Antiangiogenic mechanisms of Pj-8, a novel inhibitor of vascular endothelial growth factor receptor signaling

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Angiogenesis occurs not only during tissue growth and development but also during wound healing and tumor progression. Angiogenesis is a balanced process controlled by proangiogenic and antiangiogenic molecules. As a critical factor in the induction of angiogenesis, vascular endothelial growth factor (VEGF) has become an attractive target for antiangiogenic and cancer therapeutical agents. In an effort to develop novel inhibitors to block VEGF signaling, we selected Pj-8, a benzimidazole derivative, and investigated its inhibitory mechanisms in human umbilical vascular endothelial cells (HUVECs). Pj-8 concentration-dependently inhibited VEGF-induced proliferation, migration and tube formation of HUVECs. Pj-8 also suppressed VEGF-induced microvessel sprouting from aortic rings ex vivo and suppressed neo-vascularization of implanted matrigel plugs in vivo. Pj-8 inhibited VEGF-induced phosphorylation of VEGF receptor (VEGFR) 2 (F) and the downstream protein kinases, including Akt, focal adhesion kinase, extracellular signal-regulated kinases and Src. Results from in vitro kinase assay further demonstrated that Pj-8 suppressed the kinase activity of 3-phosphoinositide-dependent kinase 1 (PDK1). Using xenograft tumor angiogenesis model, Pj-8 markedly eliminated tumor-associated angiogenesis. Taken together, our findings suggest that Pj-8 inhibits VEGF and tumor cells MDA-MB-231-induced angiogenesis and it may be a potential drug candidate in anticanter therapy. Downregulation of VEGFR2-mediated signaling may contribute to its antiangiogenic actions.

Introduction

Angiogenesis, the formation of new vessels from preexisting vasculature, is a complex process, including endothelial cell proliferation, migration, adhesion and basement membrane degradation. It not only participates in a variety of physiological processes but also plays important roles in several diseases, typically in tumor progression (1,2). The newly formed vessels not only supply oxygen and nutrients for the survival of tumor cells but also provide the route for metastatic spread (3). Thus, tumor-induced angiogenesis has been an attractive target for cancer therapy (3,4). Numerous growth factors and cytokines are involved in tumor angiogenesis. These growth factors include vascular endothelial growth factor (VEGF) (5), basic fibroblast growth factor (bFGF) (6), epidermal growth factor, platelet-derived growth factor (PDGF) (7) and angiopoietin (8). Among these known angiogenic molecules, VEGF is the most critical and specific mediator that promotes angiogenesis (9,10). The VEGF family comprises of six subgroup of proteins: VEGF-A, B, C, D, E and placenta growth factor. These proteins exert their biological effects by binding to and activating receptors, VEGF receptor (VEGFR) 1 (Flt-1), VEGFR2 (KDR/Flik-1) and VEGFR3. Among them, VEGFR2 is a major receptor transducing VEGF-A-induced angiogenesis in endothelial cells (11). Binding of VEGF-A to VEGFR2 results in autophosphorylation of the receptor and leads to the activation of downstream signaling molecules including phosphoinositide 3-kinase, 3-phosphoinositide-dependent kinase 1 (PDK1), focal adhesion kinase (FAK), extracellular signal-regulated kinases (ERK), Src and Akt (12). ERK pathway activated by VEGF has been implicated in the regulation of cell motility and survival (13). Activation of Src by VEGFR2 has been shown to promote cell proliferation, migration and angiogenesis (14). FAK also participates in a number of processes that have impact on the malignant phenotype (15). Accumulating reports indicated that PDK1 can be a promising target for anticancer therapy because PDK1 acts as an oncogenic kinase in various cancers (16). In addition, phosphoinositide 3-kinase–PKD1–Akt cascade plays a causal role in VEGF-stimulated proliferation, migration and sprouting of endothelial cells in vitro and angiogenesis in tumor (11,17–19). Therefore, VEGF and VEGFR2 have been recognized as the most important targets for the antiangiogenesis therapy of cancers (20). A variety of approaches to inhibit VEGF activation of VEGFR are currently being assessed in clinical trials. These include soluble receptors that sequester VEGF (21), monoclonal antibodies targeting VEGF or VEGFR (22) and small molecule inhibitors that inhibit protein kinase activity (23). These inhibitors including bevacizumab (Avastin®), sorafenib (Nexavar®), sunitinib (Sutent®, SU11248) inhibit VEGF-VEGFR signaling and have been approved by the United States Food and Drug Administration for indication of specific types of cancer (24). However, most of these inhibitors possess some adverse effects, such as bleeding complications (25), which prompt the need for developing more VEGF–VEGFR signaling cascade inhibitors with less toxicity. In an effort to discover more VEGF–VEGFR inhibitors, we evaluated a series of benzimidazole derivatives. Pj-8 (Figure 1) was selected from these compounds and displayed potent and specific inhibition of VEGF–VEGFR2 signaling in vitro. In the present study, we demonstrated that Pj-8 inhibited VEGF-induced VEGFR2 phosphorylation and activation of various downstream signaling molecules. Pj-8 significantly inhibited VEGF-induced endothelial cell proliferation, migration and tube formation in vitro. Pj-8 also attenuated VEGF-induced neovascularization in mouse matrigel implantation model. By use of MDA-MB-231 breast tumor cells xenograft angiogenesis model, Pj-8 was further shown to antagonize tumor-associated angiogenesis. Taken together, our data suggest that Pj-8 may function as a novel VEGF–VEGFR2 inhibitor and suppresses tumor-induced angiogenesis.

