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Research Paper

In vitro anti-angiogenic effects of *Hemidesmus indicus* in hypoxic and normoxic conditions

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ABSTRACT

Ethnopharmacological relevance: The decoction of the roots of *Hemidesmus indicus* is widely used in the Indian traditional medicine for many purposes and poly-herbal preparations containing *Hemidesmus* are often used by traditional medical practitioners for the treatment of cancer. In the context of anticancer pharmacology, anti-angiogenic therapy has become an effective strategy for inhibiting new vessel formation and contrast tumor growth. These considerations are supported by the evidence that most tumors originate in hypoxic conditions and limitation of oxygen diffusion stimulates the formation of tumor abnormal microvasculature. Aim of this study was to evaluate the *in vitro* anti-angiogenic potential of *Hemidesmus indicus* (0.31–0.93 mg/mL) on human umbilical vein endothelial cells and delineate the main molecular mechanisms involved in its anti-angiogenic activity both in normoxia and hypoxia.
Materials and methods: The decoction of *Hemidesmus indicus* was subjected to an extensive HPLC phytochemical characterization. Its *in vitro* anti-angiogenic potential was investigated in normoxia and hypoxia. Cell proliferation, apoptosis induction, and inhibition of endothelial cell migration and invasion were analyzed by flow cytometry. The endothelial tube formation assay was evaluated in matrix gel. The capillary tube branch points formed were counted using a Motic AE21 microscope and a VisiCam videocamera. The regulation of key factors of the neovascularization process such as VEGF, HIF-1α and VEGFR-2 was explored at mRNA and protein level by real time PCR and flow cytometry, respectively.
Results: Treatment with *Hemidesmus* resulted in a significant inhibition of cell proliferation and tube formation in both normoxia and hypoxia. *Hemidesmus* differently regulated multiple molecular targets related to angiogenesis according to oxygen availability. In normoxia, the inhibition of VEGF was the main responsible for its anti-angiogenic effect; the angiogenesis inhibition induced in hypoxia was regulated by a more complex mechanism involving firstly HIF-1α inhibition, and then VEGF and VEGFR-2 down-regulation. Additionally, the inhibition of endothelial cell migration and invasion by *Hemidesmus* was more pronounced in normoxia than in hypoxia, possibly due to the physiological enhanced induction of invasion characteristic of hypoxia.
Conclusions: Our results indicate that *Hemidesmus* might represent a promising therapeutic strategy for diseases in which the inhibition of angiogenesis could be beneficial, such as cancer. The antiangiogenic activity of *Hemidesmus* is based on multiple interactions with critical steps in the angiogenic cascade. VEGF expression stimulated by HIF-1α as well as endothelial cell migration and differentiation represent important targets of *Hemidesmus* action and might contribute to its cancer therapeutic efficacy that is presently emerging and offer a scientific basis for its use in traditional medicine.

1. Introduction

Angiogenesis covers a key role in the development and spread of tumor. Cancer cells are not able to grow in diameter more than 1–2 mm^3 and metastasize without blood circulation. Tumor cells need blood vessels that bring oxygen and nutrients and remove metabolic wastes to spread. In absence of vascular support, tumors may become necrotic or even apoptotic (Parangi et al., 1996). Most tumors originate in hypoxic conditions and limitation of oxygen diffusion stimulates the formation of tumor abnormal microvasculature (Jain, 2005). The hypoxic condition enhances the transcription of vascular endothelial growth factor (VEGF) by hypoxia

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inducible factor-1α (HIF-1α) (Kuschel et al., 2012). An improved activity of VEGF has been reported in most aggressive cancers and it is related with a poor prognosis (Foekens et al., 2001).

The pivotal role of angiogenesis in tumor spread and metastasis formation provides the rationale for using anti-angiogenic strategies as a form of anticancer treatment. Thus, the inhibition of VEGF-signaling pathway is an interesting therapeutic strategy in the treatment of cancer and the most validated anti-angiogenic strategy targets the VEGF axis. Bevacizumab, the first clinically available angiogenesis inhibitor, directly blocks VEGF, and other drugs such as sunitinib, sorafenib and pazopanib indirectly inhibit VEGF receptor (VEGFR) activity (Cesca et al., 2013). Several angiogenesis inhibitors have been approved by FDA for cancer treatment, but their use is associated with many side effects, among them bleeding is one of the most severe (Elice and Rodeghiero, 2012).

Hemidesmus indicus (L.) R.Br. (HI) belongs to the family of *Asclepiadaceae* and is an Indian weed widely used in the traditional medicine. The plant is a bush, woody, with thick and brown bark, which grows from the upper Gangetic plains East-wards to Assam, throughout Central, Western and Southern India. The decoction of the roots of HI is traditionally used for the treatment of blood diseases, dyspepsia, loss of taste, dyspnea, cough, poison, menorrhagia, fever, and diarrhea (Mary et al., 2003). Moreover, poly-herbal preparations containing HI are used by traditional medical practitioners for the treatment of cancer (Thabrew et al., 2005) and different studies demonstrated the anticancer potential of HI (Costa-Lotufo et al., 2005; Fimognari et al., 2011; Samarakoon et al., 2012; Zarei and Javarappa, 2012; Ferruzzi et al., 2013). Aim of this study was to evaluate the *in vitro* anti-angiogenic potential of HI on human umbilical vein endothelial cells (HUVECs) and delineate the main molecular mechanisms involved in its anti-angiogenic activity both in normoxia and hypoxia.

