

# Antiangiogenic activity of herboxidiene via downregulation of vascular endothelial growth factor receptor-2 and hypoxia-inducible factor-1 $\alpha$

Hye Jin Jung<sup>1</sup> · Yonghyo Kim<sup>2</sup> · Ju Yong Shin<sup>1</sup> · Jae Kyung Sohng<sup>1</sup> ·  
Ho Jeong Kwon<sup>2,3</sup>

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**Abstract** Antiangiogenesis is now thought of as one of the most important approaches for anticancer therapy. In this study, we determined the antiangiogenic property of herboxidiene, a polyketide natural product. Herboxidiene effectively inhibited the proliferation of human umbilical vein endothelial cells (HUVECs) at concentrations not exhibiting cytotoxicity. Furthermore, the natural product significantly suppressed vascular endothelial growth factor-induced invasion and tube formation in HUVECs as well as neovascularization of the chorioallantoic membrane in developing chick embryos. We also identified an association between the antiangiogenic activity of herboxidiene and the downregulation of both the phosphorylation of VEGF receptor 2 (KDR/Flk-1) and the expression of hypoxia-inducible factor-1 $\alpha$  at the transcriptional level. These results suggest that herboxidiene functions as a potential antiangiogenic agent and may be applicable for anticancer therapy by targeting tumor angiogenesis.

**Keywords** Antiangiogenesis · Herboxidiene · VEGFR2 · HIF-1 $\alpha$

Hye Jin Jung and Yonghyo Kim contributed equally to this work.

✉ Ho Jeong Kwon  
kwonhj@yonsei.ac.kr

<sup>1</sup> Department of BT-Convergent Pharmaceutical Engineering, Sun Moon University, 70, Sunmoon-ro 221, Tangeong-myeon, Asan-si, Chungnam 336-708, Republic of Korea

<sup>2</sup> Department of Biotechnology, Translational Research Center for Protein Function Control, College of Life Science and Biotechnology, Yonsei University, 50 Yonsei-ro, Seodaemun-gu, Seoul 120-749, Republic of Korea

<sup>3</sup> Department of Internal Medicine, College of Medicine, Yonsei University, Seoul 120-752, Republic of Korea

## Introduction

Angiogenesis, also known as neovascularization, is the generation of new blood vessels from pre-existing vasculature (Bussolino et al. 1997; Folkman 1995). Because tumors require vascular supply for their survival, growth, and metastasis, angiogenesis has become an important therapeutic target in most human cancers (Carmeliet and Jain 2000; Hanahan and Folkman 1996). Vascular endothelial growth factor (VEGF) is the most important stimulator of tumor angiogenesis and therefore antiangiogenic therapy has focused on inhibitors of the VEGF signaling pathway (Carmeliet 2005; Ferrara 2004). Drugs targeted against the VEGF pathway have demonstrated therapeutic efficacy in several clinical studies on cancer therapy (Cardones and Banez 2006; Hoeben et al. 2004).

VEGF induced angiogenesis mainly by interacting with VEGFR2 (KDR/Flk-1) (Holmes et al. 2007; Matsumoto and Claesson-Welsh 2001; Olsson et al. 2006). Binding of VEGF to VEGFR2 causes dimerization and autophosphorylation of the receptor. Activation of VEGFR2 leads to phosphorylation and activation of specific downstream signal transduction effectors, including Akt and mitogen-activated protein kinases (MAPK), which regulate endothelial cell survival, proliferation, migration, and invasion. Therefore, blocking the kinase activity of VEGFR2 can be used as a rational therapeutic approach for the suppression of VEGF-induced angiogenic signaling pathways (Faivre et al. 2007; Hanrahan and Heymach 2007).

An alternative approach involves the regulation of hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) activation, which is critical for VEGF gene transcription (Forsythe et al. 1996). Tumors become hypoxic as their size increases; hence, tumor cells have evolved to survive and grow in microenvironments with very low concentrations of

oxygen. Tumor hypoxia activates HIF-1 $\alpha$ , a master regulator of the mammalian transcriptional response to oxygen deprivation (Höckel and Vaupel 2001; Semenza 2003). Increased HIF-1 $\alpha$  levels have been observed in many human cancers, resulting in overexpression of VEGF and other genes that are involved in the induction of angiogenesis (Pugh and Ratcliffe 2003). In this context, a dual small-molecule inhibitor that simultaneously targets the VEGFR2 tyrosine kinase and HIF-1 $\alpha$  could represent a more efficient drug to target the VEGF pathway and perturb tumor angiogenesis.

