Journal of Cancer Research and Practice

journal homepage: www.ejcrp.org

Original Article

Anticancer Effect of *Andrographis paniculata* by Suppression of Tumor Altered Hypoxia Signaling Cascade in Mouse Melanoma Cells

Tanmoy Paul[1], Sharmita Basu[2], Nimai Chandra Saha[3]*

[1]Division of Pathophysiology, National Institute of Cholera and Enteric Diseases, Kolkata, West Bengal, India
[2]Department of Biological Sciences, Joygram J. N. High School, Kolkata, West Bengal, India
[3]Fishery and Ecotoxicology Research Laboratory (Vice-Chancellor's Research Group), Department of Zoology, The University of Burdwan, Burdwan, West Bengal, India

Abstract

Background: Intratumor hypoxia, the main factor responsible for the angiogenic switch, represents one of the major events leading to tumor progression. Tumor hypoxia leads to the stabilization of hypoxia-inducible factor-1α (HIF-1α) which influences tumor angiogenesis. The new blood vessels formed by the HIF-1α-vascular endothelial growth factor (VEGF) signaling axis create the tumor microenvironment, which inhibits drug delivery to solid tumors. The present study aimed to investigate the effect of *Andrographis paniculata* leaf extract as a powerful anticancer agent targeting the HIF-1α-VEGF signaling axis in mouse melanoma cell. **Materials and Methods:** We induced hypoxia-mimicking conditions in mouse B16 melanoma cell with cobalt chloride. Total RNA was isolated followed by reverse transcriptase polymerase chain reaction to study the transcriptional expressions of HIF-1α and VEGF. Further confirmation of the transcriptional profiling was done at the protein level with Western blot analysis. Expression profiling of transcriptional factors involved in the hypoxia signaling cascade was done. An immunofluorescence study was also used to confirm the results obtained from transcriptional and translational analyses. **Results:** *A. paniculata* leaf extract significantly downregulated the expressions of HIF-1α and VEGF both at the transcriptional and translational level. Sp1, p300, CBP expressions were also downregulated, whereas the expression of Sp3 was significantly upregulated by *A. paniculata* leaf extract in B16 melanoma cells. **Conclusion:** In the present study, *A. paniculata*-treated cells demonstrated lower expressions of VEGF and HIF-1α both at the transcriptional and translational level. The mechanism of the downregulation of HIF-1α was probably through the altered expressions of transcriptional factors involved in the hypoxia-signaling cascade.

Keywords: *Andrographis*, anticancer agent, hypoxia signaling cascade

Introduction

Andrographis paniculata, popularly known as Kalmegh in India, is widely regarded to be a beneficial plant due to its diverse

Received: 30-Jan-2019 **Revised:** 02-Apr-2019 **Accepted:** 21-Apr-2019

Access this article online

Quick Response Code:

Website: www.ejcrp.org

DOI: 10.4103/JCRP.JCRP_9_19

Address for correspondence: Prof. Nimai Chandra Saha, Fishery and Ecotoxicology Research Laboratory (Vice-Chancellor's Research Group), Department of Zoology, The University of Burdwan, Burdwan - 713 104, West Bengal, India.
E-mail: prof.ncsaha@gmail.com

How to cite this article: Paul T, Basu S, Saha NC. Anticancer effect of *Andrographis paniculata* by suppression of tumor altered hypoxia signaling cascade in mouse melanoma cells. J Cancer Res Pract 2019;6:117-23.

medicinal applications. It belongs to the family *Acanthaceae*[1] colloquially known as "king of bitters," and is abundant throughout India and Sri Lanka.[2] The therapeutic potential of *A. paniculata* is due to its immense effect on gastrointestinal, upper respiratory infections, fever, herpes, and a variety of other chronic and infectious diseases.[3] The therapeutically important active principal of *A. paniculata* found in a specific part of the plant is known as andrographolide. A previous study showed the effect of the aqueous extract of *A. paniculata* on the antioxidant defense system in lymphoma in a mice model;[4] whereas the methanolic extract has shown antioxidant and anti-inflammatory properties inhibiting the formation of oxygen-derived free radicals.[5] Earlier studies have also reported its inhibitory effect on lipopolysaccharide-induced increases in TNF-α and granulocyte-macrophage colony stimulating factor.[6]

