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BRN-103, a novel nicotinamide derivative, inhibits VEGF-induced angiogenesis and proliferation in human umbilical vein endothelial cells

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ABSTRACT

Anti-angiogenesis is regarded as an effective strategy for cancer treatment, and vascular endothelial growth factor (VEGF) plays a key role in the regulations of angiogenesis and vasculogenesis. In the present study, the authors synthesized five novel nicotinamide derivatives which structurally mimic the receptor tyrosine kinase inhibitor sunitinib and evaluated their anti-angiogenic effects. Transwell migration assays revealed that 2-(1-benzylpiperidin-4-yl) amino-*N*-(3-chlorophenyl) nicotinamide (BRN-103), among the five derivatives most potently inhibited VEGF-induced human umbilical vein endothelial cells (HUVECs). In addition, BRN-103 dose-dependently inhibited VEGF-induced migration, proliferation, and capillary-like tube formation of HUVECs and vessel sprouting from mouse aortic rings. To understand the molecular mechanisms responsible for these activities, the authors examined the effect of BRN-103 on VEGF signaling pathways in HUVECs. BRN-103 was found to suppress the VEGF-induced phosphorylation of VEGF receptor 2 (VEGR2) and the activations of AKT and eNOS. Taken together, these results suggest that BRN-103 inhibits VEGF-mediated angiogenesis signaling in human endothelial cells.

Angiogenesis is a crucial regulator of tumor growth and metastases,[1] and one of the major clinical advances in cancer treatment made recently involves the use of anti-angiogenic drugs like sunitinib. Sunitinib is a multi-targeting inhibitor of receptor tyrosine kinases (RTKs) and has been shown to be effective clinical for the treatment of various human cancers.[2,3] Recently, the US Food and Drug Administration (FDA) approved sunitinib for the treatments of clear cell renal cell carcinoma (ccRCC) and gastrointestinal stromal tumors,[4,5] and it is also being investigated intensely for the treatment for several other cancers, including breast cancer, colorectal cancer, and nonsmall cell lung cancer.[6,7] Therefore, the suppression of angiogenesis offers a promising approach to the treatment of various types of solid tumors. During our continued efforts to screen natural and synthetic compounds for anti-tumor effects, we examined the anti-angiogenic effects of five novel synthetic nicotinamide derivatives (BRN-103,[8] 218, 228, 229, and 279; Fig. 1A), which mimicked sunitinib and were prepared using synthetic methods as shown in Scheme 1 for the discovery of novel and potent anti-angiogenic compounds.

Tumor angiogenesis is regulated by the productions of angiogenic stimulators, such as, vascular endothelial growth factor (VEGF), which is a key regulatory factor in the prognostic indicator in several cancers. For this reason, the inhibition of the VEGF signaling pathway has been studied as a potential therapeutic strategy. VEGF receptors (VEGFRs) initiated pathways control various steps in physiological angiogenesis,[9,10] and it has been demonstrated that VEGFR2 is the primary receptor that mediates the angiogenic activity of VEGF via distinct signal transduction pathways that regulate endothelial cell proliferation, migration, differentiation, and tube formation.[2] Binding of VEGF to its major receptor VEGFR2 activates receptor tyrosine kinase (RTK), which is responsible for playing critical roles in angiogenesis. Furthermore, Tyr-1175 is the major autophosphorylation site on VEGFR2[2], and phosphorylation at this site is required for the activation of AKT, which is critically required for the subsequent stimulation of endothelial cell migration and proliferation.[11]

Angiogenesis is the process of forming blood vessels from preexisting vessels.[12] In response to VEGF (an angiogenic factor),

