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A new anti-angiogenic small molecule, G0811, inhibits angiogenesis via targeting hypoxia inducible factor (HIF)-1α signal transduction

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ABSTRACT

Regulation of hypoxia inducible factor (HIF)-1α stabilization, which in turn contributes to adaptation of tumor cells to hypoxia has been highlighted as a promising therapeutic target in angiogenesis-related diseases. We have identified a new small molecule, G0811, as a potent angiogenesis inhibitor that targets HIF-1α signal transduction. G0811 suppressed HIF-1α stability in cancer cells and inhibited *in vitro* and *in vivo* angiogenesis, as validated by tube formation, chemoinvasion, and chorioallantoic membrane (CAM) assays. In addition, G0811 effectively decreased the expression of vascular endothelial growth factor (VEGF), which is one of target genes of HIF-1α. However, G0811 did not exhibit anti-proliferative activities or toxicity in human umbilical vein endothelial cells (HUVECs) at effective doses. These results demonstrate that G0811 could be a new angiogenesis inhibitor that acts by targeting HIF-1α signal transduction pathway.

1. Introduction

Hypoxia inducible factor (HIF) is a transcription factor that adapts cells to hypoxic conditions and is a key regulator for the induction of genes that participate in angiogenesis, glucose metabolism, and cell proliferation/survival [1,2]. This protein is a heterodimer comprised of an oxygen-regulated HIF-α subunit and a constitutively expressed and stable HIF-β subunit. There are three known members of the HIF-α family; HIF-1α, HIF-2α, and HIF-3α. HIF-1α was the first to be cloned and is the best characterized isoform that is involved in cancer biology, including angiogenesis [3]. Therefore, there has been extensive interest in the development of bioactive small molecules that regulate HIF-1α function.

Under normoxic conditions, prolyl hydroxylase (PHD) hydroxylates two prolyl residues (P402 and/or P564) in the HIF-1α isoform, which are required for binding with the von Hippel–Lindau (VHL) tumor suppressor gene for proteasomal degradation [4,5]. During hypoxia, however, PHD activity is attenuated, leading to HIF-1α protein stabilization [6,7]. Of the many factors related to HIF-1α stability, mitochondrial reactive oxygen species (mROS) inhibitors, protein synthesis regulators, and other signaling molecules, such as nitric oxide, prevent hypoxia-induced HIF-1α stabilization [8–11]. Therefore, identifying new small molecules that regulate HIF-1α stability could be a promising strategy to control hypoxia-induced HIF-1α activity.

Herein, we screened 200 druggable synthetic small molecules for their effects on suppression of HIF-1α stability under hypoxic conditions and found G0811 (Fig. 1A) to be a potent hit compound. Furthermore, we found that G0811 exhibits anti-angiogenic activity by suppressing mROS generation and protein synthesis pathway under hypoxic conditions. These results imply that G0811 could be the basis for developing new drug therapies targeting HIF-1α signal transduction.

2. Materials and methods

2.1. Materials

The synthetic small molecule G0811 was purchased from Chemdiv (San Diego, CA). Endothelial basal media-2 (EBM-2) was purchased from Cambrex Bio Science (Walkersville Inc., MD). Dulbecco's modified Eagle's medium (DMEM), Roswell Park Memorial Institute (RPMI) 1640, fetal bovine serum (FBS), a dichlorodihydro-fluorescein diacetate (DCFH-DA), and Hoechst 33342 were purchased from Life Technologies (Grand Island, NY). Transwell plates, recombinant human vascular endothelial cell growth factor (VEGF), and Matrigel were obtained from Corning Stars (Cambridge, MA), KOMA Biotech Inc. (Seoul, Korea), and BD Bioscience (Bedford, MA), respectively. Anti-HIF-1α and anti-tubulin antibody were purchased from BD Bioscience (Bedford, MA) and Millipore (Billerica, MA), respectively.