Hypoxia-inducible factor (HIF) is expressed by living metazoans,[7] indicating the important roles played by HIF in the ancient world during and after the period of oxygenation. There are different isoforms of HIF among which HIF-1 is most significant. HIF-1 consists of HIF-1α and HIF-1β subunits, of which HIF-1β is expressed consecutively, whereas, HIF-1α protein levels determine HIF-1 transcriptional activities.[8] Under oxygen-rich conditions, HIF-1α is bound by the von Hippel–Lindau (VHL) protein, leading to the proteasomal degradation of HIF-1α.[9] VHL binding is dependent on the hydroxylation of a specific proline residue in HIF-1α by prolyl hydroxylase (PHD), which uses oxygen as a substrate such that its activity is inhibited under hypoxic conditions.[10] In addition, when PHD becomes inactivated, HIF1α accumulates within cells, binds with HIF1 β, and the complex binds with hypoxia response element (HRE) of the vascular endothelial growth factor (VEGF) gene, thereby upregulating VEGF expression in a hypoxic environment. The angiogenic switch is a major event in tumor progression and is triggered by the hypoxia-mediated activation of HIF-1α by inducing the expression of VEGF. VEGF is a key growth factor essential for angiogenesis. VEGF promoters contain binding sites of different transcriptional regulators such as HIF-1α and Sp1/Sp3. The interaction between HIF-1 and phosphorylated Sp1 in hypoxic conditions boosts VEGF gene transcription. Considering the myriad of modes of regulation of the VEGF promoters, an integrated view of VEGF regulation at the transcriptional level is far from being resolved; however, two major avenues for interfering with VEGF expression should focus on the action of the two transcription factors Sp1 and HIF-1. Another important factor in the hypoxia-signaling cascade is a factor inhibiting HIF, which represses HIF-1α transactivation[11] by hydroxylating an asparaginyl residue, thereby blocking the association of HIF-1α with the p300/CBP coactivator protein.[12] The coactivators p300/CBP works in binding HIF1-α and helps in the transcriptional expression of VEGF.[13] Other transcription factors regulate this HIF-1α-mediated VEGF expression in a hypoxic environment in B16 melanoma cells. SP1 is a transcription factor that after phosphorylation helps HIF1α to bind with HRE regulatory sequence. The transcription factors Sp1 and Sp3 play crucial roles in this process.[14] Sp3 is another transcription factor generally thought to be a competitive inhibitor of Sp1, that can down-regulate VEGF expression. By promoting the transcription of all these genes, HIF helps to maintain oxygen homeostasis.

In solid tumors, hypoxia, or an oxygen tension below the physiologic levels, develops as abnormal proliferation outstrips the blood supply. This hypoxic region is involved in the progression of malignancy and results in the development of resistance to radiotherapy.[15] HIF1α is overexpressed in many different types of human cancers.[16] Its expression is associated with an aggressive phenotype and is a marker for a poor prognosis for many types of tumors, including prostate tumors, oropharyngeal, esophageal, and breast cancer. HIF-1α active or hypoxic cells have been shown to play crucial roles in angiogenesis and radioresistance. This suggests that HIF-1α is a potential target for anticancer therapy. The present work aimed to investigate the role of *A. paniculata* as an anticancer agent targeting the hypoxia-signaling cascade in mouse melanoma cells. We also analyzed alterations caused by *A. paniculata* extract in the regulation of different components of the hypoxia cascade, to establish *A. paniculata* as a potential anticancer agent.

Materials and Methods

Cell culture

B16 melanoma cells, previously obtained from the National Centre for Cell Science, Pune, India, were revived from liquid nitrogen (−173°C) and thawed with 37°C water and recultured in Dulbecco's Modified Eagle Medium (DMEM) high glucose (Gibco, Invitrogen, New York, USA), supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco, Invitrogen, New York, USA) supplemented with sodium pyruvate (Sigma, Japan). The cells were cultured up to 20 passages through this study. The cells were incubated at 37°C in an incubator (HeraCell, Thermo Scientific, New York, USA) with the supply of 5% CO_2 and were passaged every 4–5 days.

