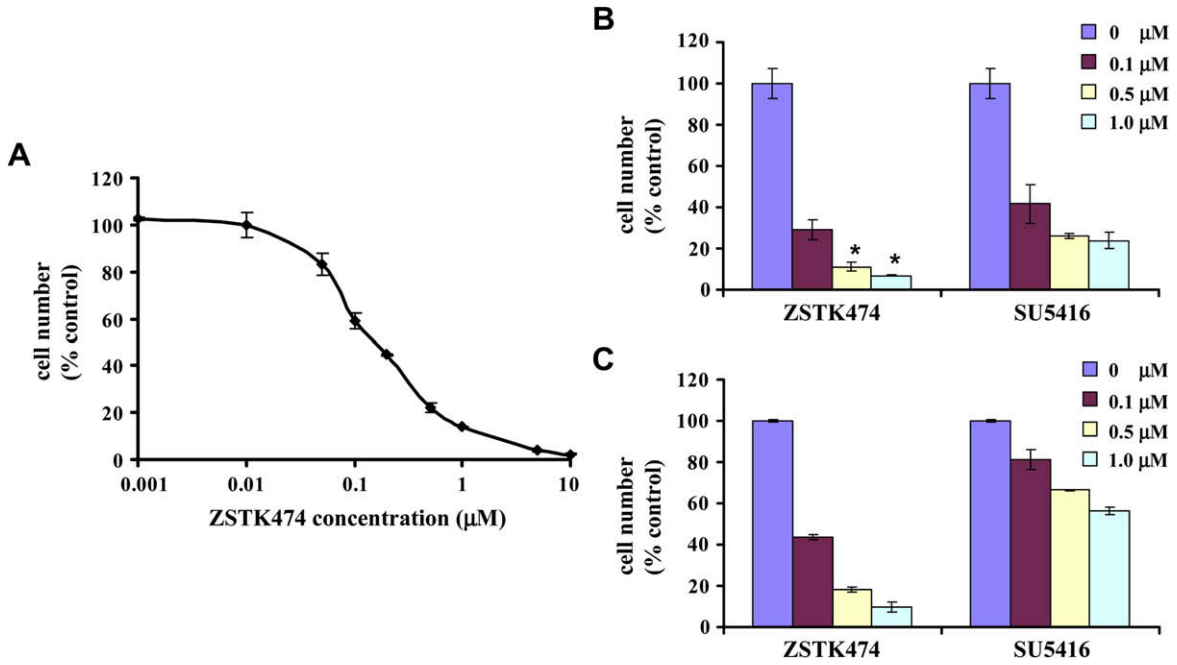

Fig. 2 – Growth inhibitory effect of ZSTK474 on HUVEC. (A) ZSTK474 inhibits the growth of HUVEC cultured in growth medium EGM2. The number of viable cells remaining after the ZSTK474 treatment was expressed as the percentage of viable control cells remaining after the DMSO treatment. Data are mean ± SD ($n = 3$) and are representative of two independent experiments. (B, C) Inhibitory effect on VEGF- (B) or bFGF- (C) induced HUVEC growth. *: $p < 0.05$, compared with the data obtained using the same concentration of SU5416 as ZSTK474. Data are mean ± SD ($n = 3$) and are representative of three independent experiments.

3.4. ZSTK474 blocked HUVEC migration

A recent study suggests that PI3K controls the endothelial cell migration,[19] a critical step for angiogenesis. Therefore, we next examined the effect of ZSTK474 on HUVEC migration using a modified Boyden Transwell Chamber assay. Fig. 3A shows the representative photographs of eosine-stained HUVECs that migrated to the bottom face of the membrane. Thus, 0.1 μM, 1 μM and 5 μM of ZSTK474 blocked the migration of HUVECs by 51%, 91% and 100%, respectively, suggesting that ZSTK474 inhibited VEGF-induced HUVEC migration in a dose-dependent manner. Results of the migration studies are quantified and shown in Fig. 3B. As shown, SU5416, which was used as positive control, blocked the HUVEC migration by 78% at 1 μM, indicating that it is a less potent inhibitor of endothelial cell migration than the ZSTK474 ($p < 0.01$).