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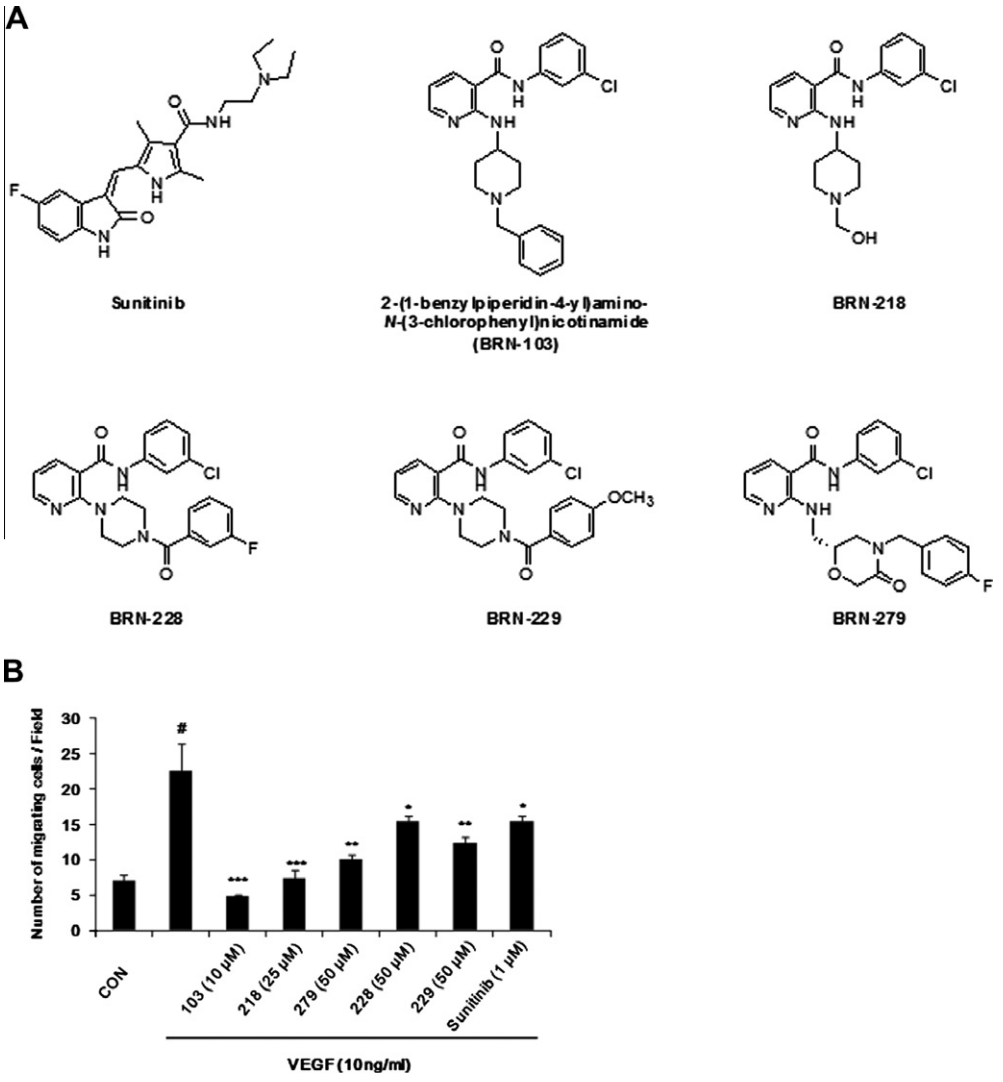
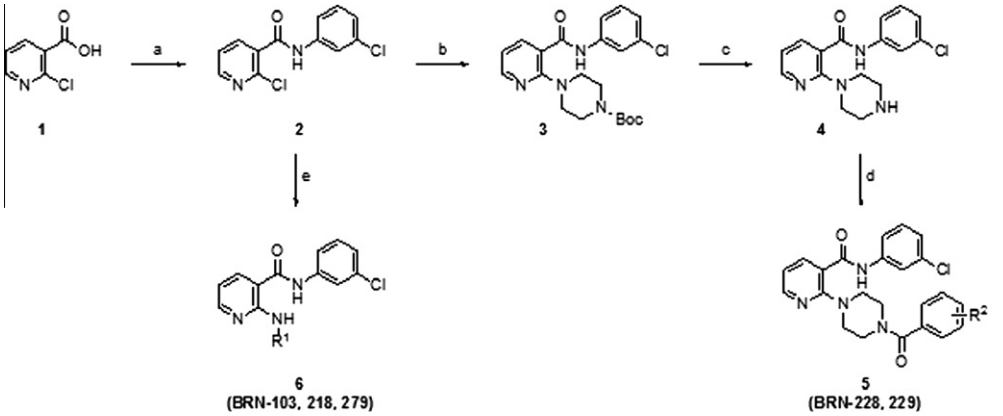