Preparation of *Andrographis paniculata* leaf extract

The whole plant was collected, and the leaves were separated. Leaves of the same size and color (indicative of the same age) were then washed with distilled water and dried completely. The leaves were then powdered into a mixture, and the hydroalcoholic extract was prepared by refluxing with double distilled water (DDH_2O) and alcohol (3:1) from 1 g of *A. paniculata* leaf powder in 10 mL solvent in a round bottom flask for 48 h at 60°C. The liquid extract was filtered, cooled, and concentrated by evaporating its liquid contents in a vacuum evaporator (Savant SPD 111V, Thermo Scientific, New York, USA) and collected. Extracts were diluted in DMEM to produce a concentration of 100 μg/mL. To ensure sterility, the extract was filter sterilized. The obtained extract of *A. paniculata* was screened for various constituents (alkaloids, steroids, flavonoids, tannins, quinines, sugars, and proteins) utilizing the standard procedure.[17]

Cobalt chloride-induced hypoxia mimicking condition

The B16 melanoma cells were treated with different concentrations of cobalt chloride ($CoCl_2$) (Sigma, USA) (50, 100, and 150 μM/ml concentrations of $CoCl_2$ solution). Stock solutions of $CoCl_2$ (800 μM/10 ml DMEM) were filter-sterilized (0.22 μm), and the resultant solutions were kept at 4°C and used within 24 h for the assay.[18] Among the different concentrations, 100 μM $CoCl_2$ was observed to induce maximum HIF1α and VEGF expressions without substantial cell death. Cellular hypoxia in the B16 melanoma cells was induced by 100 μM $CoCl_2$.

Induction of cellular severe hypoxia

A modular incubator chamber was used to induce cellular physiological hypoxia. The chamber was flushed with a gas mixture (2.2% O_2, 5.5% CO_2, and 92.3% N_2) for 4 min at a flow rate of 20 L/min (according to the manufacturer's manual) to purge the chamber and achieve an oxygen level of 0.5%–2% for severe hypoxia.

RNA isolation and semi-quantitative reverse-transcriptase polymerase chain reaction

Total RNA was isolated from a single-cell suspension of cultured B16 melanoma cells using Tri-reagent (Invitrogen, Carlsbad CA, USA). The cDNA synthesis was carried out using a RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Thermo Scientific, USA) following the manufacturer's protocol and polymerase chain reaction (PCR) was carried out using gene-specific primers. PCR products were identified by image analysis software for gel documentation (Gel Doc™ XR + system, BioRad) the following electrophoresis on 2% agarose gels stained with ethidium bromide. The transcriptional expressions of the studied genes were analyzed by densitometric analysis using picture management software.

Western blotting

Cell lysates were prepared by incubating cells from different treatment groups with RIPA buffer (GCC biotech, WB, India) for 30 min and clarified by centrifugation. The cellular lysates were separated on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane (Millipore, USA) using the BioRad Gel Transfer system. The membrane was first blocked with 5% BSA for 2 h at room temperature, followed by incubation overnight at 4°C with the primary antibodies for the respective proteins. Finally, blots were incubated with peroxidase-conjugated secondary antibody for 2 h at room temperature. Immunoreactive proteins were detected by the addition of the HRP color development reagent according to the manufacturer's protocol. The membrane was immersed into the solution for 1 min, wrapped with a Saran wrap, exposed to X-ray film and developed. The protein expressions of HIF and VEGF were analyzed by densitometric analysis using picture management software.

Immunofluorescence analysis

The B16 melanoma cells were grown in chambered slides in DMEM high glucose medium. After incubation, the medium was removed, and the slides were fixed in 4% paraformaldehyde followed by blocking with 5% BSA and staining with anti-mouse HIF1α (1:500, SC53546; Santa Cruz, USA) and anti-mouse VEGF (1:400, SC507; Santa Cruz, USA) antibodies. After incubation overnight, the chamber slides were washed three times with PBS-T-20 and incubated for 30 min with anti-mouse IgG-FITC (1:500) and anti-mouse IgG-PE (1:500). Sections were washed followed by mounting in 4′,6-diamidino-2-phenylindole (DAPI) (F6057, Sigma) and images were acquired using a Zeiss Confocal Microscope (Carl Zeiss, USA).

Statistical analysis

All results represent the average of three independent *in vitro* experiments. For all assays, values of individual observations were presented as mean ± standard deviation. We compared all pairs of columns using one-way ANOVA with GraphPad Prism software (GraphPad Software, California, USA) with differences between groups attaining a $P < 0.05$ considered as statistically significant.

Results

The hypoxia-inducible factor-1α and vascular endothelial growth factor expressions in RNA and protein levels in mouse melanoma cells treated with *Andrographis paniculata* leaf extract in normoxia and $CoCl_2$-induced hypoxia-mimicking conditions

B16 melanoma cells were treated with both *A. paniculata* and $CoCl_2$ (100 μM). RT-PCR and Western blot analysis was performed to study the expression levels of VEGF and HIF-1α at both the transcriptional and translational level. It was observed from the RNA and protein level study that, VEGF and HIF-1α were both downregulated at the transcriptional and translational level by *A. paniculata* leaf extract, under $CoCl_2$-induced cellular hypoxia [Figure 1].