3.5. ZSTK474 inhibited capillary-like tube structure formation of HUVECs

Finally, we investigated whether or not ZSTK474 affects angiogenesis *in vitro*. For this purpose, we used a well known *in vitro* angiogenesis model by observing the capillary-like tube structure formation by HUVECs on matrigel. Accordingly, HUVECs on matrigel were incubated in the absence or presence of various concentrations of ZSTK474 for 18 h, and the capillary-like tube formation was visualised under a microscope and photographed. The representative networks of tube structure formed are shown in Fig. 4A. In the presence of 0.1 μM, 1 μM and 5 μM of ZSTK474, the tube formation was inhibited by 30%, 83% and 100%, respectively, exhibiting a dose-dependent inhibition pattern (Fig. 4B). Furthermore, at 1 μM concentration, ZSTK474 inhibited the tube formation in HUVECs more potently than SU5416 ($p < 0.05$).

3.6. ZSTK474 inhibited phosphorylation of Akt in HUVEC

Our previous report indicated that ZSTK474 inhibited PI3K/Akt pathway in cancer cells as a PI3K inhibitor.[11] To determine whether ZSTK474 also affects the same pathway in HUVEC, we examined its effect on phosphorylation of Akt in HUVECs. Western blot analysis of the cell lysates prepared from the ZSTK474-treated and control HUVECs clearly dem-

