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A natural small molecule voacangine inhibits angiogenesis both *in vitro* and *in vivo*

Yonghyo Kim, Hye Jin Jung, Ho Jeong Kwon *

Chemical Genomics National Research Laboratory, Department of Biotechnology, Translational Research Center for Protein Function Control, College of Life Science & Biotechnology, Yonsei University, Seoul 120-749, Republic of Korea

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ABSTRACT

Angiogenesis, the formation of new blood vessels from pre-existing ones, plays a critical role in normal and pathological phenotypes, including solid tumor growth and metastasis. Accordingly, the development of new anti-angiogenic agents is considered an efficient strategy for the treatment of cancer and other human diseases linked with angiogenesis. We have identified voacangine, isolated from *Voacanga africana*, as a novel anti-angiogenic agent. Voacangine inhibits the proliferation of HUVECs at an IC_{50} of 18 μM with no cytotoxic effects. Voacangine significantly suppressed *in vitro* angiogenesis, such as VEGF-induced tube formation and chemoinvasion. Moreover, the compound inhibits *in vivo* angiogenesis in the chorioallantoic membrane at non-toxic doses. In addition, voacangine decreased the expression levels of hypoxia inducible factor-1α and its target gene, VEGF, in a dose-dependent manner. Taken together, these results suggest that the naturally occurring compound, voacangine, is a novel anti-angiogenic compound.

1. Introduction

Angiogenesis, the formation of new blood vessels from existing microvessels, is important in embryogenesis, wound healing, and tissue or organ regeneration [1,2]. However, pathological angiogenesis can lead to solid tumor growth and metastasis, diabetic retinopathy, and other diseases [3,4]. Accordingly, the inhibition of angiogenesis is considered a promising strategy for the treatment of cancer and other human diseases linked with angiogenesis [2,5].

Natural compounds have played a positive role in the advancement of new bioactive small molecules as leads for drug development [6]. Some natural compounds act as anti-viral, anti-bacterial, and anti-cancer agents. For instance, etoposide, a topoisomerase inhibitor derived from podophyllotoxin, a toxin found in the *Podophyllum peltatum*, prevents the re-ligation of DNA strands. Accordingly, it is used as a chemotherapeutic agent for the treatment of cancers such as Ewing's sarcoma, lung cancer, testicular cancer, lymphoma, non-lymphocytic leukemia, and glioblastoma multiforme [7].

As part of our continuous efforts to discover new anti-angiogenic agents from the natural plants, using cell-based screening, we screened 300 crude extracts of natural plants for their effects on HUVEC proliferation. We discovered that voacangine, a new natural small molecule, possesses anti-angiogenic properties. Voacangine (12-methoxyibogamine-18-carboxylic acid methyl ester), an indole alkaloid, was isolated from root bark of the *Voacanga africana* and *Tabernaemontana catharinensis* trees (Fig. 1A). A crude extract of *T. catharinensis*, which contained voacangine, was reported to be a potent anti-cancer agent [8]. Voacangine has also been shown to inhibit capsaicin contraction in a dose-dependent manner [9]. However, there have been no reports demonstrating the anti-angiogenic activity of the compound. Here, we report for the first time that voacangine is a new natural small molecule that inhibits angiogenesis *in vitro* and *in vivo* at a non-toxic dose.

2. Materials and methods

2.1. Materials

Voacangine (12-methoxyibogamine-18-carboxylic acid methyl ester) was purchased from THC Pharm (Frankfurt, Germany). Endothelial growth medium-2 (EGM-2) was purchased from Lonza (Walkersville, MD). RPMI 1640 and fetal bovine serum (FBS) were purchased from Invitrogen (Grand Island, NY). Vascular endothelial growth factor (VEGF), Matrigel and Transwell chamber systems were obtained from KOMA Biotech (Seoul, Korea), BD Bioscience (Bedford, MA) and Corning Costar (Corning, NY), respectively. Anti-HIF-1α, anti-cyclin D1 and anti-tubulin antibody were purchased from BD Bioscience, Cell Signaling (Beverly, MA) and Millipore (Billerica, MA), respectively.