Immunofluorescence analyses of the hypoxia-inducible factor-1α and vascular endothelial growth factor expressions in mouse melanoma cells treated with *Andrographis paniculata* extract in normoxia and $CoCl_2$-induced hypoxia-mimicking conditions

The immunofluorescence analyses indicated that HIF-1α was upregulated in $CoCl_2$-treated B16 melanoma cells, mimicking cellular hypoxia. The analyses also showed that when treated with *A. paniculata* extract, the B16 cells showed downregulation of HIF-1α and VEGF. An immunofluorescence study also confirmed the results of RT-PCR and Western Blotting analyses [Figure 2].

Andrographis paniculata extract altered the expressions of different molecular mediators of the hypoxia signaling cascade in mouse B16 melanoma cells in severe hypoxic conditions

The severe physiological hypoxic conditions altered the expressions of different signaling molecules of the hypoxia-signaling cascade. The expressions of HIF-1α, Sp1,

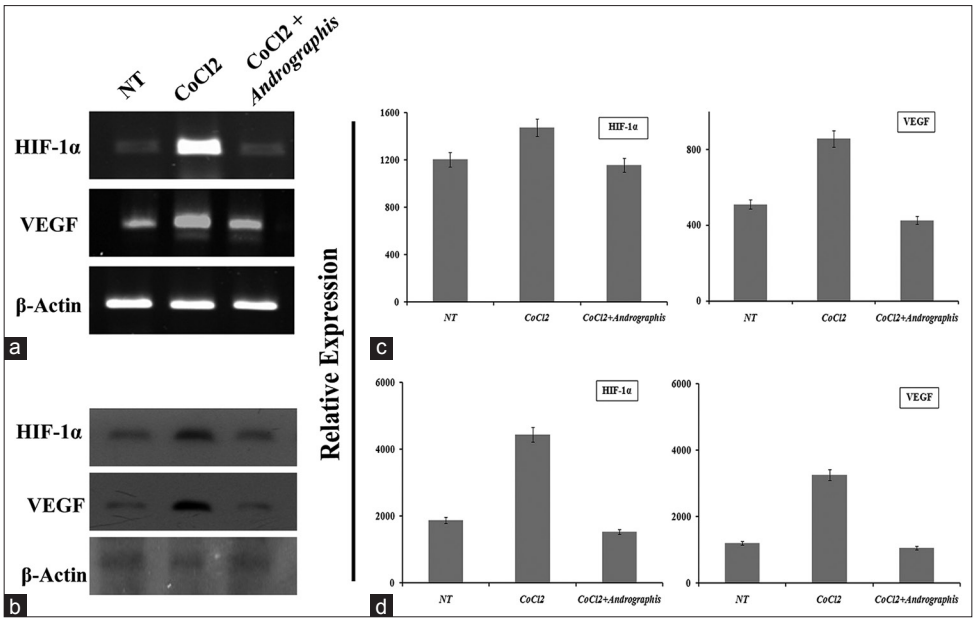

Figure 1: Expression profile of HIF-1α and VEGF in $CoCl_2$ induced cellular hypoxia. The effect of *Andrographis paniculata* leaf extract (100 μg/ml) treatment in B16 melanoma cell for HIF and VEGF expressions for 24 h at the transcriptional level (a) with densitometric analysis of the observed transcriptional expression (c). The expressions were further confirmed at the protein level by Western blotting (b) with densitometric analysis (d). HIF-1α: Hypoxia-inducible factor-1α, VEGF: Vascular endothelial growth factor

