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Identification and biological activities of a new antiangiogenic small molecule that suppresses mitochondrial reactive oxygen species

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ABSTRACT

Mitochondrial reactive oxygen species (ROS) are associated with multiple cellular functions such as cell proliferation, differentiation, and apoptosis. In particular, high levels of mitochondrial ROS in hypoxic cells regulate many angiogenesis-related diseases, including cancer and ischemic disorders. Here we report a new angiogenesis inhibitor, YCG063, which suppressed mitochondrial ROS generation in a phenotypic cell-based screening of a small molecule-focused library with an ArrayScan HCS reader. YCG063 suppressed mitochondrial ROS generation under a hypoxic condition in a dose-dependent manner, leading to the inhibition of *in vitro* angiogenic tube formation and chemoinvasion as well as *in vivo* angiogenesis of the chorioallantoic membrane (CAM) at non-toxic doses. In addition, YCG063 decreased the expression levels of HIF-1α and its target gene, VEGF. Collectively, a new antiangiogenic small molecule that suppresses mitochondrial ROS was identified. This new small molecule tool will provide a basis for a better understanding of angiogenesis driven under hypoxic conditions.

1. Introduction

During mitochondrial respiration, mitochondrial electron transport chain (ETC) generates ROS [1]. Mitochondrial ROS, produced mainly at complex III of mitochondrial ETC, might be required for the hypoxic stabilization of HIF-α through iron-mediated inhibition of PHD [2]. Terpestacin and stigmatellin, inhibitors of complex III, are known to block the generation of mitochondrial ROS and attenuate the expression level of HIF-1α [3,4]. As HIF-α plays a key role in angiogenesis by regulating the gene expression of angiogenic factors [5,6], small molecules suppressing mitochondrial ROS production could be new agents for treating angiogenesis-related diseases.

Phenotypic cell-based assays are powerful tools to obtain information regarding molecular events occurring in the living cellular environments. To identify new antiangiogenic small molecules that suppress mitochondrial ROS production, a simple cell-based assay that exposes cells to hypoxia and monitors mitochondrial ROS generation with a fluorescence probe, a DCFH-DA in this case, was constructed. In addition, the ArrayScan HCS reader was utilized to facilitate automated image acquisition and data analysis, which provide complex sets of data from single cells [7]. Indeed, the ArrayScan HCS reader has been actively used to analyze high-content information concerning changes in the cell cycle, gene expression, cell motility, receptor internalization, and in the trafficking of intracellular components [8].

In the present study, a new antiangiogenic small molecule that suppresses mitochondrial ROS generation was identified in a phenotypic cell-based assay under hypoxia with systemic analysis of high-content information in the cells using the ArrayScan HCS reader. We found that a new small molecule, YCG063, suppresses mitochondrial ROS under hypoxia, leading to the inhibition of angiogenesis. These results demonstrate that mitochondrial ROS plays a crucial role in regulating HIF-α and its resulting biological phenotype, angiogenesis. The unique structure of YCG063 differs from those of other known antiangiogenic small molecules, and its biological activity during mitochondrial ROS generation under hypoxia will provide better insight into the role of mitochondrial ROS in angiogenesis and serve as a basis for the development of new antiangiogenic agents.

2. Materials and methods

2.1. Reagents

Endothelial growth medium-2 (EGM-2) was purchased from Lonza (Walkersville, MD). Dulbecco's modified Eagle medium (DMEM), RPMI 1640, and fetal bovine serum (FBS) were purchased from Invitrogen (Grand Island, NY). DCFH-DA and Hoechst 33342 were purchased from Invitrogen (Grand Island, NY). Vascular endothelial growth factor (VEGF), Matrigel and Transwell chamber

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systems were obtained from KOMA Biotech. (Seoul, Korea), BD Bioscience (Bedford, MA) and Corning Costar (Corning, NY). Anti-HIF-1α, anti-cyclin D1 and anti-tubulin antibody were purchased from BD Bioscience (Bedford, MA), Cell Signaling (Berverly, MA) and Millipore (Bilerica, MA), respectively. A focused small molecule library that could target mitochondria electron transport chain (ETC) was purchased from Equispharm (Suwon, Korea).