Figure 1. BRN compounds and the inhibition of VEGF-induced migration of HUVECs. (A) The chemical structures of the five BRN compounds examined; (B) Migration and the Transwell assay: HUVECs were cultured in a Boyden chamber with VEGF (10 ng/ml) in the lower chamber and various BRN compounds at concentrations less than their IC_{90} concentrations in the upper chamber. After 24 h, cellular migrations were determined by counting cells that had migrated through the pores. The bar graphs show the quantitative results of the average numbers of HUVECs that migrating through the membrane. Data are presented as means ± SD of the results of three independent experiments. $^{\#}p$ <0.05 versus nontreated control group, $^{*}p$ <0.05, $^{**}p$ <0.01, $^{***}p$ <0.001 versus VEGF-stimulated group; statistical significances were determined using ANOVA and Dunnett's post-hoc test.



Scheme 1. Reagents and conditions: (a) (i). $SOCl_2$, DMC, reflux, 2 h; (ii). Et_3N, DMC, 3-chloroaniline, reflux, 4 h, 81% (two-step yield); (b) 1-Bocpiperazine, K_2CO_3, xylene, 130 °C, 24 h, 89%; (c) CF_3COOH, EtOH, reflux, 3 h, 95%; (d) R^2-benzoyl chloride, Et_3N, DMC, rt, 4–6 h, 75–83%; (e) R^1-NH_2, K_2CO_3, xylene, 130 °C, 24 h, 67–92%.

endothelial cells migrate into the surrounding extracellular matrix where they form blood capillaries, and thus, cell migration is a crucial step in angiogenesis and invasion. In this study, five novel nicotinamide derivatives were preliminary screened using a Transwell migration assay, the most popular in vitro test of angiogenesis[13] Cells were seeded onto the upper surface of an 8 μm pore