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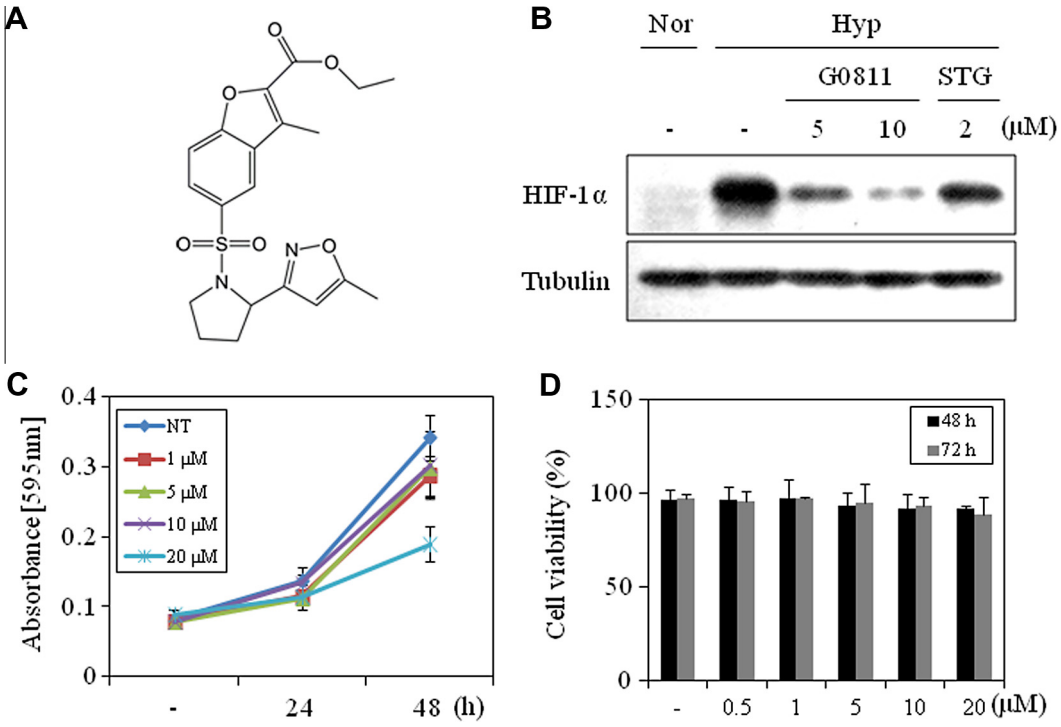

Fig. 1. Chemical structure and biological activities of G0811. (A) The chemical structure of G0811 ($C_{20}H_{22}N_2O_6S$, MW 418.46). (B) The expression level of HIF-1α was detected by western blot. HepG2 cells were pretreated with G0811 (5–10 μM) or stigmatellin (*STG*, 2 μM) for 1 h and then exposed to 1% O_2 for 4 h. *Nor*, normoxia; *Hyp*, hypoxia. (C) The effect of G0811 on cell proliferation. HUVECs were treated with G0811 (0.5–20 μM) for 2 days, and cell growth was measured using the MTT colorimetric assay. (D) The effect of G0811 on cell viability. Cell viability was examined using the trypan blue assay.

2.2. Cell culture and hypoxic conditions

Human umbilical venous endothelial cells (HUVECs) were grown for 4–10 passages in EBM-2 medium supplemented with 10% FBS. Human liver carcinoma (HepG2) and human cervical carcinoma (HeLa) cells were grown in DMEM and RPMI 1640 containing 10% FBS and 1% antibiotics, respectively. All cell lines were maintained at 37 °C in an incubator under a humidified atmosphere of 5% CO_2. For hypoxic conditions, cells were incubated in an anaerobic chamber (Forma) at 5% CO_2 with 1% O_2 balanced with N_2.

2.3. Cell growth and viability assay

HUVECs were seeded onto 96-well plates, incubated for 24 h, and treated with various concentrations of G0811 for 48–72 h. Cell growth was measured using a 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT; Sigma–Aldrich, Saint Louis, MO) colorimetric assay, and viability was assessed using trypan blue staining.

2.4. Measurement of ROS levels

ROS levels were determined by DCFH-DA assay. 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. DCFH-DA and Hoechst were observed under a microscope (IX71, Olympus), and fluorescent intensity was measured by Image J.

2.5. In vitro capillary tube formation assay

Matrigel (150 μL, 10 mg/mL) was coated in a 48-well culture plate and allowed to polymerize for 1 h at 37 °C. HUVECs (6×10^4 cells/well) were seeded on the surface of matrigel and treated with VEGF (50 ng/mL). Then, G0811 was 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).