2.2. Cell culture and hypoxic conditions

Human umbilical vascular endothelial cells (HUVECs) were grown for 7–11 passages in EGM-2 medium supplemented with 10% FBS. HeLa (human cervical carcinoma) cells were grown in DMEM with 10% FBS and 1% antibiotics. HCT15 (human colon carcinoma) cells and HepG2 (human river 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. For hypoxic conditions, cells were incubated at a CO_2 level of 5% with 1% O_2 balanced with N_2 in an anaerobic chamber (Forma).

2.3. Cell growth and viability assay

Cell growth was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay and cell viability assay was assessed using trypan blue staining [9].

2.4. Measurement of mitochondrial ROS levels

Mitochondrial ROS levels were assayed using DCFH-DA. After incubation with DCFH-DA (10 μM) and Hoechst 33342 for 10 min, the cells were washed once with washing buffer and fixed with 4% formaldehyde. Cell image and data of the cells were automatically obtained using the ArrayScan V^{TI} HCS reader, produced by Cellomics, Inc. (Pittsburgh, PA). For these experiments, the Compartmental Analysis BioApplication used the Hoechst-labeled nuclei to identify individual cells and then automatically analyzed the DCF fluorescence intensity changes with each cell.

2.5. Capillary tube formation assay

Matrigel (150 μL, 10 mg/mL) was coated in a 48-well plate and allowed to polymerize for 1 h at 37 °C. The HUVECs (6×10^4 cells) were seeded on the surface of the Matrigel, and treated with VEGF (50 ng/mL). Then small molecules were added for 3–16 h at 37 °C. Morphological changes of cells and tube formations were observed under a microscope (IX71, Olympus) and photographed at 100× magnification (DP70, Olympus) [10].

2.6. Chemoinvasion assay

The invasiveness of HUVECs was examined *in vitro* using a Transwell chamber system with 8.0-μm pore polycarbonate filter inserts [11]. 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). YCG063 were added to the lower chamber in the presence of VEGF (50 ng/mL) and HUVECs (7×10^5 cells) were placed in the upper chamber of the filter. The invasiveness of cells, fixed with 70% ethanol and stained with hematoxylin/eosin, was measured by counting the total number of whole cells in the lower side of the filter using a microscope at 100× magnification.

2.7. Chorioallantoic membrane (CAM) assay

The CAM assay was performed as using a method previously described in the literature [12]. Fertilized chick eggs were kept in a humidified incubator at 37 °C for 4 days. Approximately 4–5 mL of egg albumin were removed with a hypodermic needle, allowing the CAM and yolk sac to drop away from the shell membrane. On day 5, YCG063-loaded Thermanox coverslip (NUNC, Rochester, NY) were applied to the CAM surface. Two days later, 1 mL of Intralipose (Greencross Co., Suwon, Korea) was injected beneath the CAM, and the membrane was observed under a microscope. Because retinoic acid (RA) is a known antiangiogenic compound, it was used as a positive control.

2.8. Measurement of VEGF by ELISA

The VEGF concentration in media from YCG063-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 [13].

2.9. Western blot analysis

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, anti-cyclinD1 and anti-tubulin antibodies. Immunolabeling was detected by an enhanced chemiluminescence (ECL) kit (Amersham Life Science, Inc., Buckinghamshire, UK) according to the manufacturer's instructions.