Materials and methods

Reagents

Pj-8, 5-benzoyl-2-(5-methyl-2-furyl)-1H-benzimidazole, and Pj-6, 6-benzoyl-1-benzyl-2-(5-methyl-2-furyl) benzimidazole, were provided by Dr J.C.Lien (Graduate Institute of Pharmaceutical Chemistry, China Medical University, Taichung, Taiwan), and their purities (95%) were confirmed by 1H-nuclear magnetic resonance analysis. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) and tosylidine blue O were from Sigma (St Louis, MO). Medium 199 (M199), Dulbecco’s modified Eagle’s medium, RPMI medium 1640, fetal bovine serum (FBS) and all cell cultured reagents were purchased from Invitrogen (Carlsbad, CA). Antibody against c-Src phosphorylated at Tyr 216, platelet-derived growth factor receptor (PDGFR) phosphorylated at Tyr 1021, anti-mouse and anti-rabbit IgG-conjugated peroxidase antibodies, rabbit
polyclonal antibodies specific for α-tubulin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against VEGFR2 phosphorylated at Tyr1175, ERK1/2 phosphorylated at Tyr 204, Akt phosphorylated at Ser 473, FAK phosphorylated at Tyr 397 and FGFR phosphorylated at Tyr 655/654 were purchased from Cell Signaling (Danvers, MA). The enhanced chemiluminescence detection kit was from GE Healthcare (Little Chalfont, UK). Antibody against VEGFR1 phosphorylated at Tyr1213, recombinant human VEGF, bFGF and PDGF were purchased from R&D Systems (Minneapolis, MN). Cell proliferation ELISA, BrdU, assay kit was acquired from Chemicon (Temecula, USA). All materials for immunoblotting were purchased from Bio-Rad (Hercules, CA). All other chemicals were obtained from Sigma.

**Cell culture**

Primary human umbilical vascular endothelial cells (HUVECs) were isolated from umbilical cord veins as described previously (26). Isolated endothelial cells were identified by positive immunofluorescent staining of human von Willebrand factor antigen (DAKO, Carpinteria, CA). The cells were maintained in M199 medium containing 20% FBS, 5 U/ml heparin, 4 mM t-glutamine, 100 U/ml of penicillin G, 100 µg/ml streptomycin and 30 µg/ml endothelial cell growth supplements in a humidified 37°C incubator. MDA-MB-231, PC3, HT29, HCT116 and H68 cells line were obtained from the American Type Culture Collection (Livingstone, MT). The cells were maintained in RPMI1640 (MDA-MB-231 and PC3) or Dulbecco's modified Eagle's medium (HT29, HCT116 and H68) containing 5% fetal calf serum, 100 U/ml of penicillin G and 100 µg/ml streptomycin in a humidified 37°C incubator.

**Cell viability assay (MTT assay)**

Cell viability was measured by the colorimetric MTT assay as described previously (26).

**Cell proliferation assay (BrdU incorporation assay)**

HUVECs (2 × 10³ per well) were seeded in 96-well tissue culture plates and incubated for 24 h. Cells were then starved in M199 medium containing 2% FBS in the absence of endothelial cell growth supplements for another 16 h. After starvation, cells were pretreated for 30 min with various concentrations of pj-8, followed by the stimulation with VEGF (25 ng/ml), bFGF (25 ng/ml) or PDGF (25 ng/ml) for another 24 h. Cell proliferation was then determined by bromodeoxyuridine (BrdU) cell proliferation enzyme-linked immunosorbent assay (Chemicon, Temecula, CA) based on the colorimetric detection of the incorporation of BrdU, following the manufacturer instructions.