2.6. In vitro chemoinvasion assay

A transwell chamber system with 8.0 μm pore sized polycarbonate filter inserts was used to examine the *in vitro* invasiveness of HUVECs. The lower side of the filter was coated with 10 μL of gelatin (1 mg/mL), and the upper side was coated with 10 μL of Matrigel (3 mg/mL). G0811 was added to the lower chamber in the presence of VEGF (50 ng/mL), and HUVECs (7×10^5 cells/well) were placed in the upper part of the filter. The chamber was incubated at 37 °C for 16 h. The invasiveness of cells, fixed with 70% methanol and stained with hematoxylin and eosin, was measured by counting the total number of cells in the lower side of the filter, as seen under an Olympus IX70 microscope at 100× magnification.

2.7. Chorioallantoic membrane (CAM) assay

The CAM assay was performed as described previously [10]. Fertilized chicken eggs were kept in a humidified incubator at 37 °C for 4 days. Approximately 4–5 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, the shell membrane was peeled away, and then compound-loaded Thermanox coverslips (NUNC, Rochester, NY) were applied to the CAM surfaces. Two days later, 1 mL of Intralipose (Greencross Company, Korea) was injected beneath the CAM, and the membrane was observed under a microscope. Retinoic acid (RA), a well known anti-angiogenic compound, was used as a positive control.

2.8. Measurement of VEGF by enzyme-linked immunosorbent assay (ELISA)

The VEGF concentration in media from G0811-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.

2.9. Western blot analysis

The cell lysates were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by transfer to polyvinylidene difluoride (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 with an enhanced chemiluminescence (ECL) kit (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions.

2.10. HIF-1α half-life analysis

HepG2 cells were pretreated with G0811 for 1 h and then incubated under hypoxic conditions. After 4 h, cycloheximide (CHX, 30 μg/mL) was added for different periods of time (up to 2 h). HIF-1α and tubulin were evaluated by Western blotting and quantified, and then the ratio of HIF-1α to tubulin was determined [12].

2.11. RNA isolation, reverse transcription polymerase chain reaction (RT-PCR) analysis, and primer construction

Cells were collected with Trizol (Invitrogen, Carlsbad, CA), and total RNA was extracted according to the manufacturer's protocol. This procedure was followed by precipitation with isopropanol, washing with 70% ethanol, and elution with diethylpyrocarbonate (DEPC)-treated water. Total RNA (5 μg) was reversibly transcribed with Molony murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA) using Oligo-d(T)15 primer. A cDNA mixture (2 μL) was used for PCR amplification of each gene with exTaq (Takara Bio Inc., Japan). Primers used for HIF-1α and GAPDH are as follows: HIF-1α forward: GCTGGCCCCAGCCGCTGGAG, HIF-1α reverse: GAGTGCAGGGT CAGCACTAC, GAPDH forward: AACAGCGACACCCACTCCTC, and GAP DH reverse: GGAGGGGAGATTCAGTGTGGT.

2.12. Statistical analysis

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

3. Results and discussion

3.1. G0811 suppresses HIF-1α expression level without toxic effects on cells

To identify bioactive small molecules that regulate HIF-1α signal transduction, we examined the inhibitory activities of 200