Abbreviations: HUVECs, human umbilical vein endothelial cells; VEGF, vascular endothelial cell growth factor; HIF-1α, hypoxia inducible factor-1 alpha; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; CAM, chorioallantoic membrane.

* Corresponding author. Fax: +82 2 362 7265.
E-mail address: kwonhj@yonsei.ac.kr (H.J. Kwon).

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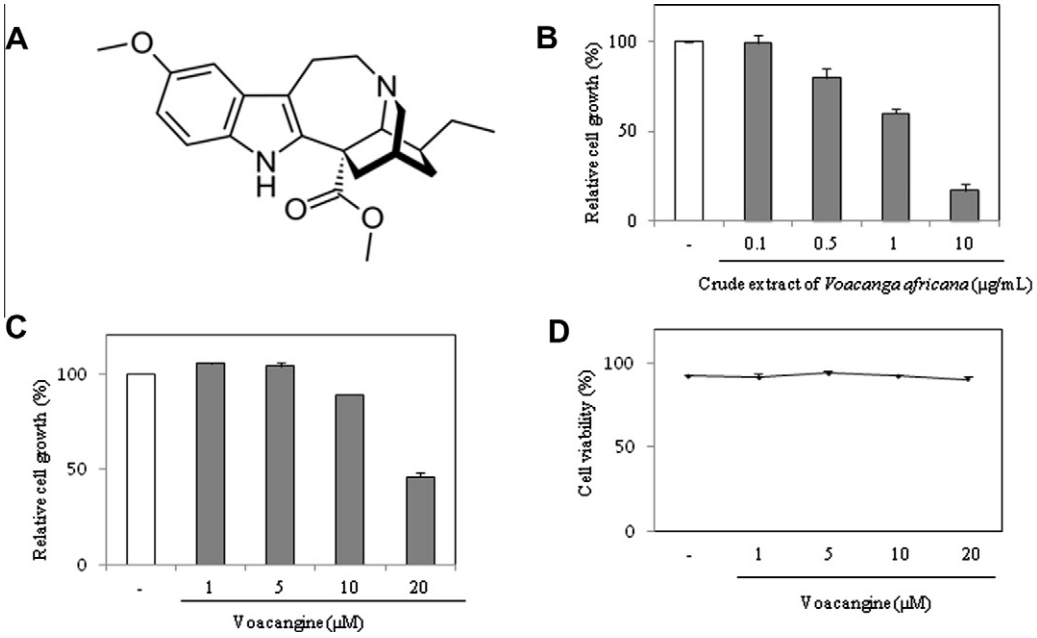

Fig. 1. Chemical structure and anti-proliferative activity of voacangine on HUVECs. (A) The chemical structure of voacangine ($C_{22}H_{28}N_2O_3$, MW 368.4). (B) The effect of crude extract of *Voacanga africana* on the proliferation of HUVECs. (C) The effect of voacangine on cell proliferation. HUVECs were treated with voacangine (1–20 μM) for 3 days, and cell growth was measured using the MTT colorimetric assay. (D) The effect of voacangine on cell viability. Cell viability was examined using the trypan blue assay.

2.2. Cell culture and proliferation assay

Human umbilical vascular endothelial cells (HUVECs) were grown for 7–11 passages in EGM-2 medium supplemented with 10% FBS. HepG2 (human liver carcinoma) cells were grown in RPMI 1640 containing 10% FBS and 1% antibiotics. All cell lines were maintained at 37 °C in a humidified 5% CO_2 incubator. Cell proliferation was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay, and cell viability was assessed using Trypan blue staining [10].