**Flow cytometric assay**

Cells were starved with 2% FBS M199 in the absence of endothelial cell growth supplements for 16 h. The cells were then incubated with pi-8 at indicated concentrations for 30 min followed by the treatment with VEGF (25 ng/ml) for another 24 h. At the end of the experiments, apoptotic cells were detected by propidium iodide (PI) and annexin V-fluorescein isothiocyanate labeling as described previously (27). The double labeling was performed at 37°C by treating cells with PI (50 µg/ml) and annexin V-fluorescein isothiocyanate (2 µg/ml) for 15 min in the dark. The samples were then immediately analyzed by the FACSscan and Cellquest program (BD Biosciences, San Jose, CA). Each experiment was repeated at least three times.

**Migration assay**

Migration assay was done as described previously with some modifications (28). Briefly, the bottom face of the filter in the transwell plate (Corning, NY) was coated with 0.2% gelatin. The bottom chambers were filled with M199 medium containing 2% FBS in the absence of VEGF (25 ng/ml) and the top chambers were seeded with 200 µl M199 medium (without growth factors) and HUVEC (10⁵ cells per well). The top chamber contained vehicle or various concentrations of Pj-8. Cells were allowed to migrate for 16 h. Non-migrated cells (on the top side of filter) were scraped with a cotton swab, and migrated cells were fixed and stained with 0.5% toluidine blue in 4% paraformaldehyde. The cells were photographed and quantified by counting the number of stained cells in three random fields at ×40 objectives under an inverted contrast phase microscope (Nikon, Japan).

**Matrigel tube formation assay**

The tube formation assay was performed as described previously (29). Matrigel, a basement membrane matrix extracted from Engelbreth-Holm Swarm mouse sarcoma (Becton Dickinson, Mountain View, CA), was polymerized for 30 min at 37°C. HUVECs suspended in M199 containing 2% FBS in the presence or absence of VEGF (25 ng/ml) were seeded onto the Matrigel. They were then treated with Pj-8 at indicated concentrations. After 16 h, cells were photographed using phase contrast microscopy at ×40 magnification.

**Aortic ring sprouting assay**

Assay was performed as described previously with modification (30). Aortic arch was dissected from 8- to 10-week-old Sprague-Dawley rats. After removing the surrounding fibroadipose tissues and thoroughly rinsing with M199 culture medium, the aortas were cut into 1 mm ring segments. The aortic rings were immersed in Matrigel in the wells of 48-well plate. VEGF (25 ng/ml) with or without Pj-8 was then added to the wells. The aortic rings were cultured in 37°C with 5% CO₂ and the cultured medium was changed every 3 days. Growing sprouts of endothelial cells were observed and photographed on day 8. The images were photographed into a computer by using an OLYMPUS Biological Microscope, and sprouting area was determined on the computer-digitized images with Image-Pro Plus (Media Cybernetics) software. The analysis of sprouting area was done by an observer who was blinded to the treatment group.

**Immunoblot analysis**

Immunoblot analyses were performed as described previously (31). Briefly, cells were lysed in an extraction buffer containing 15 mM Tris (pH 7.0), 50 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 5 mM dithiothreitol, 1% Triton X-100, 0.05 mM pepstatin A and 0.2 mM leupeptin. Samples of equal amounts of protein were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane, which was then incubated in a Tris-Buffered Saline Tween-20 buffer containing 5% bovine serum albumin. Proteins were visualized by specific primary antibodies and then incubated with horseradish peroxidase-conjugated secondary antibodies. Immunoreactivity was detected using enhanced chemiluminescence detection system following the manufacturer’s instructions. The blot images were quantitated by densitometry using the ImageQuant analysis software and normalized with the internal control (α-tubulin).

**PDK1 kinase activity assay**

This in vitro PDK1 kinase assay was performed as described previously using PDK1de as substrate (32). This cell-free assay is based on the ability of recombinant PDK1 (derived from insect sf21 cells), in the presence of vehicle or Pj-8, to phosphorylate its substrate PDK1de (KTFCGTPYALEPVRRE-PRILSEEEQEMFR DFDYIADWC) with [γ-32P]-adenosine triphosphate. The [γ-32P]-phosphorylated PDK1de was then separated from the residual [γ-32P]-adenosine triphosphate using P81 phosphocellulose paper and quantitated by a scintillation counter after three washes with 0.75% phosphoric acid.