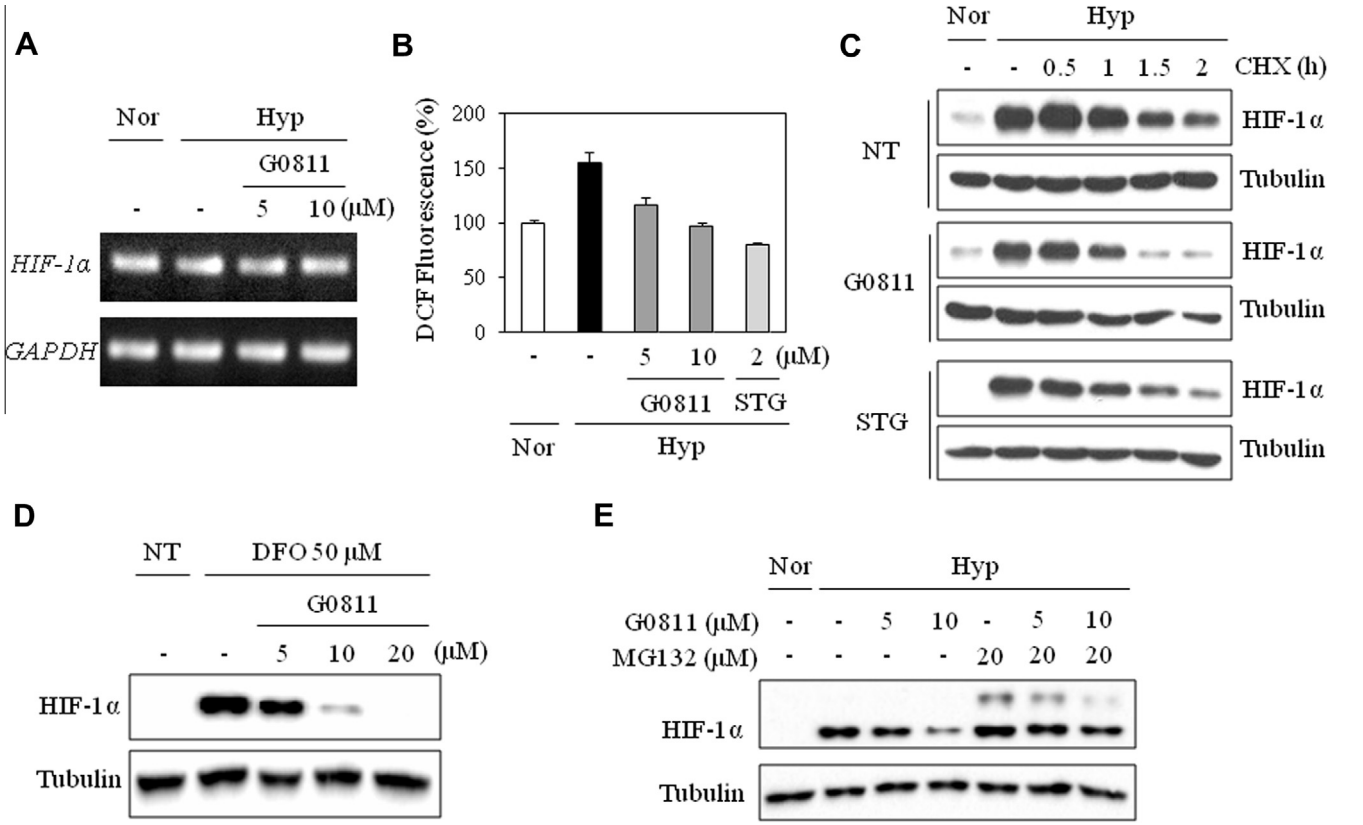

Fig. 2. Suppression of HIF-1α expression level. (A) Effect of G0811 on HIF-1α mRNA levels. HepG2 cells were pretreated with G0811 (5–10 μM) for 1 h and then exposed to 1% O_2 for 12 h. (B) Intracellular ROS levels were determined by DCFH-DA fluorescence. (C) HepG2 cells were pretreated with G0811 (10 μM) or stigmatellin (STG, 1 μM) for 1 h and then incubated under hypoxic condition for 4 h. HIF-1α half-life was measured with cycloheximide (30 μg/mL) for the indicated times to block all de novo protein synthesis. HIF-1α and tubulin levels were then analyzed by Western blot. (D) Detection of desferrioxamine (DFO)-induced HIF-1α stability. (E) HIF-1α protein accumulates in response to proteasomal inhibition. HepG2 cells were exposed to MG132 (20 μM) for 4 h.