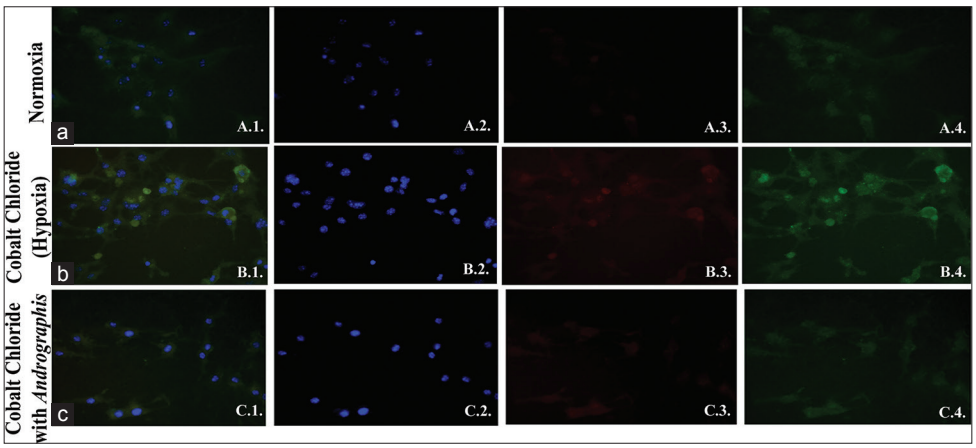

Figure 2: Immunofluorescence study of the expression of HIF-1α and VEGF in $CoCl_2$-treated mouse B16 melanoma cells ([a] HIF-1α and VEGF expression in B16 melanoma cells when incubated without $CoCl_2$ (a. 1. Composite; a. 2. 4',6-diamidino-2-phenylindole (DAPI); a. 3. HIF-1α; a. 4. VEGF). (b) The induction of severe hypoxia with $CoCl_2$in B16 melanoma cells (b. 1. Composite; b. 2. DAPI; b. 3. HIF-1α; b. 4.VEGF). (c) The effect of *Andrographis paniculata* (100 μg/ml) extract on HIF-1α and VEGF expressions in $CoCl_2$-treated B16 melanoma cells for 24 h (c. 1. Composite; c. 2. DAPI; c. 3. HIF-1α; c. 4. VEGF). HIF-1α: Hypoxia-inducible factor-1α, VEGF: Vascular endothelial growth factor

Sp3, CBP, p300, and VEGF were regulated differently by the extract of *A. paniculata*. HIF-1α, Sp1, CBP, p300, and VEGF were downregulated in *A. paniculata* extract-treated cells in hypoxic conditions, whereas Sp3 was upregulated in *A. paniculata* extract-treated cells in hypoxic condition [Figure 3].

Alterations in hypoxia-inducible factor-1α and vascular endothelial growth factor levels in B16 melanoma cells by *Andrographis paniculata* treatment under severe hypoxic conditions

Immunofluorescence analysis indicated the downregulation of HIF-1α and VEGF in B16 melanoma cells treated with *A. paniculata* extract in severe hypoxic conditions. The immunofluorescence analysis confirmed the down-regulated expression pattern observed in transcriptional and translational analyses of HIF-1α and VEGF in melanoma cells under severe hypoxic conditions treated with *A. paniculata* extract [Figure 4].

Discussion

Intratumor hypoxia is an important pathophysiological condition that has a tremendous effect on tumor growth by initiating new blood vessel formation by the process of