**Matrigel plug assay**

VEGF-induced angiogenesis. Matrigel plug assay was performed as described previously with modification (33). In one group, an aliquot (500 µl) of Matrigel containing VEGF (200 ng/ml) with or without various concentrations of Pj-8 was injected subcutaneously into the dorsal region of 6- to 8-week-old C57BL/6 mice. In another group, an aliquot (500 µl) of Matrigel containing VEGF (200 ng/ml) with heparin (20 U) was injected subcutaneously into the dorsal region of 6- to 8-week-old C57BL/6 mice. Pj-8 (1 or 3 mg/kg/day) was then administered intraperitoneally once a day. After 7 days, the animals were killed, the intact Matrigel plugs were carefully removed and hemoglobin content in the plugs was determined with Drabkin’s reagent kit (Sigma–Aldrich) according to the manufacturer’s instructions.

**Tumor-induced angiogenesis.** MDA-MB-231 breast cancer cells (5 × 10⁶ cells) were mixed with phenol red-free Matrigel and injected into both flanks of severely combined immunodeficient mice as described previously (34). For Pj-8-treated group, Matrigel was mixed with cells in the presence of Pj-8 (10 or 30 µM) and heparin (20 U). Matrigel mixed with medium alone (500 µl) was used as a negative control. Ten days after implantation, Matrigel plugs were removed and the surrounding tissues trimmed. Hemoglobin levels of the Matrigel plugs were evaluated with Drabkin’s reagent kit (Sigma–Aldrich) according to the manufacturer’s instructions. The concentration of hemoglobin was calculated based on a set of hemoglobin standards. All animal study protocol was approved by Laboratory Animal Use Committee of Collage of Medicine, National Taiwan University.

**Tail bleeding time analysis**

C57BL/6 mice (6- to 8-week-old) were used to determine tail bleeding time. Mice were administered intraperitoneally with or without Pj-8 (1 or 3 mg/kg/day) for 7 days. Mice were then anesthetized with pentobarbital (1.75%) with body temperature maintained at 37°C on a thermo-controlled pad. Bleeding was induced by amputating the tail at 2 mm from the tail tip. The bleeding tail stump was immersed in normal saline. The bleeding time was defined as the time point at which all visible signs of bleeding from the incision stopped. All animal study protocol was approved by Laboratory Animal Use Committee of Collage of Medicine, National Taiwan University.

**Statistical analysis**

Results are presented as the mean ± S.E. from at least three independent experiments. One-way analysis of variance was followed by the Newman–Keuls test.
when appropriate, to determine the statistical significance of the difference between means. A P value of <0.05 was considered statistically significant.

Results

Pj-8 inhibits cell proliferation and migration in VEGF-stimulated HUVECs

To assess the antiangiogenic activity of Pj-8 (Figure 1), we first evaluated its inhibitory effects on VEGF-induced cell proliferation of HUVECs. As shown in Figure 2A, Pj-8 concentration-dependently decreased cell viability as determined by MTT assay. Pj-8 at 30 µM significantly decreased cell viability by about 60%. In contrast, Pj-6 (Figure 1), a close chemical analog of Pj-8, only slightly decreased cell viability by about 15% at 30 µM. To determine if the action of Pj-8 in decreasing HUVEC viability was attributable to the inhibition of cell proliferation, cells were starved for 16 h before being subjected to BrdU labeling analysis. Figure 2B shows that the percentage of BrdU-labeled cells significantly increased after a 24 h VEGF treatment when compared with the vehicle-treated group. Pj-8 significantly inhibited cell proliferation in a concentration-dependent manner. To further determine whether the cytotoxic effect was attributable to Pj-8’s actions in VEGF-treated HUVECs, a lactate dehydrogenase assay was employed. As shown in Figure 2C, treatment of cells with Pj-8 at 30 µM for 48 h did not increase lactate dehydrogenase release. We also used flow cytometric analysis with PI and annexin V–fluorescein isothiocyanate labeling for the detection of apoptosis in VEGF-stimulated cells in the presence of Pj-8. Cells were starved for 16 h before being subjected to flow cytometric analysis. As shown in Figure 2D, Pj-8 did not significantly alter the VEGF effects on the percentage of the annexin V<sup>−</sup> PI<sup>−</sup> cells (lower right quadrant and early apoptotic cells) and the annexin V<sup>+</sup> PI<sup>−</sup> cells (upper right quadrant, advanced apoptotic cells and/or necrotic cells). These results suggested that Pj-8 may exert antiproliferative activity without causing cytolytic or apoptotic effect in HUVECs. Cell migration is a key step for endothelial cell in angiogenesis. We thus performed transwell assays to evaluate whether Pj-8 affects cell migration in HUVECs exposed to VEGF. As shown in Figure 2E, Pj-8 significantly reduced the number of migrated cells through the membrane barrier of the transwell using VEGF as a chemoattractant. These findings suggest that Pj-8 may exert antiangiogenic activity through inhibiting cell proliferation and migration of endothelial cells.