2.3. Capillary tube formation assay

Matrigel (10 mg/mL) was used to coat a 48-well plate and allowed to polymerize for 1 h at 37 °C. HUVECs (6×10^4 cells) were seeded on the surface of the Matrigel, and then test compounds were added for 4–16 h at 37 °C in the presence or absence of VEGF (30 ng/mL). Morphological changes in the cells and formation of tubular structures were observed under a microscope (IX71, Olympus) and photographed at 100× magnification (DP70, Olympus) [11].

2.4. Chemoinvasion assay

To examine the invasiveness of HUVECs *in vitro*, we used a Transwell chamber system with 8.0-μm pore polycarbonate filter inserts [12]. Briefly, the lower side of the filter was coated with gelatin (10 μL, 1 mg/mL) and the upper side was coated with Matrigel (10 μL, 3 mg/mL). Test compounds were added to the lower chamber in the presence of VEGF (30 ng/mL), and HUVECs (7×10^5 cells) were placed in the upper chamber of the filter. The chamber was incubated at 37 °C for 18 h, and then the cells were fixed with 70% methanol and stained with hematoxylin and eosin. The invasiveness of cells was measured by counting the number of whole cells in the lower side of the filter using a microscope at 100× magnification, and cells were photographed at 100× magnification.

2.5. Chorioallantoic membrane (CAM) assay

The CAM assay was performed as described previously [13]. Fertilized chicken eggs were kept in a humidified incubator at 37 °C for 3 days. Approximately 2–3 mL of egg albumin was removed with a hypodermic needle, allowing the CAM and yolk sac to drop away from the shell membrane. On day 5, a 2.5-cm diameter window was made with a razor and tweezers, and a compound-loaded Thermanox coverslip (NUNC, Rochester, NY) was applied to the CAM surface. After further incubation for 2 days, 2–3 mL of Intralipose (Greencross Co., Suwon, Korea) was injected beneath the CAM and the membrane was observed under a microscope. Retinoic acid (RA) was used as a positive control.

2.6. Western blot analysis and hypoxic conditions

The cell lysates were separated by 10% SDS–PAGE, followed by transfer to PVDF membranes (Millipore, Bedford, MA) using standard electroblotting procedures. Blots were then blocked and immunolabeled overnight at 4 °C with primary antibodies, including anti-HIF-1α and anti-tubulin antibodies. Immunolabeling was detected by an enhanced chemiluminescence (ECL) kit (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions. For hypoxic conditions, cells were incubated at 5% CO_2 with 1% O_2 balanced with N_2 in an anaerobic chamber (Forma).

2.7. Measurement of VEGF by ELISA

The VEGF concentration in media from voacangine-treated cells was determined using a VEGF Immunoassay kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. The results were expressed as concentration of VEGF relative to the total amount of VEGF from each well.

Table 1
IC_{50} values of voacangine on various cell lines.

	Normal cells		Cancer cells		
Cell lines	HUVECs	CHANG	HeLa	HT1080	HepG2
IC_{50} (μM)	18	26	23	33	42

2.8. In vitro tumor cell-induced angiogenesis assay (combinated-chemoinvasion assay)

To examine the invasive activity of HUVEC-induced tumor cells, a Transwell chamber system with 8.0-μm pore polycarbonate filter inserts was used. Briefly, the lower side of the filter was coated with gelatin (10 μL, 1 mg/mL) and the upper side was coated with Matrigel (10 μL, 3 mg/mL). Next, tumor cell HepG2 was added to the lower chamber. Test compounds were added to the lower chamber without VEGF and HUVECs (7×10^5 cells) were placed in the upper chamber of the filter. The chamber was incubated at 37 °C for 18 h, and then the cells were fixed with 70% methanol and stained with hematoxylin and eosin. Invasiveness was measured by counting the number of whole cells on the lower side of the filter using a microscope at 100× magnification, and cells were photographed at 100× magnification [14].

2.9. Statistical analysis

Results are expressed as means ± standard error (SE). Student's *t*-test was used to determine the statistical significance between control and test groups. A *p*-value less than 0.05 was considered statistically significant.