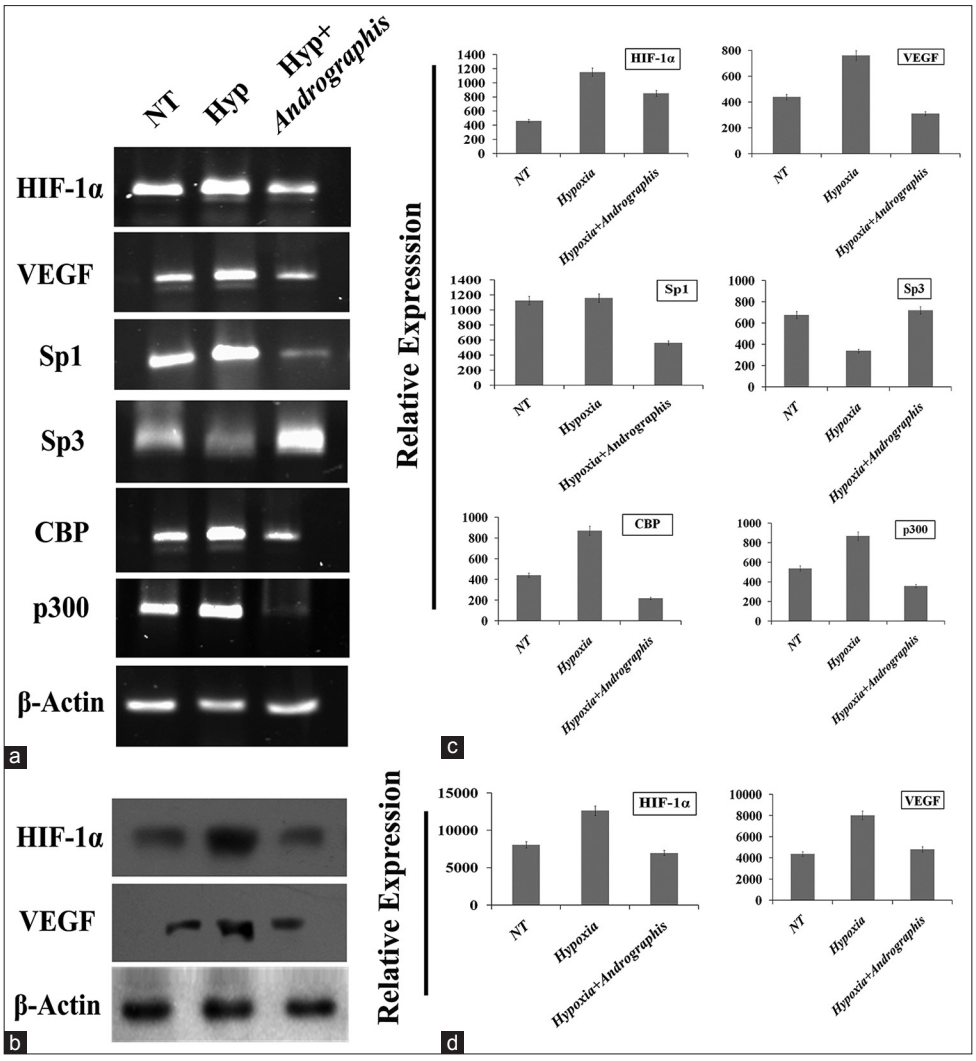

Figure 3: Expression profiles of the molecular mediators of the hypoxia signaling cascade in severe cellular hypoxia showing the effect of *Andrographis paniculata* leaf extract treatment on HIF and VEGF expressions. (a) Total RNA was isolated followed by cDNA synthesis for semi-quantitative PCR for molecular mediators involved in the hypoxia signaling cascade (HIF-1α, VEGF, Sp1, Sp3, CBP, p300) for transcriptional analysis. (b) Isolation of protein and Western blotting were performed to confirm the observed transcriptional expressions of HIF-1α and VEGF. Densitometric analyses for transcriptional (c) and translational (d) expression profiles were performed. HIF-1α: Hypoxia-inducible factor-1α, VEGF: Vascular endothelial growth factor

angiogenesis,[19] which supports the increasing demand for nutrients and oxygen supply in the tumor.[20] Tumor hypoxia can be as influential as to alter the expression of about 1.5% of the human genome[21] through alterations in different transcription factors of the hypoxia signaling cascade. Tumor hypoxia exerts its effects through the activation and stabilization of HIF-1α, in an oxygen-dependent manner.[22] On stabilization, HIF-1α binds to HRE to regulate the transcription of growth factors such as VEGF which are involved in angiogenesis. Other transcription factors, including Sp1, Sp3, CBP, and p300, are also involved in the HIF-mediated downregulation of VEGF, which influenced the angiogenesis of solid tumors. Thus, a hypoxic tumor microenvironment can cause the formation of chaotic tumor vascular architecture beyond the oxygen and nutrient supply to the tumor, which also contributes to hindering drug delivery to solid tumors such as melanoma, and its dissemination can lead toward a ubiquitous tumor sanctuary.[23] Thus, destabilizing HIF, by targeting HIF-1α and VEGF, is a promising anti-angiogenic approach to normalize tumor hypoxia.

The aim of the present study was aimed to observe the anticancer effect of *A. paniculata* leaf extract on mice melanoma cells by targeting the hypoxia signaling cascade. The melanoma cells treated with *A. paniculata* leaf extract showed down-regulated VEGF expression, which could assist in the normalization of a hypoxic tumor microenvironment through the rectification of chaotic tumor vasculature. The downregulation of VEGF by *A. paniculata* leaf extract may be mediated by the lower expression of HIF1α mediated by treatment with leaf extract in hypoxic melanoma cells. Transcriptional, translational, and immunofluorescence studies also confirmed that Sp1, CBP, and p300 transcription factors were downregulated, whereas Sp3 transcription factor was upregulated in *A. paniculata* leaf