This study is the first to demonstrate the antiangiogenic effect and the molecular mechanisms of herboxidiene, a microbial-derived natural product (Fig. 1a). Our results showed that herboxidiene could efficiently suppress tumor angiogenesis through dual inhibition of the VEGFR2 signaling and HIF-1 $\alpha$  expression.

## Materials and methods

### Materials

Endothelial growth medium-2 (EGM-2) was purchased from Lonza. RPMI 1640 and fetal bovine serum (FBS) were purchased from Invitrogen. Recombinant human vascular endothelial growth factor (VEGF), Matrigel<sup>®</sup>, and Transwell<sup>®</sup> chamber systems were obtained from Koma Biotech, BD Biosciences, and Corning Costar, respectively. Anti-hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ), anti-phospho-VEGFR2, anti-VEGFR2, and anti-beta-3 tubulin antibodies were purchased from BD Biosciences, Cell Signaling, and Millipore, respectively.

### Cell culture and hypoxic conditions

Early passages (4–8 passages) of human umbilical vein endothelial cells (HUVECs) were grown in EGM-2 supplemented with 10 % FBS. Human hepatocellular carcinoma (HepG2) cells were grown in RPMI 1640 medium containing 10 % FBS. All cells were maintained at 37 °C in a humidified 5 % CO<sub>2</sub> incubator. For hypoxic conditions, cells were incubated in a hypoxic chamber (Forma Scientific) under 5 % CO<sub>2</sub> and 1 % O<sub>2</sub> balanced with N<sub>2</sub>.

### Cell viability assay

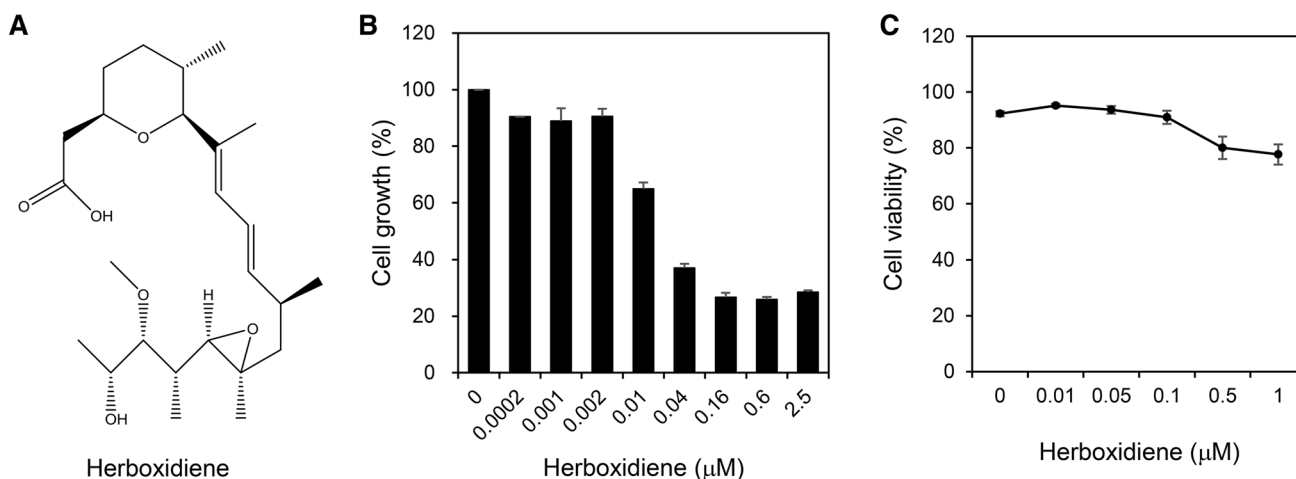
HUVECs were seeded at a density of  $1.5 \times 10^4$  cells/well in gelatin-coated 24-well culture plates (SPL Life Sciences). Herboxidiene was added to each well and the cells were incubated for up to 72 h. After 72 h, the cells were stained with Trypan blue and counted using a hemocytometer as described previously (Jung et al. 2003).