2.10. 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. Cell-based screening for small molecules that suppress mitochondrial ROS generation

To identify new small molecules that affect mitochondrial ROS generation during hypoxia, a focused small molecule library consisting of 100 compounds that could target the mitochondrial ETC was applied to the cells under hypoxia [3]. Hypoxia-induced ROS generation in HepG2 was monitored using DCFH-DA and analyzed with the ArrayScan V^{TI} HCS reader (Fig. 1A). Two compounds were identified as hit candidates that decreased DCF fluorescence intensity by more than 40%. Then, these compounds were subjected to an *in vitro* tube formation assay to investigate their antiangiogenic effects (Fig. 1B). From these candidates, YCG063 strongly inhibited tube formation and exhibited a dose-dependent inhibitory effect on mitochondrial ROS generation (Fig. 1C and D). Accordingly, YCG063 was identified as a potent inhibitor of angiogenesis that suppresses mitochondrial ROS generation and was subjected to further investigation of its antiangiogenic activities.

3.2. YCG063 is a potent growth inhibitor of HUVECs

To investigate the antiangiogenic properties of YCG063, we first examined the growth inhibitory activity of YCG063 in various cell lines. HUVECs, HeLa cells, and HCT15 cells were treated with various concentrations of YCG063 for 3 days, and cell growth was assessed by the MTT colorimetric assay. Notably, YCG063 exhibited the most potent cell growth inhibition in HUVECs (Fig. 2A). Next, the cytotoxic effect of YCG063 in HUVECs was examined in a viability assay. As shown in Fig. 2B, the viability of HUVECs was