2. Materials and methods

2.1. Materials and cell cultures

Dimethylsulfoxide (DMSO), fetal bovine serum (FBS), antibiotics (penicillin and streptomycin), trypsin-EDTA, and human recombinant VEGF were obtained from Sigma Aldrich (St. Louis, MO, USA). HUVECs were purchased from Lonza (Basel, Switzerland), cultured in EGM complete medium supplemented with SingleQuots™ (containing hydrocortisone, hEGF, FBS, VEGF, hFGF-B, R3-IGF-1, ascorbic acid, heparin and gentamycin/amphotericin-B, Lonza) and incubated at 37 °C and 5% CO_2 in normoxia (21% O_2) or hypoxia (2.5% O_2). The hypoxia was guarantee by the use of the hypoxic station InVivo$_2$ 200 (Baker Ruskinn, Sanford, MA, USA). To maintain the exponential growth, cells were divided when they reached 80% of confluence in a 25 cm^2 dish. HUVECs at passage between 3 and 8 were used for the experiments.

2.2. Plant materials

HI (voucher #MAPL/20/178) was obtained from Ram Bagh (Rajasthan, India) after its authentication by Dr. MR Uniyal, Maharishi Ayurveda Product Ltd., Noida, India. The ayurvedic crude drug was collected in 2010, in particular, following the indications of Ayurvedic Pharmacopoeia of India (2004), during the balsamic period (January). The decoction was prepared according to the method previously described and agreeing with Ayurvedic Pharmacopoeia (Fimognari et al., 2011). Briefly, 10 g of grinded roots were added to 300 mL of boiling water, and boiled until the suspension reached the volume of 75 mL. The yield of the decoction was 15%. HI decoction was filtered, lyophilized, and stored at room temperature. The experiments were performed by preparing a stock solution of 31 mg/mL. The suspension was centrifuged at 4000 rpm to discard any insoluble material.

2.3. HPLC–MS analysis of plant decoction

The main phytomarkers of HI, namely 2-hydroxy-4-methoxybenzaldehyde, 3-hydroxy-4-methoxybenzaldehyde and 2-hydroxy-4-methoxybenzoic acid (Das and Bisht, 2013), were identified and quantified by HPLC–MS analysis. The reference compounds (all obtained from Sigma) were used as external standards to set up and calculate appropriate calibration curves. The calibration graphs were provided by the regression analysis of peak area of the analytes *vs* the related concentrations.

The analyses of three different batches of HI were performed on a Jasco PU-1585 Liquid Chromatograph (Jasco Corporation, Tokio, Japan) interfaced with a Jasco 1575 UV–vis detector (λ=254 nm) and a LCQ-Duo Mass Spectrometer (Thermo Finnigan, San Jose, CA, USA), by a splitting flow *T*-valve. The mass spectrometer is equipped with heated capillary interface and electrospray ionization (ESI) source, operating with an Ion Trap (IT) analyzer. ESI system employed a 4.5 kV (positive polarity) and 5.0 kV (negative polarity) spray voltage and a heated capillary temperature of 200 °C. The sheath gas and the auxiliary gas (nitrogen) flow rates were set to 0.75 and 1.2 L/min, respectively. ESI was optimized using 3-hydroxy-4-methoxybenzaldehyde and 2-hydroxy-4-methoxybenzoic acid for positive and negative polarity, respectively. The mass chromatograms were acquired in total ion current (TIC) modality from 50 to 400 *m/z*, and in MS/MS mode (multiple reaction monitoring) on the ESI generated most abundant ion, corresponding to the pseudomolecular ion; $[M+H]^+$ at 153 *m/z* for 2-hydroxy-4-methoxybenzaldehyde and 3-hydroxy-4-methoxybenzaldehyde, and $[M-H]^-$ at 167 *m/z* for 2-hydroxy-4-methoxybenzoic acid. The relative collision energy varied for the different compounds from 18% to 23%.

Chromatographic analyses were performed on a Phenomenex Gemini C18 column (5 μm, 150 mm × 2.0 mm I.D.) by gradient elution from A (0.1% formic acid in acetonitrile)—B (0.1% formic acid in water) 28:72 (v/v) for 7 min to A-B 55:35 (v/v) in 20 min, at the flow rate of 0.3 mL/min. The re-equilibrium time between runs was 5 min. The injection volume was 50 μL.

2.4. Preparation and GC–FID and GC–MS analyses of lipidic fraction

As previously reported (Das and Bisht, 2013; Fiori et al., 2014) in HI root decoction can be detected not only hydrophilic compounds but also lipophilic ones.

Two g of decoction were exactly weighed into a 25 mL flask and then extracted with 20 mL of chloroform by ultrasound system maceration for 20 min. The residual decoction was centrifuged at 3000 rpm for 20 min. The extraction was performed three times All supernatants were transferred into a 100 mL round bottom flask, then taken to dryness with a rotary vacuum evaporator. The procedure was repeated on three different batches. The dried extracts have been mixed with 100 μL BSTFA (1% TMCS) (bis (trimethylsilyl)trifluoroacetamide + trimethylchlorosilane) (Sigma-Aldrich) for 45 min at 80 °C. Then 1 μL of solution was directly injected in GC.

Lupeol, lupeol acetate, β-sistosterol, and β-amyrin acetate were identified by GC–MS and then quantified in GC–FID by external standard method. All standards were purchased from Extrasynthese (Genay, France). After derivatization, an appropriate calibration curve was calculated for each reference compound. The calibration graphs were provided by the regression analysis of peak area of the analytes *vs* the related concentrations.

GC–MS analysis was performed by a gas chromatograph (Model Varian GC-3800, Agilent Technologies Inc., Santa Clara, California,