Pj-8 inhibits VEGF-induced tube formation, microvessel sprouting and neovascularization

Angiogenesis is a complex process and tube formation of endothelial cells is an essential step during angiogenesis. To further assess antiangiogenic effects of Pj-8, a two-dimensional Matrigel tube formation assay and a rat aortic ring microvessel sprouting assay were used. Once HUVECs seeded on the surface of Matrigel, it became elongated and formed capillary-like structures in the vehicle-treated group within 16 h. VEGF significantly increased capillary-like network. However, treatment of Pj-8 concentration-dependently decreased the formation of capillary-like network, with a complete inhibitory effect at 30 µM (Figure 3A). We next explored the potential effects of Pj-8 on VEGF-induced angiogenesis ex vivo. As shown in Figure 3B, VEGF significantly triggered microvessel sprouting, leading to the formation of a complex network of microvessels around the aortic rings, whereas treatment with Pj-8 at 10 and 30 µM antagonized the sprouting, suggesting that Pj-8 suppresses VEGF-induced angiogenesis ex vivo. We then evaluated the antiangiogenic effects of Pj-8 in vivo mouse Matrigel plug model of angiogenesis. As shown in Figure 3E, a significant microvessel formation was observed in VEGF-supplemented Matrigel plug (Figure 3Ea). The pale color of the plugs removed from Pj-8–treated mice indicated VEGF-induced less neovascularization over a 7 day period (Figure 3Eb and 3Ec). We next quantified the level of angiogenesis by determining the hemoglobin content of the plugs. A marked reduction in neovascularization was shown in plugs from Pj-8–treated mice when compared with those from vehicle-treated mice (Figure 3E). These data indicate that Pj-8 significantly suppressed angiogenesis dose-dependently in this in vivo assay.

Pj-8 inhibits VEGFR2 signaling pathway

VEGF-induced VEGFR2 phosphorylation leads to the activation of several downstream signaling molecules that are responsible for endothelial cell migration and tube formation. These signaling molecules include FAK, Akt, ERK, Src and PDK1 (17). To investigate whether Pj-8 inhibited VEGFR2 and its downstream signaling, we examined these essential protein kinases involved in the VEGFR2 signaling cascade. As shown in Figure 4, Pj-8 concentration-dependently suppressed the phosphorylation of VEGFR2 (Figure 4B), FAK (Figure 4C), Akt (Figure 4D), ERK (Figure 4E) and Src (Figure 4F) in VEGF-stimulated HUVECs. On the other hand, PDK1 activates various downstream protein kinases including Akt and S6K (p70 ribosomal S6 kinase) that have been reported highly active in many cancer cells and PDK1 inhibitor might be beneficial for treatment of cancer (35). We thus determined whether Pj-8 affects the PDK1 kinase activity using in vitro kinase assay. As shown in Figure 4G, Pj-8 concentration-dependently suppressed PDK1 kinase activity with IC50 approximately 10 µM. These results suggest that Pj-8 may not only exert its antiangiogenic activity by targeting VEGFR2-mediated signaling cascade but also by directly inhibiting PDK1.

Pj-8 inhibits tumor-induced angiogenesis in severely combined immunodeficient mice mice

To further investigate whether Pj-8 is able to inhibit tumor-induced angiogenesis, a xenograft tumor-induced angiogenesis model was employed. Human breast cancer cells (MDA-MB-231) were mixed with Matrigel and injected into the flanks of mice. Gel plugs were harvested 10 days after implantation. MDA-MB-231 cells profoundly induced blood vessel formation in the plug (Figure 5A, upper panel). However, blood vessel formation induced by MDA-MB-231 cells significantly reduced in the presence of Pj-8 (Figure 5A). We also quantified the level of angiogenesis by determining the hemoglobin content of the plugs and found that Pj-8 is an active inhibitor on tumor cells-induced angiogenesis in vivo (Figure 5A, lower panel). We also used the mouse Matrigel plug model of angiogenesis to determine whether systemic administration with Pj-8 exhibits antiangiogenic effects. As shown in Figure 5B, significant microvessel formation was observed in VEGF-supplemented Matrigel plug. The pale color of the plugs removed from the mice administered intraperitoneally with Pj-8 indicated VEGF-induced less neovascularization over a 7 day period. A marked reduction in neovascularization was shown in plugs from Pj-8–treated mice when compared with those from vehicle-treated control mice (Figure 5B). These data indicate that