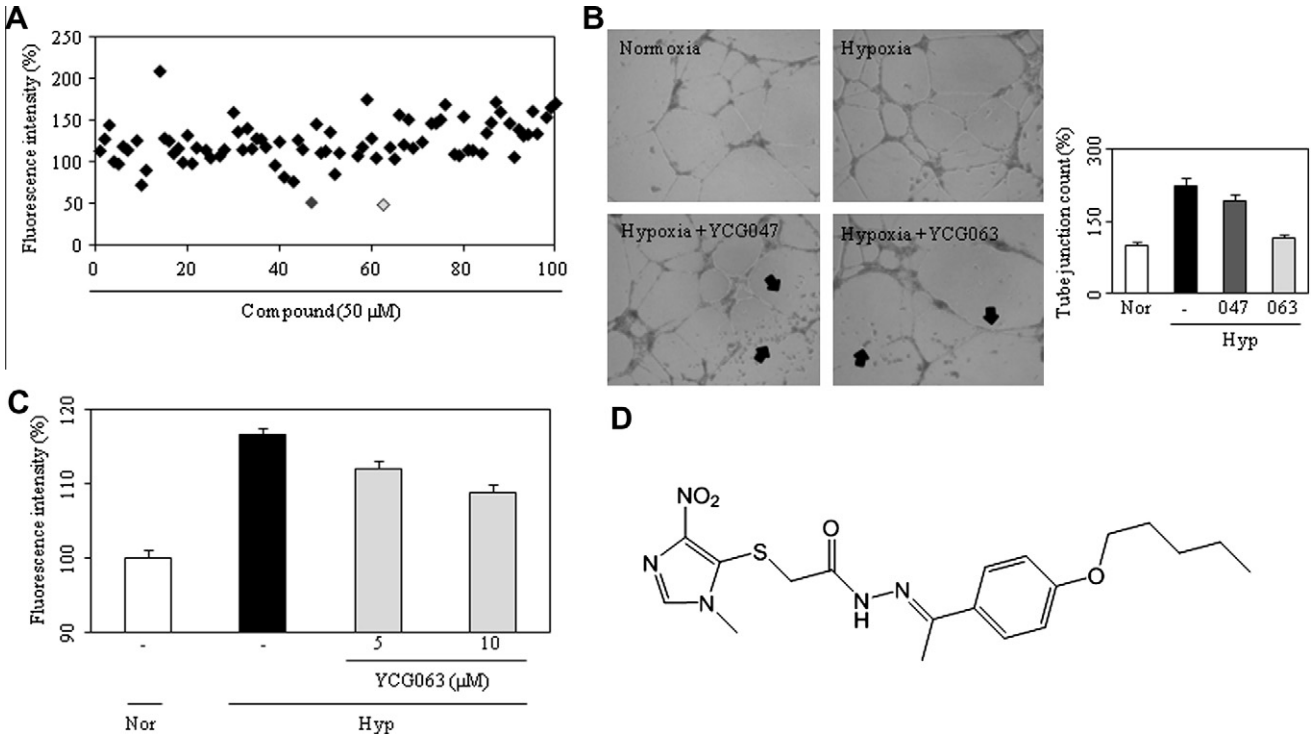

Fig. 1. Screening and chemical structure of YCG063: (A) Cell-based screening using DCFH-DA. ArrayScan V^{TI} HCS was used for quantitative analysis. HepG2 cells were pretreated with small compounds (50 μM) for 30 min and then exposed to 1% O_2 for 2 h. (B) The anti-angiogenic effect of small molecules (10 μM) on tube formation. Nor, normoxia; Hyp, hypoxia. (C) Inhibitory activity of YCG063 on mitochondrial ROS that was measured using DCFH-DA. (D) Chemical structure of YCG063.

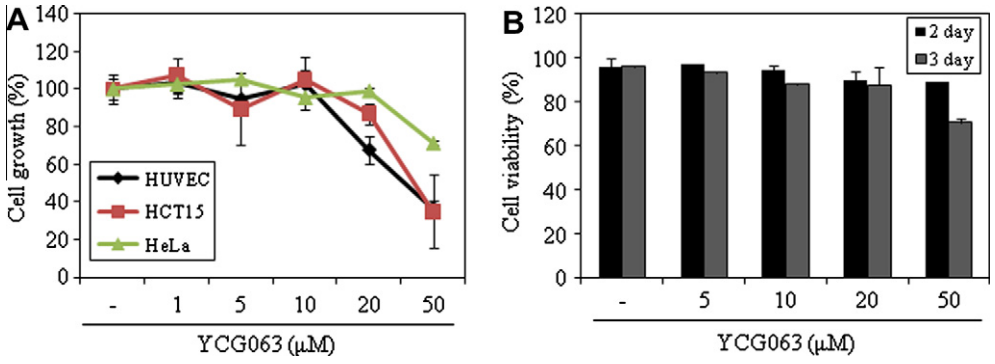

Fig. 2. Effect of YCG063 on the cell growth and viability: (A) Various concentrations of YCG063 (0–50 μM) was treated for 3 days and cell growth was measured using the MTT assay. (B) Cell viability was examined using a trypan blue assay. YCG063 exhibited no toxicity on HUVECs at concentrations up to 20 μM.

not affected by treatment with YCG063 up to a concentration of 20 μM for 3 days. Accordingly, the following studies were performed using a concentration range of 1–10 μM, which was not toxic to these cells.

3.3. YCG063 inhibits in vitro and in vivo angiogenesis

Next, we explored the activity of YCG063 on the angiogenic phenotypes of HUVECs *in vitro*, such as tube formation and chemoinvasion. Serum-starved HUVECs were stimulated by VEGF with or without YCG063. As shown in Fig. 3A, YCG063 inhibited VEGF-induced tube formation in a dose-dependent manner without producing any toxicity. Then, the effect of YCG063 on the invasive activity of HUVECs by VEGF was investigated. While VEGF-induced HUVECs exhibited enhanced invasiveness, YCG063 inhibited VEGF-induced invasiveness in a dose-dependent manner (Fig. 3B). These findings demonstrate that YCG063 effectively inhibits VEGF-induced *in vitro* angiogenesis.

The antiangiogenic activity of YCG063 was further validated *in vivo* by using the chick embryo CAM assay. Normally developed CAMs exhibited extensive networks of capillaries. However, YCG063-treated CAM exhibited inhibited capillary formation during CAM development without any sign of thrombosis and hemorrhage (Fig. 3C). These results demonstrate that YCG063 potently inhibits angiogenesis both *in vitro* and *in vivo* without producing any toxicity.