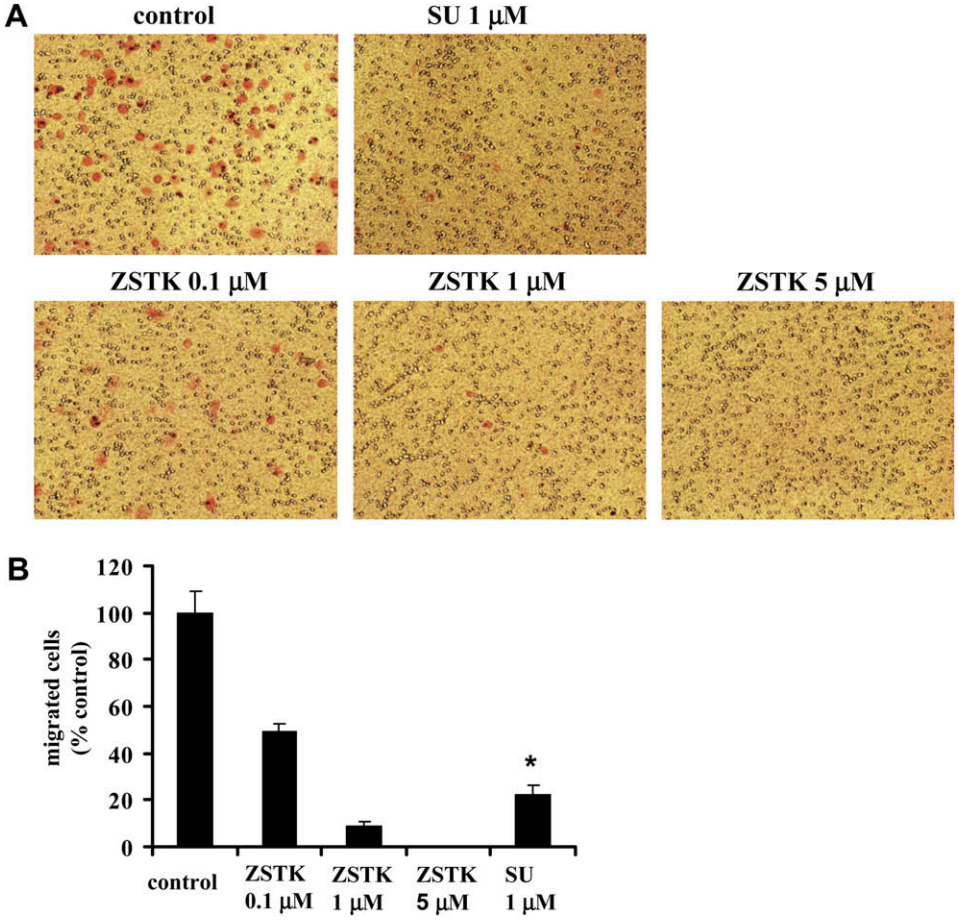

Fig. 3 – ZSTK474 inhibits migration of HUVEC. (A) Representative images depicting the effect of treatment with the indicated concentrations of ZSTK474 or SU5416 on HUVEC migration. (B) Percentage of HUVECs migrated following treatment with the indicated concentrations of ZSTK474 or SU5416 relative to those of the control cells (DMSO-treated). Data are mean ± SD ($n = 3$), and are representative of two independent experiments. *: $p < 0.01$, compared with the data obtained using 1 μM of ZSTK474. ZSTK: ZSTK474; SU: SU5416.

onstrated that ZSTK474 inhibited phosphorylation of Akt in a dose-dependent manner (Fig. 5).

3.7. *ZSTK474 inhibited the expression of HIF-1α and VEGF in RXF-631L cancer cells*

After demonstrating the antiangiogenic effect of ZSTK474 on HUVEC, we next investigated its effect on cancer cells. Since PI3K/Akt pathway is known to regulate angiogenesis via HIF-1α and its target VEGF,[18,21,32] we next asked whether ZSTK474 would inhibit angiogenesis by down-regulating the expressions of HIF-1α and VEGF. Accordingly, we examined the effect of ZSTK474 treatment on the expression level of HIF-1α in RXF-631L cells. As shown in Fig. 6A, ZSTK474 treatment for 6 h inhibited the expression of HIF-1α in a dose-dependent manner. Next, we determined the VEGF level in the culture supernatant of RXF-631L cells, which were incubated in the absence or presence of ZSTK474 for 24 h, by ELISA. Fig. 6B clearly demonstrates that ZSTK474 blocked the secretion of VEGF in a dose-dependent manner. We also determined the levels of two other angiogenesis-related growth factors, bFGF and PDGF, in the culture media. Although both growth factors were secreted by the RXF-631L cells, their levels were far lower than VEGF, and ZSTK474 treatment did not reduce their levels in the culture media (data not shown).

4. Discussion

In the present study, we demonstrated that ZSTK474 inhibited angiogenesis both *in vitro* and *in vivo*. *In vitro,* ZSTK474 inhibited the growth, migration and tube formation of HUVECs at submicromolar concentrations. ZSTK474 inhibited the growth of HUVEC with an IC_{50} of 0.146 μM, and blocked the phosphorylation of Akt in HUVECs at a similar concentration, suggesting that ZSTK474 mediates its potent growth inhibition effect via blocking the PI3K/Akt pathway of these endothelial cells. PI3K/Akt activation not only promotes endothelial cell survival but also regulates their migration and tube formation.[19,33–36] In this context it is noteworthy that, in a recent publication, a novel Akt substrate called Girdin was reported to promote migration and tube formation of endothelial cells.[35] Therefore, we think that the *in vitro* antiangiogenic effect of ZSTK474 observed in this study could be closely associated with its ability to inhibit the PI3K/Akt pathway in HUVEC.