### Cell proliferation assay

HUVECs were plated at  $3 \times 10^3$  cells/well in gelatin-coated 96-well plates (SPL Life Sciences). Herboxidiene was added to each well and the cells were incubated for 72 h. Cell proliferation was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay as described previously (Jung et al. 2003).

### Chemoinvasion assay

The invasiveness of HUVECs was determined in vitro using a Transwell<sup>®</sup> chamber system with polycarbonate



**Fig. 1** The anti-proliferative activity of herboxidiene on HUVECs. **a** Chemical structure of herboxidiene. **b** The effect of herboxidiene on the growth of HUVECs. Cells were treated with various concentrations of herboxidiene for 72 h, and cell growth was assayed by MTT colorimetric assay. **c** The effect of herboxidiene on the viability of HUVECs. Cells were treated with herboxidiene and incubated for 72 h. Cell viability was measured by the Trypan blue assay

filter inserts with a pore size of 8.0  $\mu\text{m}$  as described previously (Jung et al. 2003). Briefly, the lower side of the filter was coated with 10  $\mu\text{L}$  gelatin (1 mg/mL) and the upper side was coated with 10  $\mu\text{L}$  Matrigel<sup>®</sup> (3 mg/mL). HUVECs ( $1 \times 10^5$  cells) were placed in the upper chamber of the filter and herboxidiene was added to the lower chamber in the presence of VEGF (30 ng/mL). The chamber was incubated at 37 °C for 18 h, and the cells were subsequently fixed with methanol and stained with hematoxylin/eosin. The total number of cells that invaded the lower chamber was counted on the filter using an optical microscope (Olympus, Center Valley) at a 100 $\times$  magnification.

### Capillary tube formation assay

Capillary tube formation by HUVECs in vitro was assessed as described previously (Jung et al. 2003). Briefly, HUVECs ( $1 \times 10^5$  cells) were inoculated on a surface containing Matrigel<sup>®</sup> and were incubated with herboxidiene for 6–18 h in the presence or absence of VEGF (30 ng/mL). Morphological changes of the cells and tube formation were visualized under a microscope (Olympus) and photographed at a 100 $\times$  magnification. Tube formation was quantified by counting the total number of branched tubes in randomly selected fields at a 100 $\times$  magnification.

### Chorioallantoic membrane assay

Fertilized chick eggs were maintained in a humidified incubator at 37 °C for 3 days. Approximately 2 mL egg albumin was removed with a hypodermic needle, allowing the chorioallantoic membrane (CAM) and yolk sac to drop away from the shell membrane. On day 3.5, the shell was punched out and removed, and the shell membrane was peeled away. Thermanox<sup>®</sup> coverslips (NUNC) coated with herboxidiene were air-dried and applied to the CAM surface at embryonic day 4.5. Two days later, 10 % fat emulsion (2 mL, Greencross Co.) was injected into the chorioallantois and the CAM was visualized under a microscope. The response was scored as positive when herboxidiene-treated CAM showed an avascular zone compared to that of a control coverslip. The response was calculated as the percentage of positive eggs relative to the total number of eggs tested.

### Western blot analysis

Cell lysates were separated by 10 % SDS-PAGE electrophoresis and the separated proteins were transferred to polyvinylidenedifluoride membranes (Millipore) using standard electroblotting procedures. The blots were blocked and immunolabeled with primary antibodies against HIF-

1 $\alpha$ , phospho-VEGFR2, VEGFR2, and beta-3 tubulin overnight at 4 °C. Immunolabeling was detected with an enhanced chemiluminescence (ECL) kit (GE Healthcare), according to the manufacturer's instructions. Images were quantified with Image Lab<sup>TM</sup> software (Bio-Rad).

### RNA isolation and reverse transcription polymerase chain reaction (RT-PCR)

Total cellular RNA was isolated using TRIzol reagent (Invitrogen) and then reverse transcribed by Moloney murine leukemia virus reverse transcriptase (Invitrogen) using Oligo-d(T)<sub>15</sub> primers. To determine the expression of *HIF-1 $\alpha$*  pre-mRNA and mRNA, standard PCR was performed using 5'-TTGAAGATGACATGAAAGCA-3' and 5'-TTTCTGTGTGTAAGCATTCTC-3' as specific primers. The PCR products were resolved by 1 % agarose gel electrophoresis and visualized using a DNA staining dye. The mRNA level of *GAPDH* was used as an internal control.