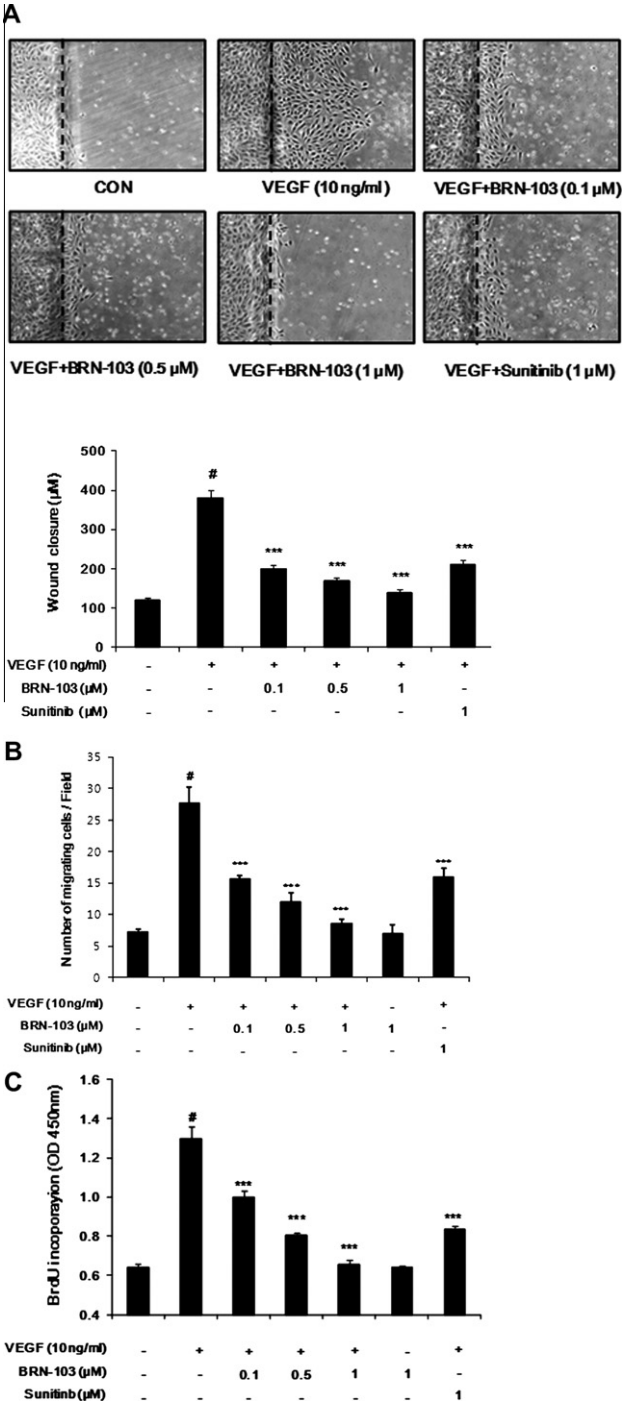


Figure 2. BRN-103 inhibited VEGF-induced migration and proliferation of HUVECs. (A) Representative photomicrographs of scratch wound healing assays. Using near confluent HUVECs in a 60 mm culture dish, scratched, and incubated in 0.1% EBM medium with 10 ng/mL VEGF for 24 h in the presence or absence of various concentrations (0.1, 0.5, 1 μM) of BRN-103 for 1 h. Representative photomicrographs of cells treated with VEGF and cells treated with VEGF together with BRN-103. Dotted lines, area occupied by the initial scraping. Columns show mean values of three different experiments and bars represent SD. $^{\#}p$ <0.05 versus nontreated control group, $^{***}p$ <0.001 versus VEGF-stimulated group; statistical significances were compared using ANOVA and Dunnett's post-hoc test; (B) Migration in the Transwell assay: Different concentrations (0.1, 0.5, 1 μM) of BRN-103 were treated and tested as described in Figure 1B. Columns represent the means of three different experiments, and bars represent SD. $^{\#}p$ <0.05 versus nontreated control group, $^{***}p$ <0.001 versus VEGF-stimulated group; statistical significances were compared using ANOVA and Dunnett's post-hoc test; (C) VEGF-induced cell proliferation was quantified using BrdU incorporation assays to quantify DNA synthesis in 0.1% serum-starved HUVECs in the presence or absence of BRN-103. Columns represent the means of three different experiments, and bars represent SD. $^{\#}p$ <0.05 versus nontreated control group, $^{***}p$ <0.001 versus VEGF-stimulated group; statistical significances were compared using ANOVA and Dunnett's post-hoc test.

size membrane separating upper and lower chambers. The upper chamber contained BRN-103, BRN-218, BRN-228, BRN-229, BRN-279, or sunitinib (as a positive control) in 0.1% endothelial basal medium (EBM), and cellular migration through the membrane was induced when VEGF was present in the lower chamber. As shown in Figure 1B, treatment of HUVECs with BRN compounds at concentrations less than their IC_{90} values (Supplementary Table 1) prevented cell migration. BRN-103 was found to have