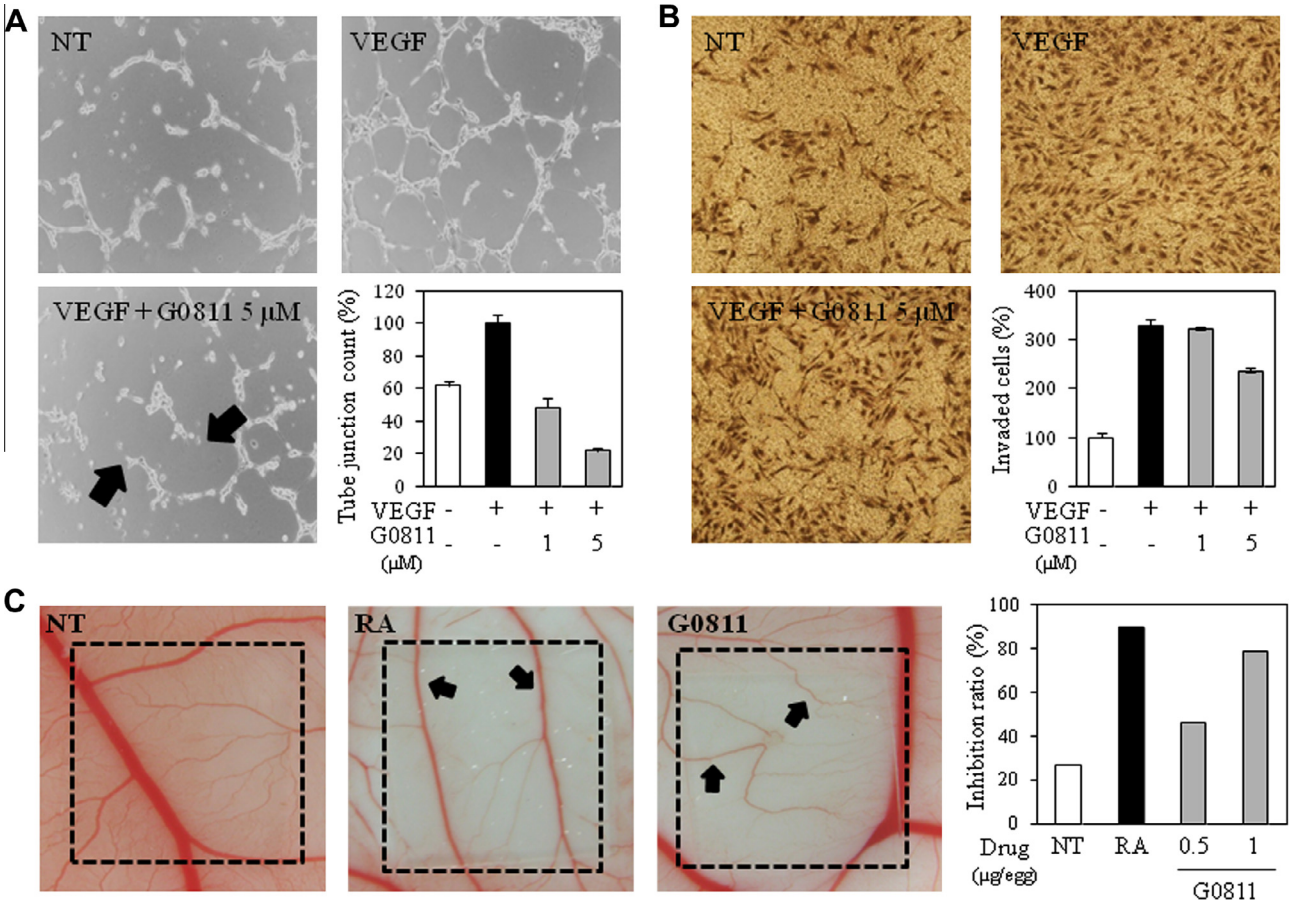

Fig. 3. Anti-angiogenic activity of G0811 *in vitro* and *in vivo*. (A) Inhibitory activity of G0811 on tube formation of HUVECs. Arrows indicates broken tubes formed by VEGF-stimulated HUVECs. (B) Effect of G0811 on invasion of HUVECs. (C) Anti-angiogenesis activity of G0811 *in vivo*. G0811 and retinoic acid (RA) were applied to the CAM, and the neovessel formation was observed. Arrows indicate inhibition of neovascularization of CAM by G0811. Calculations were based on the proportion of positive eggs relative to the total number of eggs tested.