### Statistical analysis

The results are expressed as the mean  $\pm$  standard error (SE). Student's *t* test was used to determine statistical significance between the control and test groups. A *P* value of <0.05 was considered statistically significant.

## Results

### The effect of herboxidiene on the proliferation of HUVECs

To explore the antiangiogenic activity of herboxidiene, we first examined the effect of herboxidiene on endothelial cell proliferation. HUVECs were treated with a concentration range of herboxidiene for 72 h, and cell growth was measured using the MTT colorimetric assay. Herboxidiene inhibited the proliferation of HUVECs in a dose-dependent manner, with an IC<sub>50</sub> of 0.026  $\mu\text{M}$  (Fig. 1b). To determine whether the antiproliferative effect of herboxidiene is owing to its cytotoxic effect, the viability of the HUVECs was measured by Trypan blue staining. As shown in Fig. 1c, 72 h exposure of up to 0.1  $\mu\text{M}$  herboxidiene was not cytotoxic. These results demonstrate that herboxidiene suppresses the proliferation of HUVECs by cytostasis.

### The antiangiogenic activity of herboxidiene in vitro and in vivo

To evaluate the effects of herboxidiene on the characteristic angiogenic phenotypes of endothelial cells, in vitro angiogenesis assays measuring cell invasion and tube

formation were performed in an optimal dose range (0.01–0.1  $\mu\text{M}$ ) of herboxidiene, at which no cytotoxicity was observed. Serum-starved HUVECs were stimulated by VEGF with or without herboxidiene, and assessed for chemoinvasion and capillary tube formation. As shown in Figs. 2 and 3, VEGF strongly induced the invasion and tube formation of HUVECs, both of which were inhibited by herboxidiene in a dose-dependent manner. No cytotoxicity was observed at the tested concentrations as shown by Trypan blue staining performed in parallel to the in vitro angiogenesis assays (data not shown).

The antiangiogenic activity of herboxidiene was further validated in vivo using a CAM assay. Thermanox<sup>®</sup> coverslips coated with herboxidiene were placed on the CAM surface and neovascularized zones were visualized under a microscope. The angiogenesis inhibition on control coverslips was 13 % ( $n = 8$ ), whereas herboxidiene very efficiently inhibited the neovascularization of the CAM (71 % at 0.1  $\mu\text{g}/\text{egg}$ ,  $n = 14$ ; 85 % at 0.2  $\mu\text{g}/\text{egg}$ ,  $n = 13$ ) without any evidence of rupture of, or toxicity against, pre-existing vessels (Fig. 4). In conclusion, these results demonstrate that herboxidiene possesses potent antiangiogenic activity, both in vitro and in vivo, without cytotoxicity for endothelial cells.