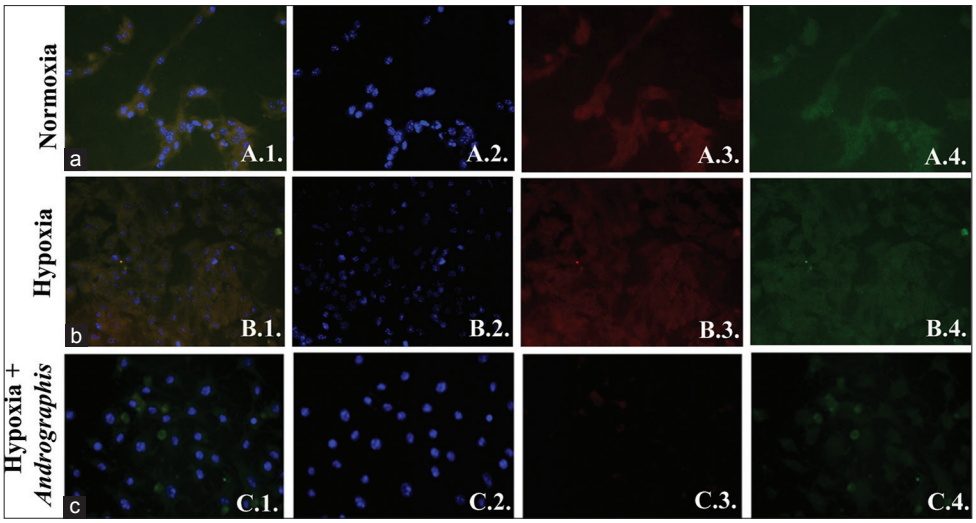

Figure 4: Immunofluorescence study of the expressions of HIF-1α and VEGF in melanoma cells under the condition of severe physiological hypoxia. (a) HIF-1α and VEGF expressions in B16 melanoma cells when incubated in normal oxygen concentrations (a. 1. Composite; a. 2. 4',6-diamidino-2-phenylindole (DAPI); a. 3. HIF-1α; a. 4. VEGF). (b) The effect of severe hypoxia on HIF-1α and VEGF expressions in B16 melanoma cells (b. 1. Composite; b. 2.DAPI; b. 3.HIF-1α; b. 4.VEGF). (c) The HIF-1α and VEGF expressions in B16 melanoma cells when incubated with *Andrographis paniculata* extract for 24 h under severe hypoxia (c. 1. Composite; c. 2.DAPI; c. 3.HIF-1α; c. 4.VEGF). HIF-1α: Hypoxia-inducible factor-1α, VEGF: Vascular endothelial growth factor. HIF-1α: Hypoxia-inducible factor-1α, VEGF: Vascular endothelial growth factor. HIF-1α: Hypoxia-inducible factor-1α, VEGF: Vascular endothelial growth factor

extract treated melanoma cells in both hypoxia-mimicking and physiological hypoxic conditions. This could be a novel therapeutic approach, as downregulating Sp1, CBP, and p300 and up-regulating Sp3 with *A. paniculata* leaf extract can ultimately down-regulate VEGF expression indirectly by preventing HIF-1α binding to the HRE.[24] Thus, *A. paniculata* can significantly down-regulate HIF-1α and VEGF in tumor cells. The down-regulated expressions of Sp1, CBP, and p300 and up-regulated expression of Sp3 mediated by *A. paniculata* were primarily responsible for the HIF-1α-mediated downregulation of VEGF in hypoxic melanoma cells. Sp3 is a transcription factor which generally acts as a competitive inhibitor of Sp1,[25] thus it can downregulate the expression of VEGF.

Earlier studies have indicated the anticancer effect of *A. paniculata* on tumor cells in different tissues.[26-28] Previous studies have investigated the effect of *A. paniculata* extract on the expression of VEGF,[26,28] and here, it is of interest to understand the molecular mechanism of *A. paniculata*-mediated destabilization of HIF-1α, responsible for the downregulation of VEGF, to ensure a normalized tumor vasculature even in the situation of intratumor hypoxia. The present study demonstrated that the downregulation of HIF-1α and VEGF in melanoma cells was dependent on the differential expressions of various transcription factors involved in the hypoxia signaling cascade. This could be a novel therapeutic approach, as down-regulating Sp1 and up-regulating Sp3 with *A. paniculata* could ultimately downregulate VEGF expression indirectly by preventing HIF1α binding. We are very optimistic about our findings of the CBP and p300 levels after *A. paniculata* treatment. These experiments were performed in both hypoxia-mimicking and physiological hypoxic conditions.