3. Results and discussion

3.1. Voacangine potently inhibits the proliferation of HUVECs

We found that the crude extract of *V. africana* inhibits the proliferation of HUVECs in a dose-dependent manner (Fig. 1B). Voacangine is a known principal component of this extract. Therefore, we investigated whether voacangine is responsible for the observed anti-proliferative activity of the extract. As shown in Fig. 1C, voacangine inhibited cell growth at 10 μM. Notably, it exerted a greater growth inhibition effect on HUVECs than on other normal and cancer cell lines (Table 1). To determine the optimum dose of voacangine without cytotoxic side effects, various concentrations of voacangine (1–20 μM) were applied to HUVECs, and cell viability was determined using the trypan blue exclusion method. Voacangine exhibited no cytotoxicity on HUVECs at doses up to 20 μM for 3 days. Accordingly, the following studies were performed using a concentration range of 10–20 μM (Fig. 1D).

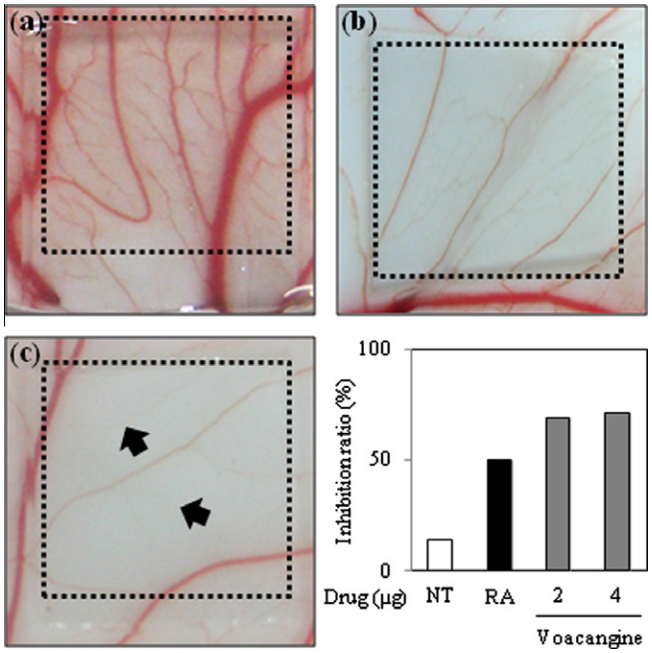

Fig. 3. Anti-angiogenesis activity of voacangine *in vivo*. (a) EtOH control, (b) RA (1 μg/egg), and (c) voacangine (4 μg/egg) were applied to the CAM, and the membrane was observed. (d) The inhibition ratio was calculated as the percentage of inhibited eggs to the total number of eggs tested. Arrows indicate inhibition of neovascularization of CAM by voacangine.