![Chemical structure of Pj-6 and Pj-8.](https://example.com/structure.png)
Fig. 2. Pj-8 inhibits proliferation and migration in VEGF-stimulated HUVECs. (A) HUVECs were starved in 2% FBS containing M199 without endothelial cell growth supplement for 16 h. After starvation, cells were pretreated with indicated concentrations of Pj-6 or Pj-8 followed by the stimulation with VEGF (25 ng/ml) for another 24 h. Cell viability was then determined by MTT assay. Each column represents the mean ± SEM of at least three independent experiments performed in duplicate (*P < 0.05 compared with the group treated with VEGF alone). (B) Cells underwent mitogenic quiescence as described in (A). After starvation, cells were subsequently stimulated with VEGF (25 ng/ml) in the presence or absence of Pj-8 for another 24 h. Cell proliferation was then determined as described in the Materials and methods. Each column represents the mean ± SEM of five independent experiments performed in duplicate (*P < 0.05 compared with the group treated with VEGF alone). (C) Cells were treated with indicated concentrations of Pj-8 in 2% FBS containing M199 medium without endothelial cell growth supplement for 24 h. Cytotoxicity of Pj-8 was then determined by lactate dehydrogenase assay. Cells were also treated with cell lysis buffer (total lysis) to serve as positive control. (D) Cells underwent mitogenic quiescence as described in (A). After starvation, cells were pretreated with indicated concentrations of Pj-8 followed by the stimulation with VEGF (25 ng/ml) for another 24 h. The percentage of apoptotic cells was then analyzed by flow cytometric analysis as described in the Materials and methods. The lower left quadrant of each panel (annexin V⁻/PI⁻) shows the viable cells, which exclude PI and are negative for annexin V binding. The lower right quadrant (annexin V⁺/PI⁻) represents the early apoptotic cells, annexin V positive and PI negative, demonstrating cytoplasmic membrane integrity. The upper right quadrant (annexin V⁺/PI⁺) contains advanced apoptotic cells and necrotic cells, which are positive for annexin V binding and for PI uptake. Results shown are representative of four independent experiments. (E) Cells were starved as described in (A), cell migration was then determined in the absence or presence of indicated concentrations of Pj-8 as described in the Materials and methods using VEGF as stimulant. Each column represents the mean ± SEM of at least three independent experiments (*P < 0.05 compared with the group treated with VEGF alone).
systemic administration with Pj-8 significantly suppressed angiogenesis in this in vivo assay. On the other hand, we also examined whether Pj-8 affects cell proliferation of MDA-MB-231, PC3, HT29 and HCT116 tumor cells. Cells were starved with serum-free medium for 24 h and then incubated in serum (10% FBS) containing medium in the presence or absence of Pj-8. As shown in Figure 5C, treatment of cells with Pj-8 for 24 h only slightly decreased serum-induced cell proliferation of MDA-MB-231 breast cancer and HCT116 colon cancer cells (P > 0.05). However, Pj-8 significantly decreased serum-induced cell proliferation of PC3 prostate cancer and HT29 colon cancer cells. Furthermore, Pj-8 did not affect serum-induced cell proliferation of non-tumor HS68 fibroblasts (Figure 5C). These results suggest that the antitumor effects of Pj-8 may vary among different type of tumors. However, Pj-8 may primarily inhibit MDA-MB-231 tumor cells-induced angiogenesis by targeting to proliferating endothelial cells rather than to tumor. We then determined whether Pj-8 affects blood clotting system using tail bleeding time assay since most of the VEGF–VEGFR signaling inhibitors possess some adverse effects, such as bleeding complications (25). As shown in Figure 5D, intraperitoneal administration with Pj-8 (1 or 3 mg/kg/day) for 7 days did not alter tail bleeding time as compared with the vehicle-treated group.