3.4. YCG063 attenuates the expression levels of HIF-1α and its target gene, VEGF

To address the antiangiogenic activities of YCG063, the effects of YCG063 on the expression levels of angiogenic factors were investigated. To do this, we conducted a Western blot analysis to examine the effect of YCG063 on the HIF-1α expression levels under hypoxia. The expression level of HIF-1α in HepG2 was dose-dependently reduced by YCG063 without affecting the other cell

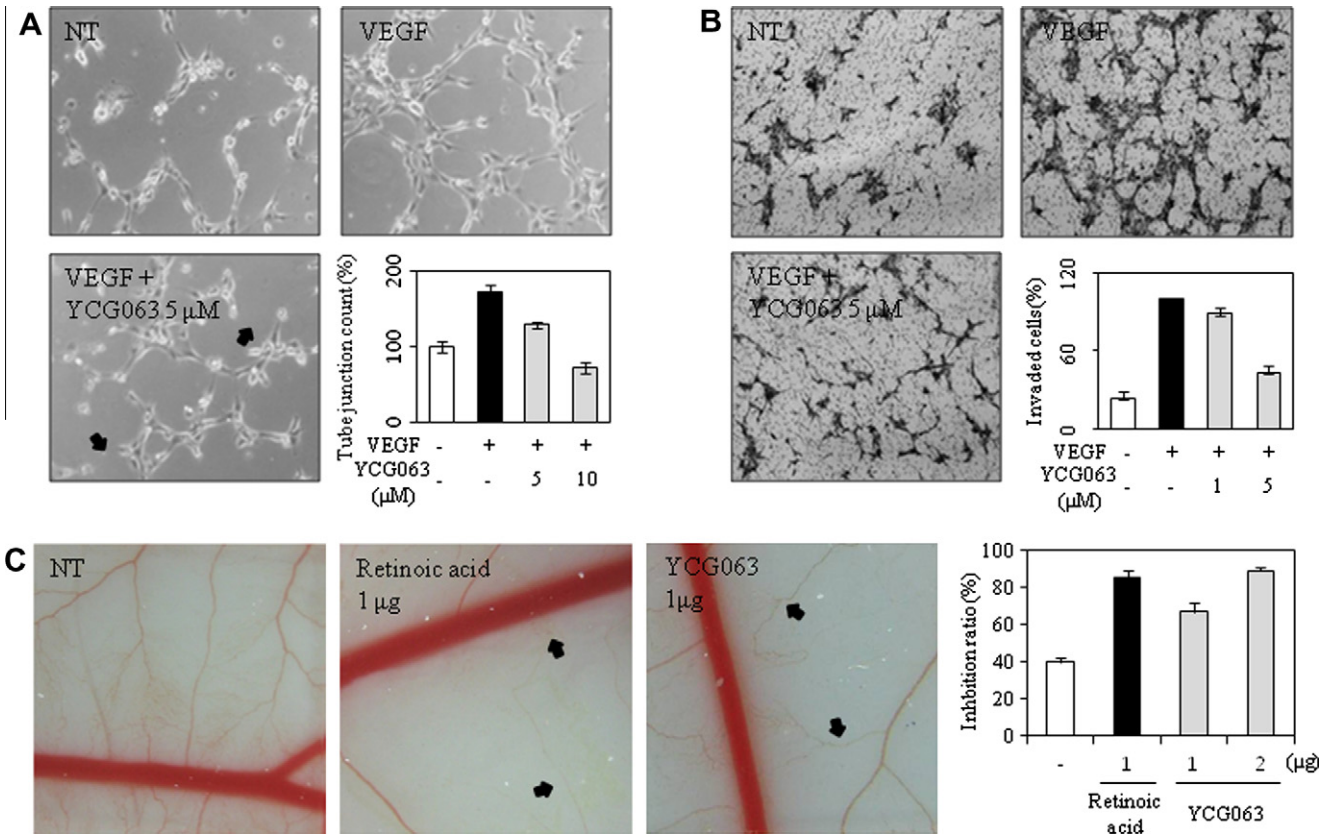

Fig. 3. Anti-angiogenic activities of YCG063 both *in vitro* and *in vivo*: (A) Tube formation assay: YCG063 inhibited tube formation induced by VEGF. Arrows indicate broken tubes formed by VEGF-stimulated HUVECs. (B) Chemoinvasion assay: YCG063 inhibited VEGF-induced invasion of HUVECs in a dose-dependent manner. (C) CAM assay: YCG063 and retinoic acid (RA) were applied to the CAM and the neovessel formation was observed.