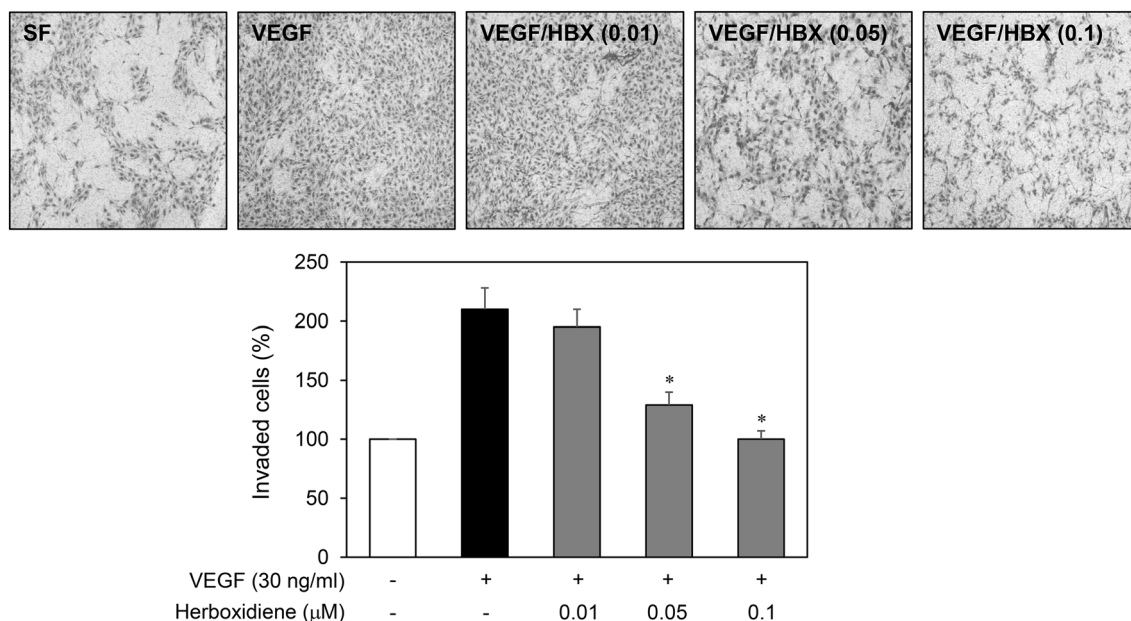
#### Down regulation of VEGFR2 signaling and HIF-1 $\alpha$ expression by herboxidiene

To explore the mechanisms by which herboxidiene inhibits angiogenesis, we measured the effect of herboxidiene on

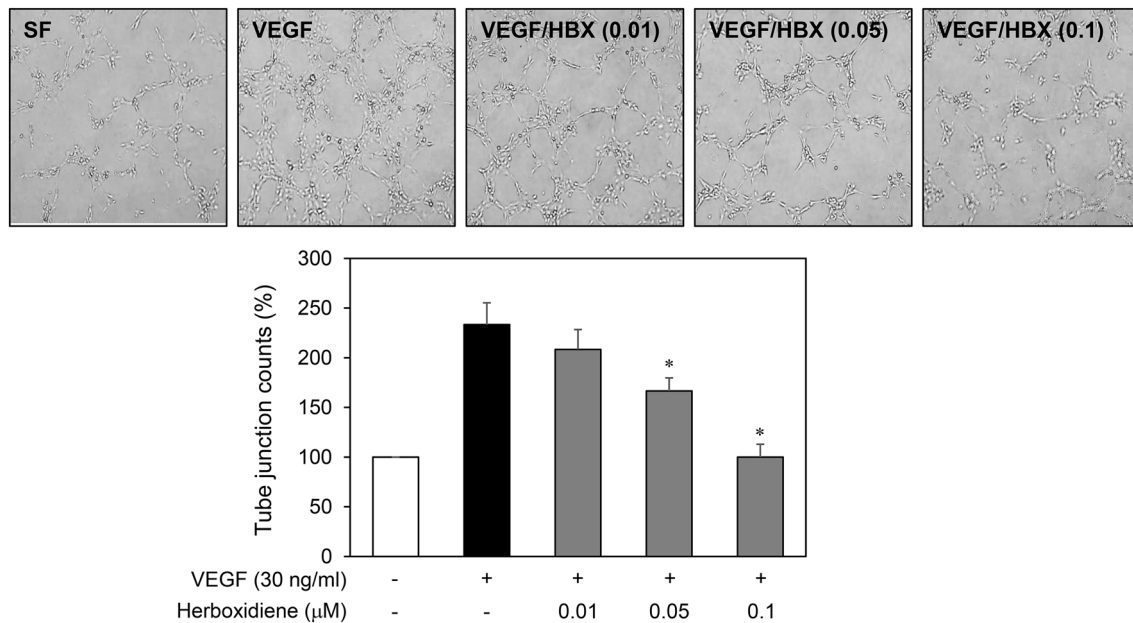
the phosphorylation of VEGFR2 in HUVECs. As shown in Fig. 5, herboxidiene significantly suppressed the phosphorylation of VEGFR2 induced by VEGF, without affecting the total protein level, suggesting that herboxidiene may exert its antiangiogenic activity by antagonizing downstream VEGFR2-mediated signaling cascades.