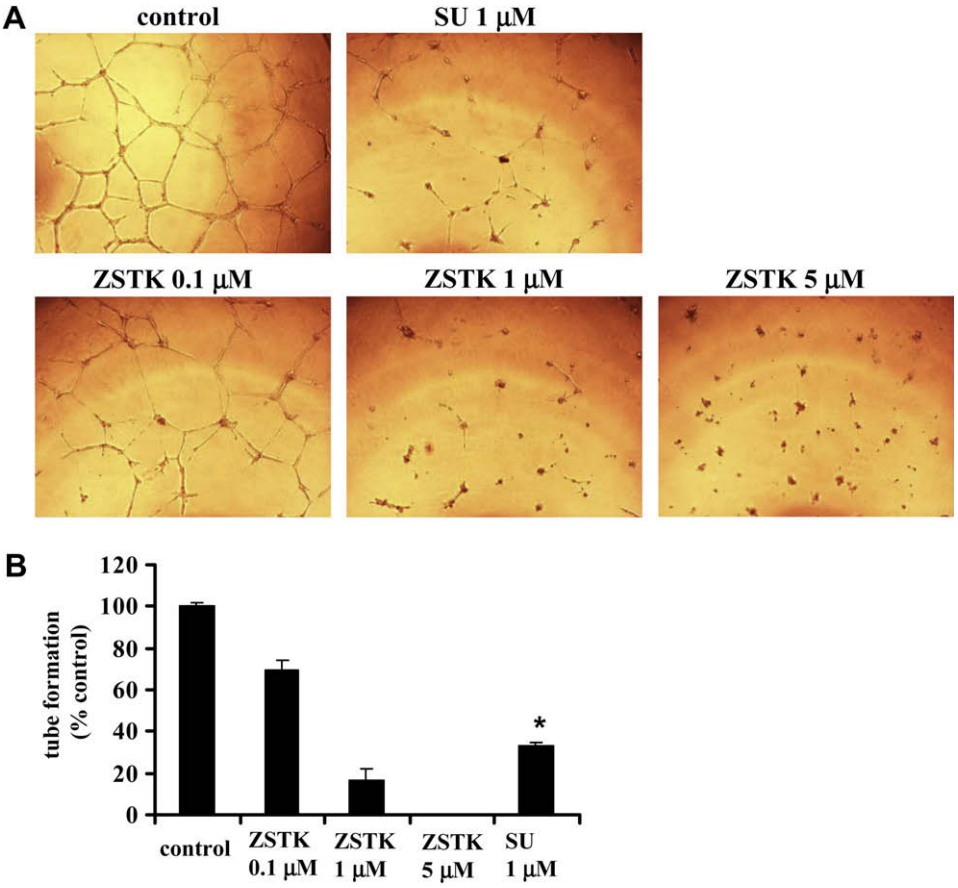

Fig. 4 – ZSTK474 blocks capillary-like tube formation by HUVEC. (A) Representative images depicting the tube formation by HUVEC following treatment with the indicated concentrations of ZSTK474 or SU5416. (B) Percentage of the tube formation following treatment with the indicated concentrations of ZSTK474 or SU5416 relative to the DMSO-treated control. Data are mean ± SD ($n = 3$), and are representative of three independent experiments. *: $p < 0.05$, compared with the data obtained with 1 μM of ZSTK474. ZSTK: ZSTK474; SU: SU5416.

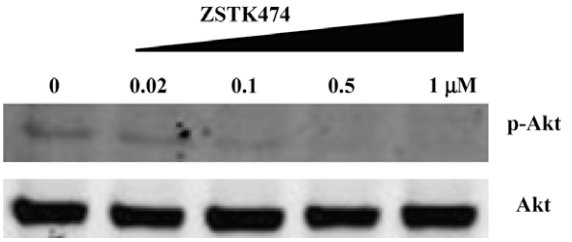

Fig. 5 – ZSTK474 inhibits phosphorylation of Akt in HUVECs. The expression levels of p-Akt and total Akt in the cell lysates prepared from the DMSO-treated and ZSTK474-treated HUVECs were determined, respectively, by western blot analysis.