cycle (cyclin D1) and cytoskeleton (tubulin) related protein synthesis (Fig. 4A). Suberoylanilide hydroxamic acid (SAHA), a histone deacetylase (HDAC) inhibitor, was used as a positive control [14].

In addition, the effect of YCG063 on VEGF expression level was also investigated. As the consequence of HIF-1α reduction by the compound, YCG063 treatment blocks VEGF expression induced by hypoxia in a dose-dependent manner (Fig. 4B).

Inhibition of angiogenesis is a promising strategy for the treatment of the cancer growth and metastasis, as well as other angiogenesis-related diseases. In this study, simple and effective phenotypic cell based assay with hypoxic setting and focused small molecule library targeting mitochondria ETC were designed to identify new antiangiogenic small molecules. As a result, a new antiangiogenic small molecule, YCG063, suppressing mitochon-

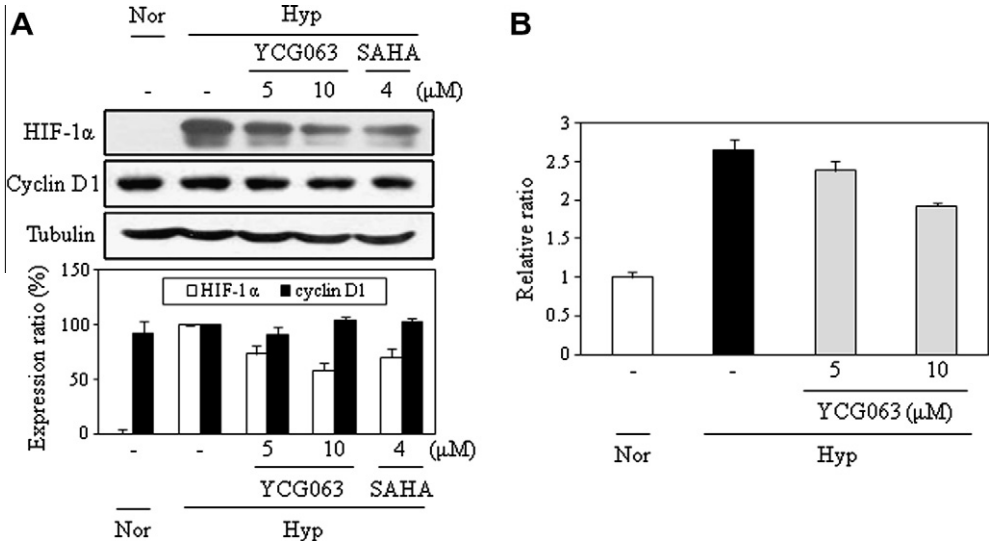

Fig. 4. Effect of YCG063 on the expression of angiogenic factors: (A) The expression level of HIF-1α and cyclin D1 were detected by Western blot. The level of tubulin was used as an internal control. (B) The relative expression level of VEGF protein in HepG2 cells was determined by a VEGF immunoassay. Results shown are representative of three independent experiments.

drial ROS was successfully identified. Our data demonstrated that YCG063 efficiently inhibited hypoxia- and VEGF-induced angiogenesis in a dose-dependent manner. We also found that YCG063 attenuated expression levels of HIF-1α and its target gene, VEGF. Following investigations on target protein identification of YCG063 and its role in hypoxia induced angiogenesis will provide new insights into the role of mitochondria ROS in angiogenesis. In conclusion, YCG063 is a new angiogenesis inhibitor targeting mitochondrial ROS and could provide a new molecular scaffold that might be useful in the development of therapeutic reagents related to angiogenesis.

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