**Fig. 3.** Pj-8 inhibits VEGF-induced tube formation, microvessel sprouting and neovascularization. (A) HUVECs were seeded on Matrigel in the presence of VEGF with or without Pj-8 at indicated concentrations. Cells were then photographed under phase contrast after 16 h. (B) Rat aortic rings were placed in Matrigel and treated with various concentrations of Pj-8 in the presence or absence of VEGF. The effect of Pj-8 on formation of vessel sprout from various aorta samples was examined on day 8. Figures shown in (A) and (B) are representative of five independent experiments with similar results. (C) Bar graphs show compiled data of average sprout arch numbers in (A) (n = 5), *P < 0.05 compared with the group treated with VEGF alone. (D) Bar graphs show compiled data of average microvessels area in (B) (n = 5), *P < 0.05 compared with the group treated with VEGF alone. (E) Matrigels in the presence of VEGF with or without Pj-8 at indicated concentrations were injected into both flank sites of male C57BL/6 mice. Hemoglobin levels in the Matrigel plug were quantified and shown in the lower panel of the figure. Each column represents the mean ± SEM of eight independent experiments (*P < 0.05 compared with the group treated with VEGF alone).
Pj-8 inhibits bFGF- or PDGF-induced cell proliferation in HUVECs

Although VEGFR2 is a major receptor for VEGF-induced angiogenesis, VEGFR1 was reported to amplify the responsiveness to VEGF during angiogenesis, including cell migration in many pathological disorders (36). Both VEGFR1 and VEGFR2 are thus indispensable for tumor angiogenesis. We thus examined whether activation of VEGFR1 by VEGF could be suppressed by Pj-8 in HUVECs. As shown in Figure 6A, Pj-8 inhibited VEGF-induced VEGFR1 Tyr1213 phosphorylation. We further evaluated whether the effects of Pj-8 on angiogenesis may attribute to its multi-target receptor tyrosine kinase (RTK) inhibition. Pj-8 inhibited the phosphorylation of FGFR (Figure 6B) and PDGFR (Figure 6C), two RTKs that play critical roles in cancer angiogenesis (37,38). Furthermore, BrdU labeling analysis was employed to determine whether Pj-8 suppression of FGFR or PDGFR phosphorylation results in the decrease of cell proliferation. The percentage of BrdU-labeled cells significantly increased after a 24 h bFGF (Figure 6D) or PDGF (Figure 6E) treatment when compared with the vehicle-treated group. Pj-8 significantly inhibited cell proliferation in a concentration-dependent manner in HUVECs exposed to bFGF (Figure 6D) or PDGF (Figure 6E). Taken

![Figure 4. Pj-8 inhibits VEGFR2 signaling pathway. Cells were pretreated with indicated concentrations of Pj-8 for 30 min, followed by the addition with VEGF (25 ng/ml) for another 5 (VEGFR2) or 30 (FAK, Akt, ERK1/2 and Src) min. Phosphorylation status of VEGFR2, FAK, Akt and ERK1/2 was then determined by immunoblotting. Figures shown in (A) are representative of four independent experiments with similar results. The compiled results of VEGFR2 (B), FAK (C), Akt (D), ERK1/2 (E) and Src (F) phosphorylations are shown. Each column represents the mean ± SEM of four independent experiments (*P < 0.05 compared with the group treated with VEGF alone). β-Tubulin level was used as a loading control. Results are representative of more than three experiments. (G) Effects of Pj-8 on PDK1 kinase activity were determined by in vitro kinase assay as described in the Materials and methods.](https://example.com/figure4.png)
together, these results suggest that Pj-8 may affect the RTKs, including VEGFR1, VEGFR2, FGFR and PDGFR.

**Discussion**

Blockade of angiogenesis is an important therapeutic strategy for cancer treatment and prevention. Several angiogenesis inhibitors have been shown to potentially prevent tumors from growing and spreading to other organs (1). In addition, antiangiogenesis therapy may improve chemotherapy targeting tumors through vascular remodeling. Thus, VEGF, a critical factor in inducing angiogenesis, has emerged as an attractive target for antiangiogenesis treatment. We have screened and identified a chemical Pj-8 from series of benzimidazole derivatives as a potent inhibitor of VEGF signaling. We demonstrated in this study that Pj-8 inhibits cell proliferation, migration and tube formation of HUVECs in a concentration-dependent manner. Pj-8 was also shown to suppress VEGF- or tumor-induced angiogenesis in vivo. Furthermore, we demonstrated for the first time that suppression of VEGFR2-mediated signaling may contribute to Pj-8 inhibitory actions on angiogenesis.