HIF-1 $\alpha$  plays an important role in the regulation of a large number of genes involved in tumor angiogenesis such as VEGF. To assess the effect of herboxidiene on the expression of HIF-1 $\alpha$  under hypoxic condition, the level of HIF-1 $\alpha$  protein was determined in human hepatoma HepG2 cells treated with herboxidiene. As shown in Fig. 6a, exposure of these cells to herboxidiene significantly reduced the level of HIF-1 $\alpha$  protein under hypoxic condition. To further evaluate the inhibitory ability of herboxidiene on HIF-1 $\alpha$  expression, we next investigated whether herboxidiene affects the accumulation of HIF-1 $\alpha$  when the HIF-1 $\alpha$  protein degradation pathway is blocked. HepG2 cells were treated with herboxidiene in the presence or absence of MG132, a specific proteasome inhibitor that blocks ubiquitin-dependent HIF-1 $\alpha$  degradation. Treatment with MG132 resulted in an increase of HIF-1 $\alpha$  protein in hypoxic condition, but MG132 did not prevent the herboxidiene-mediated reduction in HIF-1 $\alpha$  protein levels (Fig. 6b). These data indicate that herboxidiene may affect HIF-1 $\alpha$  transcription or translation, but not the HIF-1 $\alpha$  protein degradation pathway.

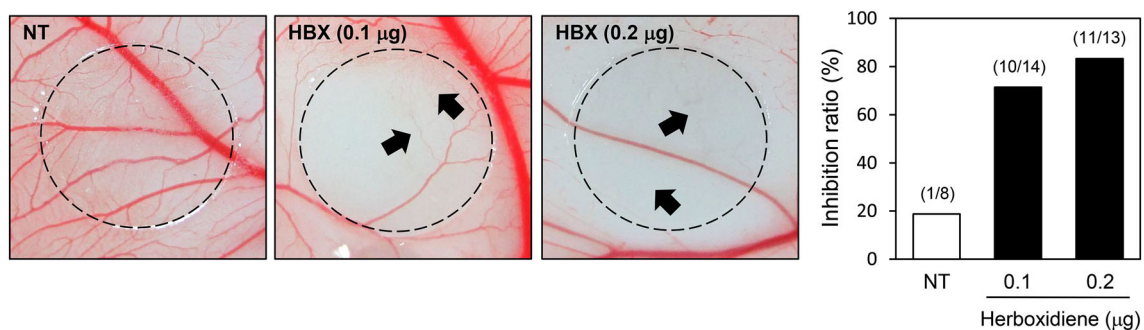
Recent studies have shown that herboxidiene exhibits anti-tumor activity by targeting splicing factor 3B subunit 1



**Fig. 2** The effect of herboxidiene on the invasion of HUVECs. Serum-starved HUVECs were stimulated with VEGF (30 ng/mL) in the presence or absence of herboxidiene. The invasiveness of HUVECs induced by VEGF in serum-free (SF) media was normalized to 100 %. \* $P < 0.01$  versus the VEGF control. Each value represents the mean  $\pm$  SE from three independent experiments



**Fig. 3** The effect of herboxidiene on the tube-forming ability of HUVECs. Serum-starved HUVECs were stimulated with VEGF (30 ng/mL) in the presence or absence of herboxidiene. The level of VEGF-induced capillary tube formation of HUVECs in serum-free (SF) media was normalized to 100 %. \* $P < 0.01$  versus the VEGF control. Each value represents the mean  $\pm$  SE from three independent experiments

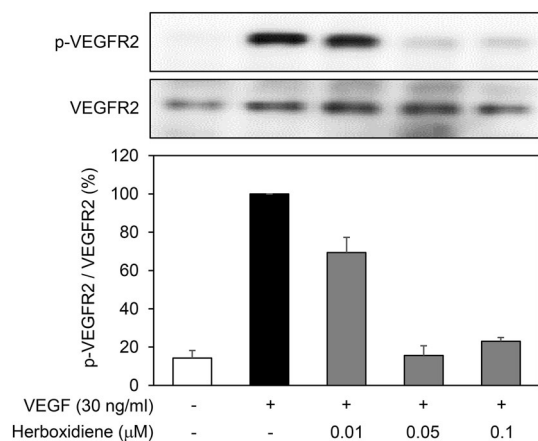


**Fig. 4** The effect of herboxidiene on angiogenesis in CAMs in vivo. Fertilized chick eggs were maintained in a humidified incubator at 37 °C. At embryonic day 4.5, herboxidiene-loaded Thermanox<sup>®</sup> coverslips were applied to the CAM surface. Two days later, the chorioallantois was visualized under a microscope. The presence of an avascular zone (arrows) in treated CAMs was scored as a positive response. The calculations were based on the proportion of positive eggs relative to the total number of eggs tested