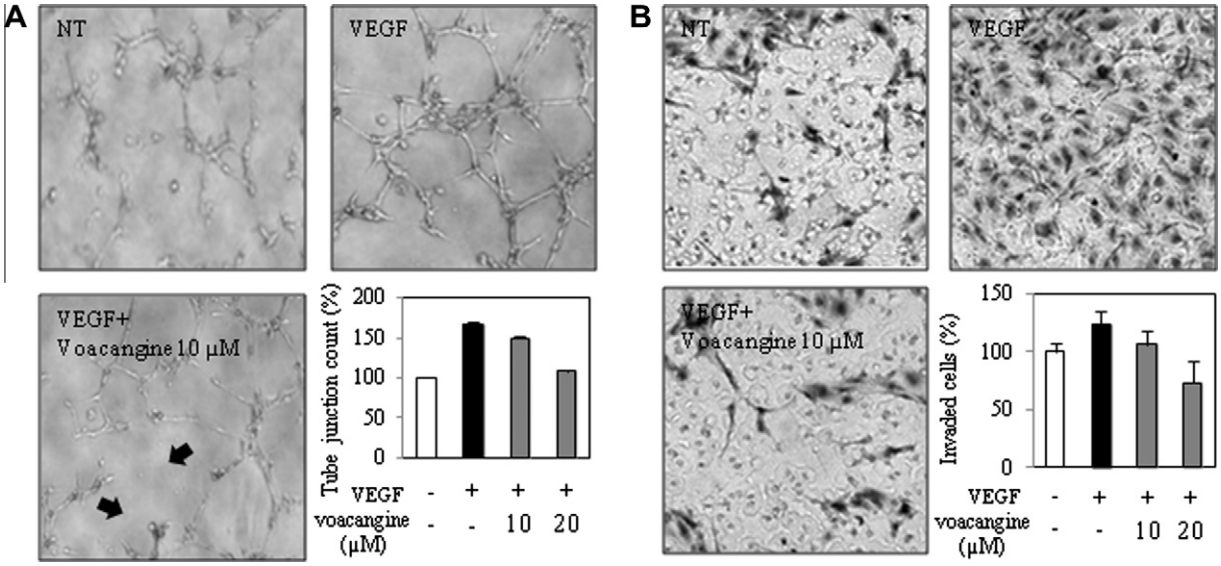

Fig. 2. Anti-angiogenic activity of voacangine *in vitro*. Serum-starved HUVECs were stimulated by VEGF (30 ng/mL) in the presence or absence of voacangine. (A) Effect of voacangine on the tube forming ability of HUVECs. Arrows indicates broken tubes formed by VEGF-stimulated HUVECs. (B) Inhibitory activity of voacangine on endothelial cell invasion. The basal level capillary tube formation (A) and invasiveness (B) of HUVECs that remained in serum-free media were normalized to 100%.

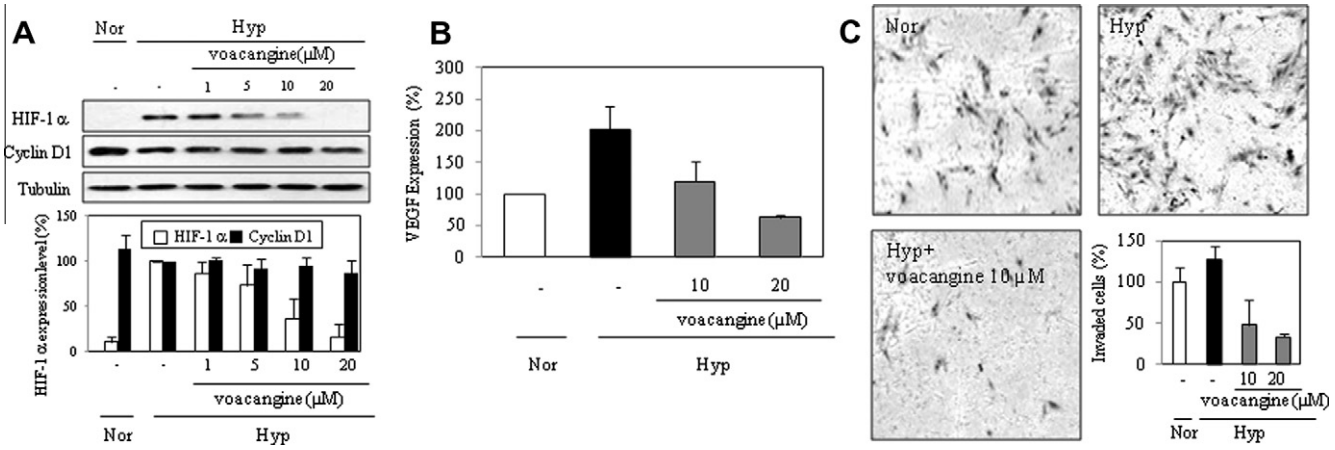

Fig. 4. Effect of voacangine on the expression of angiogenic factors. (A) The expression level of HIF-1α and cyclin D1 were detected by Western blot. The level of tubulin was used as an internal control. (B) The expression level of VEGF protein in HepG2 cells was determined by a VEGF immunoassay. (C) Tumor cell-induced angiogenesis. HUVECs were seeded in the upper chamber, and HepG2 was added to the lower chamber without VEGF. *Nor*, Normoxia; *Hyp*, Hypoxia.