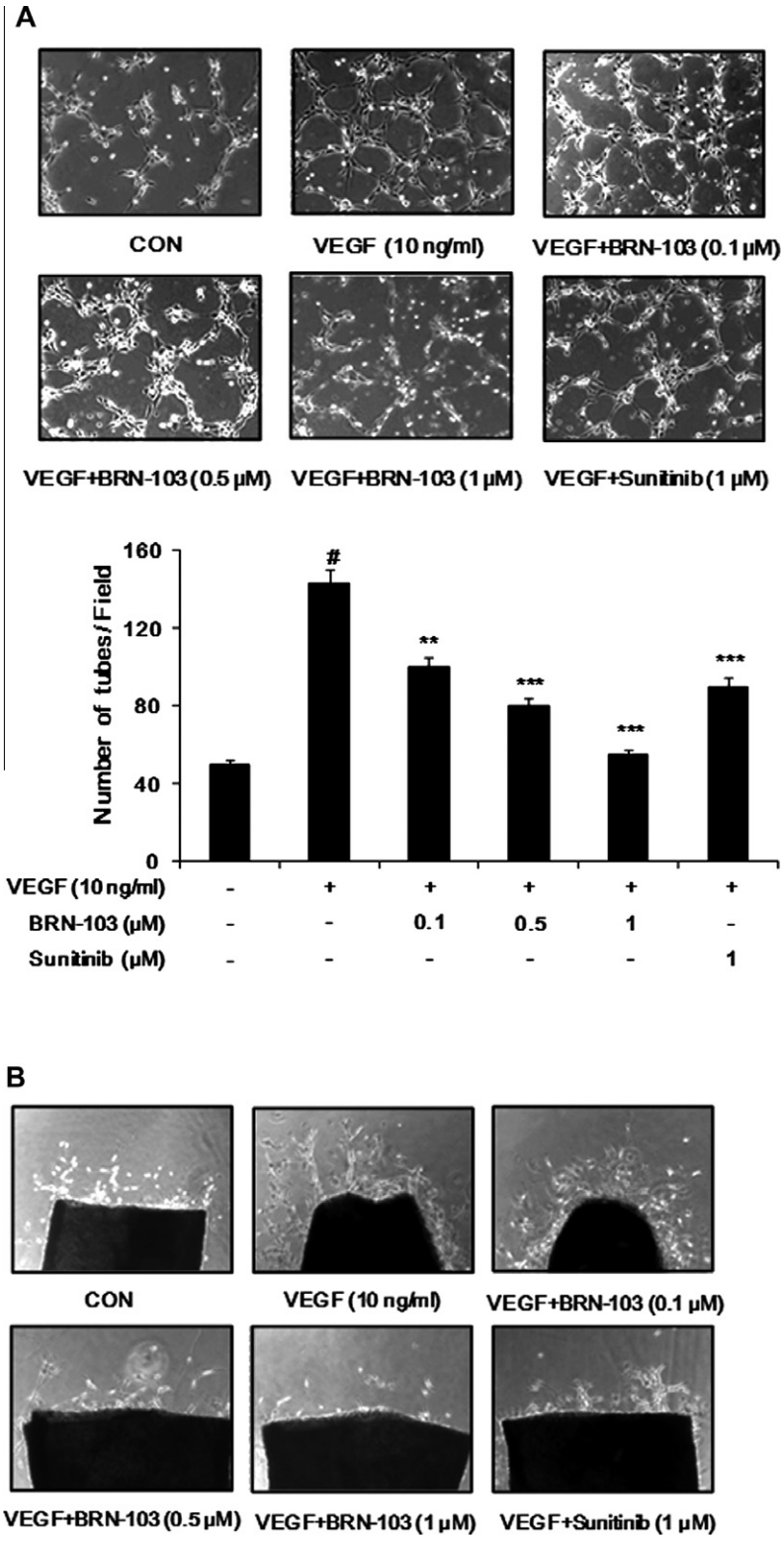


Figure 3. BRN-103 inhibited VEGF-induced capillary structure formation and microvessel sprouting ex vivo. (A) After being incubated with BRN-103, HUVECs were fixed, and tubular structures were photographed (magnification, ×100). Tube-like structures were quantified by manual counting in low power fields. Columns represent the means of three different experiments, and bars represent SD; (B) Aortic segments isolated fromC57BL/6 mice were placed in Matrigel-covered wells and treated with VEGF in the presence or absence of BRN-103. The data shown are representative of three independent experiments, and the photographs of microvessel sprouts from the margins of aortic rings are representative of three separate experiments.