druggable synthetic small molecules on HIF-1α stability. Among the compounds screened, G0811 was identified as the most potent hit (Fig. 1A). G0811 suppressed HIF-1α expression level under hypoxic conditions in HepG2 cells (Fig. 1B). We next investigated the effect of G0811 on the proliferation and viability of HUVECs and whether the inhibitory activity on HIF-1α stability induced by G0811 had any toxic effect on the cells. HUVECs were treated with G0811 for 2 days, and proliferation was measured by the MTT colorimetric assay. As shown in Fig. 1C, the proliferation of HUVECs reaches to 3 doubling times at 48 h. Notably, G0811 did not inhibit proliferation of the cells at concentrations up to 10 μM, implying that G0811 does not significantly affect cell proliferation. The effect of various concentrations of G0811 (1–20 μM) applied to HUVECs on cell viability was also determined using the trypan blue assay. G0811 exhibited no cytotoxicity on HUVECs at doses up to 20 μM for 3 days (Fig. 1D). Accordingly, the following studies were performed using a concentration of G0811 <20 μM.

3.2. G0811 inhibits HIF-1α signal transduction

HIF-1α mRNA expression was measured using RT-PCR to further examine whether the reduction of HIF-1α protein by G0811 was caused by inhibition of HIF-1α transcription. As shown in Fig. 2A, G0811 did not affect the mRNA level of HIF-1α, suggesting that the decrease of HIF-1α protein level by G0811 is not due to the transcriptional inhibition of the HIF-1α gene.

Many studies indicated that mROS is responsible for hypoxia-dependent stabilization of HIF-1α [13,14]. To evaluate whether G0811 affects mROS generation during hypoxia, we measured ROS levels in HeLa cells using the oxidant-sensitive fluorescent probe, DCFH-DA. Notably, G0811 suppressed the hypoxia-induced mROS in HeLa cells in a dose-dependent manner (Fig. 2B). To clarify that G0811 affects HIF-1α stability by suppressing mROS, we further examined the HIF-1α half-life by blocking de novo protein synthesis with cycloheximide. When HepG2 cells were incubated during hypoxia, HIF-1α half-life was ~1.5 h. However, when HepG2 cells were pretreated with G0811, HIF-1α half-life was decreased to <1 h (Fig. 2C). Next, we examined whether G0811 affects desferrioxamine (DFO)-induced HIF-1α stability, because DFO-induced HIF-1α accumulation does not require mROS [15]. G0811 also inhibited HIF-1α stability during DFO treatment (Fig. 2D). To further verify how G0811 regulates HIF-1α stability, the effect of G0811 on HIF-1α protein synthesis was evaluated using MG132. As shown in Fig. 2E, treatment with G0811 under hypoxia in the presence of MG132 exhibited a decrease of ubiquitinylated HIF-1α protein. Together, these results indicated that G0811 might inhibit HIF-1α stability through not only mROS-mediated protein degradation and but also protein synthesis pathway.

3.3. G0811 inhibits angiogenesis both in vitro and in vivo

Next, we investigated the anti-angiogenic activities of G0811, including tube formation and chemoinvasion, on the angiogenic phenotypes of HUVECs *in vitro*. To evaluate inhibitory activities of G0811 on angiogenesis induced by VEGF, serum-starved HUVECs were stimulated by VEGF with or without G0811. G0811