(SF3B1), the core spliceosome component which is responsible for pre-mRNA splicing (Hasegawa et al. 2011). To confirm that herboxidiene affects the pre-mRNA splicing of *HIF-1 $\alpha$* , we performed RT-PCR using primers designed to identify both spliced and unspliced mRNA simultaneously. As shown Fig. 6c, herboxidiene decreased the levels of spliced mRNA levels, while the levels of unspliced mRNA levels increased, implying that herboxidiene downregulates the expression of *HIF-1 $\alpha$*  mRNA by blocking the pre-mRNA splicing of *HIF-1 $\alpha$* . In conclusion, these findings demonstrate that herboxidiene could attenuate tumor angiogenesis through the dual inhibition of the VEGFR2 signaling and the *HIF-1 $\alpha$*  mRNA expression.

## Discussion

Herboxidiene, a microbial secondary metabolite, originally isolated from *Streptomyces chromofuscus* A7847, has been shown to possess several biological properties including herbicidal, anticholesterol, and antitumor activities (Isaac et al. 1992; Koguchi et al. 1997; Sakai et al. 2002a, b). However, to the best of our knowledge, no evaluation of the antiangiogenic activity of herboxidiene has been reported to date. In this study, we found that herboxidiene exhibits potent antiangiogenic activity both in vitro and in vivo with no cytotoxicity. Furthermore, our results showed that herboxidiene may inhibit tumor angiogenesis by at least two

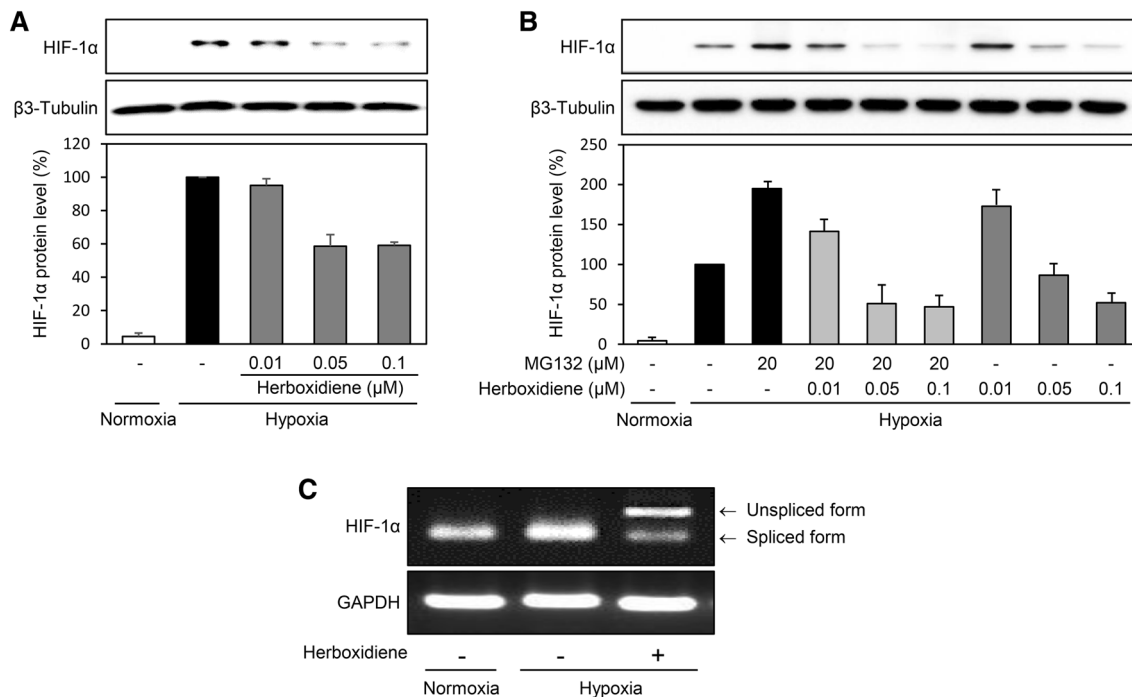


**Fig. 5** The effect of herboxidiene on the phosphorylation of VEGFR2. HUVECs were pretreated with herboxidiene for 6 h at the indicated concentrations and then stimulated with VEGF (30 ng/mL) for 15 min. Protein levels were detected by western blot analysis. The level of VEGFR2 was used as an internal control. Ratios of phosphorylated to unphosphorylated VEGFR2 were determined by densitometry