the greatest inhibitory effect on this migration. To further assess the anti-migration activity of BRN-103, wound-healing and Transwell migration assays[13,14] were performed at BRN-103 concentrations of 0.1, 0.5, and 1 μM. As shown in Figure 2A and B, BRN-103 significantly and dose-dependently inhibited VEGF-induced HUVECs migration in both assays. Since angiogenesis requires the

proliferation of endothelial cells, we tested whether incubation with BRN-103 inhibits VEGF-induced HUVECs proliferation using the BrdU incorporation assay,[15] and it was found that BRN-103 significantly and concentration-dependently inhibited this proliferation by inhibiting DNA synthesis (Fig. 2C). Sunitinib was also found to significantly inhibit VEGF-induced cell migration and proliferation, but this was less than that exhibited by BRN-103.

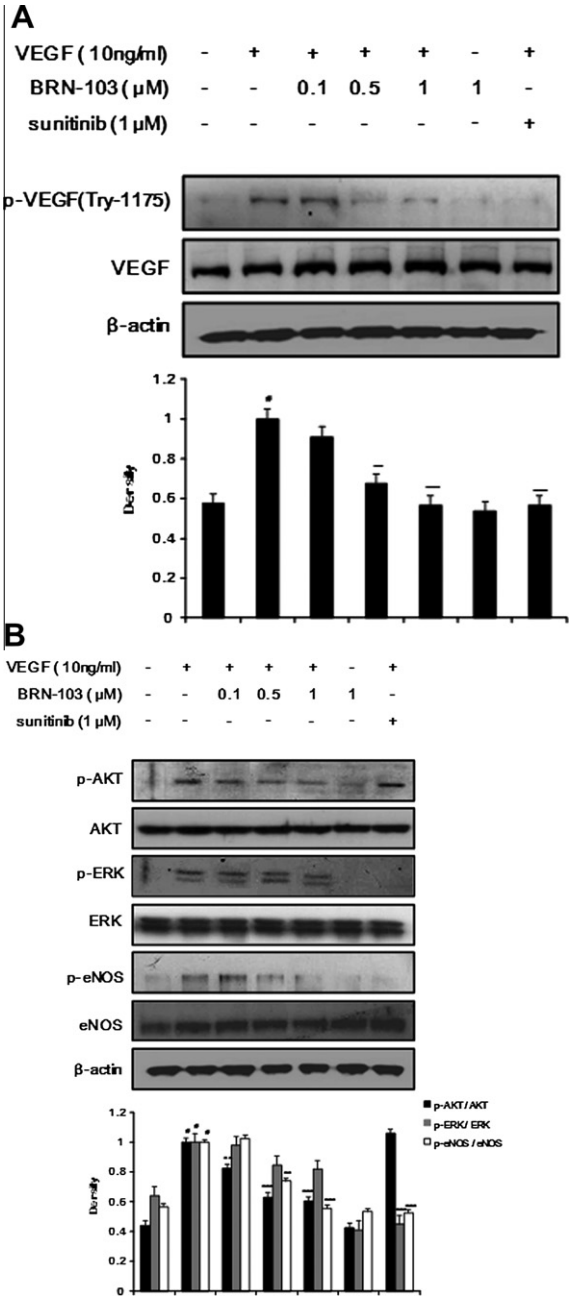


Figure 4. BRN-103 inhibited the VEGF-triggered activations of VEGFR2 and VEGFR2 signaling in HUVECs. (A) Cells were pretreated with different concentrations (0.1, 0.5, or 1 μM) of BRN-103 for 1 h, then treated with VEGF (10 ng/ml), and incubated for 5 min. Protein extracts were then harvested and subjected to Western blot analysis using specific anti-phosphorylated VEGFR2 antibody. VEGFR and β-actin were used as internal controls. The immunoblot shown is representative of three separate experiments; (B) 0.1% serum-starved HUVECs were pretreated with different concentrations (0.1, 0.5, or 1 μM) of BRN-103 for 1 h, then treated with VEGF (10 ng/ml), and incubated for 5 min (for *p*-ERK), 30 min (for *p*-AKT) and 1 h (*p*-eNOS). Protein extracts were then harvested and subjected to Western blot analysis using specific p-AKT, AKT, *p*-ERK, ERK, *p*-eNOS or eNOS antibodies. The immunoblot shown is representative of three separate experiments. Columns represent the means of three different experiments, and bars represent SD. $^{\#}p$ <0.05 versus nontreated control group, $^{**}p$ <0.01, $^{***}p$ <0.001 versus VEGF-stimulated group; statistical significances were compared using ANOVA and Dunnett's post-hoc test.

Although several types of cells participate in angiogenesis, tube formation by endothelial cells is a key step.[16] Therefore, we investigated how BRN-103 regulates capillary tube formation by HUVECs.[17] When HUVECs were seeded on growth factor-reduced two-dimensional Matrigel, robust tubular structures were formed in the presence of VEGF. However, preincubation with BRN-103 markedly and dose-dependently abolished this tube formation (Fig. 3A). To determine whether BRN-103 influences VEGF-induced angiogenesis ex vivo, the sprouting of vessels from mouse aortic rings[18] were examined in the presence or absence of BRN-103. It was found that VEGF significantly stimulated microvessel sprouting, which led to the formation of a network of vessels around the aortic rings (Fig. 3B), and that the addition of BRN-103 dose-dependently antagonized VEGF-induced sprouting.