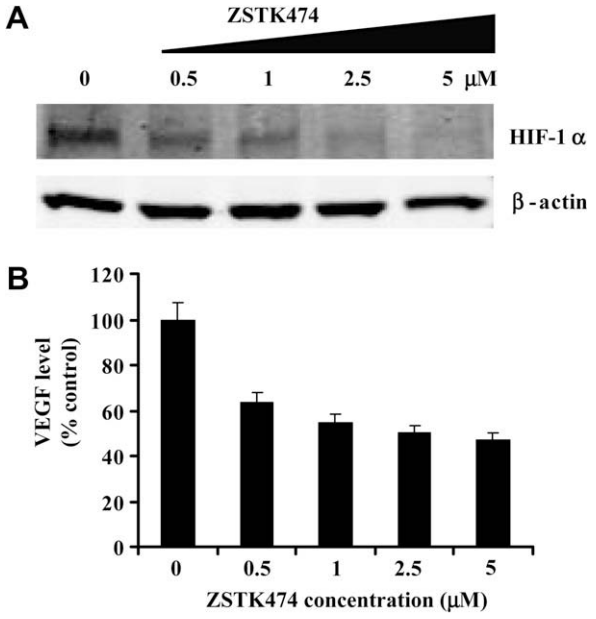

Fig. 6 – ZSTK474 inhibits the expression of HIF-1α and secretion of VEGF in RXF-631L cells. (A) Western blot analysis to determine the HIF-1α expression in RXF-631L cell lysates after ZSTK474 treatment. β-actin expression was determined for a quantitative control of protein amount. (B) VEGF secretion into the medium following treatment of RXF-631L cells with the indicated concentrations of ZSTK474. Data are mean ± SD (n = 3) and are representative of two independent experiments.

VEGF is known to play an important role in angiogenesis. VEGF regulates angiogenesis by binding to its own receptors in endothelial cells.[37] There are three types of VEGF receptors, namely VEGF-R1 (Flt-1), VEGF-R2 (flk-1/kdr) and VEGF-R3 (Flt-4). Several known angiogenesis inhibitors, such as SU5416 and sunitinib, directly target the VEGF-receptors.[4,38] Since our previous study showed that ZSTK474 did not inhibit the activities of all three of the above receptor kinases,[11] the anti-angiogenic effect of ZSTK474, therefore, could not be attributed to the direct inhibition of VEGF receptor. Therefore, we believe that ZSTK474 exerts its antiangiogenic effect on HUVEC by targeting the PI3K, which is known to be located downstream of the VEGF-receptors.[19,36]

We also investigated the antiangiogenic effect of ZSTK474 on the RXF-631L tumour cells. It has been reported that tumours produce various angiogenic factors over the course of their growth,[39] and blockade of their secretion is thought to have efficient antiangiogenic effect. Therefore, we investigated how ZSTK474 would affect the secretion of VEGF, bFGF and PDGF by the RXF-631L cells. As described above, we found that the RXF-631L cells only secreted VEGF at a high level, and ZSTK474 treatment selectively blocked secretion of VEGF. Since HIF-1α was known to trans-activate VEGF downstream of the PI3K-Akt pathway,[23,24] we also investigated the effect of ZSTK474 on the expression of HIF-1α. Western blot analysis demonstrated that the expression of HIF-1α in RXF-631L cells was reduced by ZSTK474 treatment in a dose-dependent manner. Therefore, we suggest that ZSTK474 exerts its anti-angiogenic effect on RXF-631 cells by inhibiting the expression of HIF-1α and secretion of VEGF.

Based on the above described effects of ZSTK474 on HUVEC and RXF-631L, we conclude that ZSTK474 exhibits its antiangiogenic effect via blocking the PI3K in both tumour and endothelial cells: inhibition of VEGF secretion by RXF-631L cells and inhibition of angiogenesis by endothelial cells. These dual effects are likely to contribute to the antiangiogenic activity of ZSTK474 *in vivo*. However, the antitumour efficacy of ZSTK474 *in vivo* could not be attributed solely to the antiangiogenic effect of ZSTK474, since our previous report showed that ZSTK474 inhibited the proliferation of a panel of human tumour cells, including the RXF-631L, via blocking the PI3K/Akt pathway.[11] Therefore, we believe that ZSTK474 exhibits its antitumour efficacy by inhibiting two cellular processes: tumour cell proliferation and tumour angiogenesis.

In conclusion, ZSTK474 was shown to inhibit angiogenesis *in vitro* and *in vivo*. This antiangiogenic effect of ZSTK474 might be attributed to its dual-target inhibition properties: inhibition of VEGF secretion by cancer cells and inhibition of PI3K in endothelial cells.

Conflict of interest statement

Takao Yamori has a research fund from Zenyaku Kogyo Co. Ltd, which is the proprietary company of ZSTK474. No conflict of interest is declared for the other authors.

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