mechanisms: the blockade of VEGFR2 signaling in endothelial cells and suppression of the expression of the key transcription factor HIF-1α in tumor cells.

The activation of VEGFR2 plays a critical role in mediating VEGF-induced signaling in endothelial cells (Holmes et al. 2007; Matsumoto and Claesson-Welsh 2001; Olsson et al. 2006). Therefore, VEGFR2 has been recognized as one of the most important targets for antiangiogenesis cancer therapy. Currently, several VEGFR2 inhibitors, including vandetanib and sunitinib, have been approved for use in cancer patients (Faivre et al. 2007; Hanrahan and Heymach 2007). However, most of these inhibitors produced limited responses and were accompanied by adverse effects in clinical trials; hence, the identification of new VEGFR2 inhibitors for chemotherapy is still an unmet need (Eskens and Verweij 2006; Verheul and Pinedo 2007). Our results indicate that herboxidiene inhibits the phosphorylation of VEGFR2 in endothelial cells and may therefore, exert its antiangiogenic effect by antagonizing downstream VEGFR2-mediated signaling cascades.

Recent efforts to identify the molecular target of herboxidiene have revealed that herboxidiene exerts antitumor activity by targeting SF3B1 (SAP155), an essential component of the spliceosome (Hasegawa et al. 2011; Lagisetti et al. 2014). A macromolecular complex spliceosome, catalyzing the pre-mRNA splicing process, has emerged as



**Fig. 6** The HIF-1α inhibitory activity of herboxidiene. **a** The effect of herboxidiene on the levels of HIF-1α protein. HepG2 cells were pretreated with herboxidiene for 1 h at the indicated concentrations and then exposed to 1 % O<sub>2</sub> for 6 h. The levels of HIF-1α and tubulin were measured by western blot analysis. The level of tubulin was used as an internal control. **b** The effect of herboxidiene on HIF-1α protein degradation. HepG2 cells were exposed to 1 % O<sub>2</sub> for 6 h in the presence of MG132 or herboxidiene as indicated, and the levels of HIF-1α and tubulin were analyzed by Western blot. **c** The effect of herboxidiene on HIF-1α mRNA levels. HepG2 cells were pretreated with herboxidiene (0.05 μM) for 1 h and then exposed to 1 % O<sub>2</sub> for 6 h. The levels of HIF-1α pre-mRNA and mRNA were detected simultaneously by RT-PCR using specific primers spanning two exons of HIF-1α. The mRNA level of GAPDH was used as an internal control

a potential target for cancer therapy since several natural products, including pladienolide B and spliceostatin A, were discovered (Alphen et al. 2009; Kaida et al. 2007; Kotake et al. 2007). Both these compounds impair pre-mRNA splicing by binding to the SF3B1 subunit of the spliceosome thereby inhibiting tumor cell survival and growth. Herboxidiene also interacts with SF3B1 and has similar antitumor effects (Gao et al. 2013). In this study, we found that herboxidiene inhibits the transcription and splicing of *HIF-1 $\alpha$*  mRNA in tumor cells, suggesting that HIF-1 $\alpha$  is another possible target of herboxidiene for blocking tumor angiogenesis.

In summary, our findings suggest that herboxidiene has potential as an improved antiangiogenic therapy for cancer treatment through the dual inhibition of the signaling mediated by VEGFR2 and the expression of HIF-1 $\alpha$ . Further studies on how herboxidiene concurrently regulates VEGFR-2 and HIF-1 $\alpha$  will help our understanding of the natural product's action mechanism as well as to discover the upstream cellular mediators of tumor angiogenesis.

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#### Compliance with Ethical Standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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