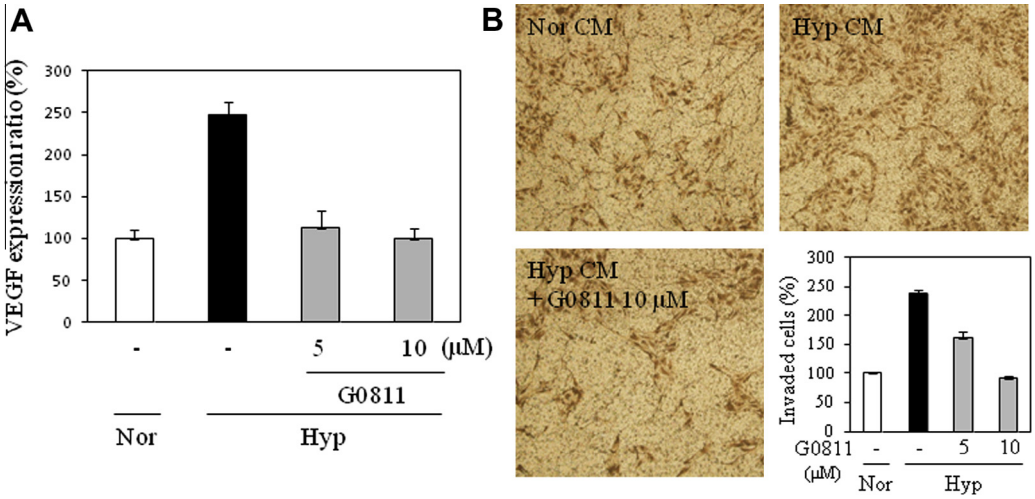

Fig. 4. Effect of G0811 on the VEGF expression and VEGF-induced angiogenesis. (A) The expression level of VEGF on HepG2 cells was determined by a VEGF ELISA assay. (B) Tumor conditioned media (CM)-induced angiogenesis of invasion. Conditioned media derived from HepG2 cells with or without G0811 was added to the lower chamber and incubated for 16 h.

inhibited VEGF-induced tube formation of HUVECs in a dose-dependent manner with no cytotoxic effects (Fig. 3A). Furthermore, the effect of G0811 on the invasive activity of HUVECs induced by VEGF was investigated. G0811 inhibited the VEGF-induced invasion of HUVECs in a dose-dependent manner whereas VEGF-induced cells enhanced invasion of HUVECs (Fig. 3B). These data demonstrate that G0811 effectively inhibits VEGF-induced angiogenesis *in vitro*.

We also evaluated *in vivo* anti-angiogenic activity of G0811 by using the chick embryo CAM assay. Normally developed CAMs elicited an angiogenic response, forming new capillaries from the exiting vascular network. In contrast, chick embryos exposed to G0811 showed weak angiogenic activity during CAM development, without any sign of thrombosis and hemorrhage (Fig. 3C). These results demonstrate that G0811 acts as an inhibitor of angiogenesis, both *in vitro* and *in vivo*, without cytotoxic effects.

3.4. G0811 suppresses VEGF expression level and inhibits tumor-conditioned media-induced in vitro angiogenesis

HIF-1α acts by binding to the HIF-responsive element (HRE) of the VEGF promoter region and upregulates VEGF expression by hypoxic tumor cells [16,17]. VEGF expression in tumors has been associated with high vascularity and poor clinical prognosis [18,19]. Accordingly, we next examined the effect of G0811 on VEGF expression levels under hypoxic conditions. Hypoxia-induced VEGF was reduced by G0811 in a dose-dependent manner (Fig. 4A). Moreover, tumor-conditioned media-induced invasiveness (Fig. 4B) of HUVECs was inhibited by G0811 in a dose-dependent manner. These data indicate that G0811 inhibits hypoxia-induced tumor angiogenesis via the suppression of HIF-1α-mediated VEGF expression.

Angiogenesis, an essential step in tumor proliferation, expansion, and metastasis, results from the expression of angiogenic factors stimulated by hypoxia, oncogenes, cytokines, and hormones. Among these factors, hypoxia is one of key physiological signals for the induction of angiogenesis, and accumulation of HIF-1α is thus induced by hypoxia [20,21]. In addition, recent studies have provided evidence about HIF-1α-mediated resistance to chemotherapy [8,22]. Moreover, toxicity of anti-angiogenesis chemotherapies must be taken into account [23,24]. Therefore, inhibition of HIF-1α activity could be a promising means of anti-angiogenesis therapy under physiological conditions [25].

In this study we identify a new synthetic small molecule, G0811 that suppresses hypoxia-induced HIF-1α stability. Notably, this bioactive small molecule exhibits anti-angiogenic activities in both *in vitro* and *in vivo* assays. G0811 also suppresses the expression levels of VEGF in a dose-dependent manner and inhibits tumor-conditioned media-induced invasion of HUVECs. Interestingly, G0811 inhibits angiogenesis via suppression of HIF-1α stability but does not inhibit proliferation and viability of HUVECs. It is also noteworthy that G0811 effectively suppresses HIF-1α stability by regulating mROS-mediated protein degradation and protein synthesis pathway. Therefore, further investigations on identifying and validating the targets of G0811 will provide the better understanding of the biological activities of this compound and new insight into the underlying mechanisms of HIF-1α signal transduction. In conclusion, G0811 is a potent angiogenesis inhibitor targeting HIF-1α signal transduction and provide a new molecular scaffold for the development of therapeutics related to angiogenesis.

Acknowledgments

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