3.2. Voacangine showed anti-angiogenic activity in vitro and in vivo

We next investigated the effect of voacangine on the angiogenic phenotypes of HUVECs *in vitro*, such as tube formation and chemoinvasion. Serum-starved HUVECs were stimulated by VEGF with or without voacangine. As shown Fig 2A, voacangine inhibited VEGF-induced tube formation in a dose-dependent manner with no cytotoxic effects. The effect of voacangine on the invasive activity of HUVECs induced by VEGF was also investigated. Voacangine inhibited the VEGF-induced enhanced invasiveness of HUVECs in a dose-dependent manner (Fig. 2B). These data indicate that voacangine effectively inhibits VEGF-induced angiogenesis *in vitro*.

The anti-angiogenic activity of voacangine was further validated *in vivo* by using a chick embryo chorioallantoic membrane (CAM) assay. After treatment with voacangine for 2 days, the CAM was observed under a microscope. Normally, developed CAMs exhibit an extensive capillary network. However, voacangine dose-dependently inhibited capillary formation during CAM development with no apparent signs of thrombosis or hemorrhage (Fig. 3). These results demonstrate that voacangine potently inhibits angiogenesis both *in vitro* and *in vivo* without cytotoxic effects.

3.3. Voacangine inhibits tumor cell-induced angiogenesis

Hypoxia-inducible factor-1α (HIF-1α) plays a key role in tumor angiogenesis by regulating the expression of angiogenic factors, including VEGF [15]. HIF-1α overexpression has been implicated in many human cancers. Thus, we examined the effect of voacangine on HIF-1α expression levels under hypoxic conditions. The expression level of HIF-1α in human hepatocellular carcinoma (HepG2) cells during hypoxia was dose-dependently reduced by voacangine without inhibiting the synthesis of other proteins related to the cell cycle (cyclin D1) and cytoskeleton (tubulin) (Fig. 4A).

As the result of reduced HIF-1α expression, voacangine treatment inhibited the hypoxia-induced expression of VEGF, a HIF-1α target gene, in a dose-dependent manner (Fig. 4B). Moreover, tumor cell-induced invasiveness of HUVECs by hypoxia was dose-dependently inhibited by voacangine (Fig. 4C). These results demonstrate that voacangine potently inhibits tumor cell-induced angiogenesis through suppression of the angiogenic factor, HIF-1α, with no observed cytotoxic effects.

In this study, our results clearly demonstrate that voacangine, an active principal component of *V. africana* extract, exhibits anti-angiogenic activity *in vitro* and *in vivo*. In HUVECs, the expression levels of HIF-1α and its target gene, VEGF, were dose-dependently suppressed by voacangine. In addition, voacangine inhibited tumor cell-induced invasiveness in a dose-dependent manner. Overall, these results suggest that this compound might provide the basis for the development of novel anti-angiogenic agents. It is noteworthy that voacangine effectively suppresses VEGF- and hypoxia-induced angiogenesis at lower doses than are necessary to inhibit HUVEC growth, suggesting that the compound may specifically perturb angiogenic signaling pathways. Moreover, the unique chemical structure of voacangine (with an iboga alkaloid as a core moiety) may provide new insights into the mechanisms underlying angiogenesis signaling pathways. Further investigation identifying and validating the targets of the small, naturally occurring molecule voacangine will help to decipher the interesting anti-angiogenic mechanisms of the compound and open a new gate into angiogenesis biology.

Acknowledgments

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