VEGFR2-mediated signaling pathways play an essential role in the pathophysiological responses in vascular endothelial cells. Tyr1175 is the major autophosphorylation site within VEGFR2, and its phosphorylation leads to the activation of downstream signaling events in endothelial cells (39). Phosphorylated Tyr1175 of VEGFR2 mediates activation of the mitogen-activated protein kinase/ERK cascade and was shown to contribute to cell proliferation in endothelial cells. It has also been linked to VEGF-induced activation of Src, which regulates cell migration and vascular permeability (40). Other signaling molecules that have been involved in VEGF-induced migration through VEGFR2 include FAK and its substrate paxillin, which are participated in focal adhesion during cell migration (41,42). In addition, PDK1–Akt cascade has been reported to play an essential role in VEGF-induced vascular remodeling (43). Recent studies also indicated that inhibition of ERK, phosphoinositide 3-kinase, PDK1/Akt and FAK downstream of VEGFR2 may be beneficial for cancer therapy (44). In agreement with these observations, we noted in this study that Pj-8 inhibited VEGFR2 phosphorylation and the subsequent phosphorylation of FAK, ERK, c-Src and Akt in HUVECs exposed to VEGF. Furthermore, Pj-8 was shown to directly inhibit PDK1 activity. PDK1 inhibitors have been recently reported as a novel agent for cancer chemotherapies because PDK1 plays a key modulator in cancer cell growth and tumor angiogenesis (45–47). Using two types of Matrigel plug assays, we also noted that Pj-8 not only inhibited...
VEGF-induced angiogenesis but also attenuated tumor-elicited angiogenesis in vivo. These findings together with the reports described above suggest that its inhibitory effects on VEGFR2 phosphorylation and the subsequent VEGFR2-mediated signaling may be crucial for its antiangiogenic activity.

In addition to VEGFR2 signaling blockade and antiangiogenic activity in vitro, we also noted that Pj-8 at 10 μM significantly inhibited MDA-MB-231 breast cancer cells-induced angiogenesis in vivo. However, Pj-8 at 30 μM only slightly affected cell proliferation in MDA-MB-231 cells, indicating that the attenuation of Pj-8 on MDA-MB-231 cells-induced angiogenesis is not due to the blockade of cancer cell viability. On the other hand, benzimidazole has been reported as an important pharmacophore in anticancer drug development (47,48). Benzimidazole derivatives were demonstrated to suppress tumor growth by inhibiting the hypoxia-induced factor-1 (HIF-1) pathway (49). Apoptotic mechanisms also contribute to benzimidazole’s anticancer actions (48). We demonstrated in this study that Pj-8, a benzimidazole derivative, only slightly affected MDA-MB-231 breast cancer and HCT116 colon cancer cell proliferation. In contrast, Pj-8 significantly suppressed cell proliferation in PC3 prostate cancer and HT29 colon cancer cells. It suggests that the antiproliferative actions of Pj-8 on tumor cells may vary among different types of tumor. However, Pj-8 did not affect cell proliferation in non-tumor HS68 fibroblasts. Hori et al. (50) further demonstrated that benzimidazole derivatives inhibited endothelial cell growth. In agreement with these observations, we noted in this study that Pj-8 inhibits VEGF- and tumor-induced angiogenesis. We also demonstrated for the first time that Pj-8 may target VEGFR2-mediated signaling pathways.

Tumor angiogenesis is a complex process that involves a variety of angiogenic molecules, including VEGF, bFGF and PDGF. The actions of these growth factors are mainly mediated through their RTKs: VEGFR1, VEGFR2, FGFR or PDGFR. Although inhibition of VEGF–VEGFR2 signaling may attenuate angiogenesis and tumor progression, targeting only one RTK may not be an effective therapy because of the complexity of tumor angiogenesis. Giavazzi et al. (51) recently demonstrated that the crosstalk between bFGF and VEGF in vascularization. It appears that agents targeting more than one angiogenic molecule or RTK may be beneficial in the treatment of tumor angiogenesis and metastasis (52). We noted in this study that Pj-8 not only inhibited VEGFR1 and VEGFR2 activation but also inhibited the activation of FGFR and PDGFR. Since more than one angiogenic molecule may be co-operating with VEGF in neovascularization, these observations in combination with the inhibition of VEGF- and tumor-induced angiogenesis by Pj-8 suggest that Pj-8 may act as a multi-target RTK inhibitor. Several lines of evidence indicated that most of the VEGF–VEGFR2 signaling inhibitors possess some adverse effects, such as bleeding complications (25). However, we noted that intraperitoneal administration of mice with Pj-8 for 7 days did not affect tail bleeding time, suggesting that Pj-8 may not affect the blood clotting system. Taken together, Pj-8 may be a good candidate as an antiangiogenic agent and a valuable lead compound in the development of anticancer agents.

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