The VEGF signaling pathway plays important roles in migration, proliferation, and tube formation by endothelial cells, which are all required for angiogenesis.[2,19] VEGF binds to its receptors, VEGFR1 and VEGFR2 (KDR/Flk), which has stronger tyrosine kinase activity than VEGFR1, and also transduces the major signals for vascular endothelial responses during angiogenesis.[20,21] The PI3K/AKT and RAF/MEK-ERK pathways have been demonstrated to regulate endothelial cell migration, proliferation, growth, and survival.[22,23] In particular, AKT regulates endothelial nitric oxide synthase (eNOS) activation,[24] which stimulates vasodilation, vascular remodeling, and angiogenesis,[25] whereas ERK (extracellular signal-related kinase) is required for the regulations of the endothelial cell cycle and endothelial proliferation, growth, migration, and apoptosis.[26] In addition, after being stimulated by extracellular growth factor, activated ERK is also required for eNOS activation.[22,27]

To elucidate the mechanism that underlies the anti-angiogenic effect of BRN-103, we examined signaling molecules and pathways using Western blotting assays. As shown in Figure 4A, the VEGF-induced phosphorylation of VEGFR2 at Tyr-1175 was suppressed dose-dependently by BRN-103 (Fig. 4A), suggesting that the anti-angiogenic properties of BRN-103 may be at least partially due to VEGR2 inhibition.. In addition, BRN-103 significantly and concentration-dependently suppressed the VEGF-triggered phosphorylations of AKT (Ser^{473}) and eNOS (Ser^{1172}), whereas it only mildly inhibited VEGF-induced phosphorylation of ERK (Thr^{202}/Tyr^{204}) in HUVECs. Therefore, we propose that BRN-103 inhibits angiogenesis by blocking the AKT and eNOS signaling pathways. In contrast, sunitinib inhibited the VEGF-mediated phosphorylations of VEGFR2, ERK and eNOS, but not the VEGF-mediated phosphorylation of AKT.

In conclusion, the novel nicotinamide derivative BRN-103 was found to inhibit endothelial cell migration, proliferation, tube formation, and capillary formation by interfering with the activation of the VEGF receptor and its downstream signaling of AKT, ERK, and eNOS. These results suggest that BRN-103 should be considered a potential lead compound for the development of novel anti-angiogenic drugs.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.09.022.

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