Conclusion

The study deals with the anti-tumor effect of *Andrographis paniculata* on hypoxic tumor microenvironment of mouse melanoma cells. The tumor cells treated with *A. paniculata* extract demonstrated lower expressions of VEGF and HIF-1α-the two key regulator of hypoxia signalling cascade. The anti-tumor effect of *A. paniculata* was due to its effect on the transcriptional regulators involved in hypoxia signalling cascade. The present research work establishes *Andrographis paniculata* as a potential anti-cancer agent normalizing the chaotic hypoxic tumor microenvironment.

Acknowledgment

We sincerely acknowledge the University of Burdwan for providing the necessary infrastructure for executing the present research.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

References

1. Okhuarobo A, Falodun JE, Erharuyi O, Imieje V, Falodun A, Langer P. Harnessing the medicinal properties of *Andrographis paniculata* for diseases and beyond: A review of its phytochemistry and pharmacology. Asian Pac J Trop Dis 2014;4:213-22.
2. Neeraja C, Krishna PH, Reddy CS, Giri CC, Rao KV, Reddy VD. Distribution of *Andrographis* species in different districts of Andhra Pradesh. Proc Natl Acad Sci India Sect B Biol Sci 2015;85:601.

3. Chopra RN, Nayar SL, Chopra IC. Glossary of Indian Medicinal Plants. New Delhi, India: NISCOM, CSIR; 1956. p. 18.
4. Verma N, Vinayak M. Antioxidant action of *Andrographis paniculata* on lymphoma. Mol Biol Rep 2008;35:535-40.
5. Sheeja K, Shihab PK, Kuttan G. Antioxidant and anti-inflammatory activities of the plant *Andrographis paniculata* nees. Immunopharmacol Immunotoxicol 2006;28:129-40.
6. Abu-Ghefreh AA, Canatan H, Ezeamuzie CI. *In vitro* and *in vivo* anti-inflammatory effects of andrographolide. Int Immunopharmacol 2009;9:313-8.
7. Loenarz C, Coleman ML, Boleininger A, Schierwater B, Holland PW, Ratcliffe PJ, *et al.* The hypoxia-inducible transcription factor pathway regulates oxygen sensing in the simplest animal, *Trichoplax adhaerens*. EMBO Rep 2011;12:63-70.
8. Semenza GL, Jiang BH, Leung SW, Passantino R, Concordet JP, Maire P, *et al.* Hypoxia response elements in the Aldolase A, enolase 1, and lactate dehydrogenase A gene promoters contain essential binding sites for hypoxia-inducible factor 1. J Biol Chem 1996;271:32529-37.
9. Kaelin WG Jr. Ratcliffe PJ. Oxygen sensing by metazoans: The central role of the HIF hydroxylase pathway. Mol Cell 2008;30:393-402.
10. Epstein AC, Gleadle JM, McNeill LA, Hewitson KS, O'Rourke J, Mole DR, *et al.* C. Elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell 2001;107:43-54.
11. Mahon PC, Hirota K, Semenza GL. FIH-1: A novel protein that interacts with HIF-1alpha and VHL to mediate repression of HIF-1 transcriptional activity. Genes Dev 2001;15:2675-86.
12. Lando D, Peet DJ, Gorman JJ, Whelan DA, Whitelaw ML, Bruick RK. FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. Genes Dev 2002;16:1466-71.
13. Arany Z, Huang LE, Eckner R, Bhattacharya S, Jiang C, Goldberg MA, *et al.* An essential role for p300/CBP in the cellular response to hypoxia. Proc Natl Acad Sci U S A 1996;93:12969-73.
14. Aoyama T, Okamoto T, Fukiage K, Otsuka S, Furu M, Ito K, *et al.* Histone modifiers, YY1 and p300, regulate the expression of cartilage-specific gene, chondromodulin-I, in mesenchymal stem cells. J Biol Chem 2010;285:29842-50.
15. Xia Y, Jiang L, Zhong T. The role of HIF-1α in chemo-/radioresistant tumors. Onco Targets Ther 2018;11:3003-11.
16. Singh D, Arora R, Kaur P, Singh B, Mannan R, Arora S. Overexpression of hypoxia-inducible factor and metabolic pathways: Possible targets of cancer. Cell Biosci 2017;7:62.
17. Odebiyi OO, Sofowora EA. Phytochemical screening of Nigerian medicinal plants II. Lloydia 1978;41:234-46.
18. Okail MS. Cobalt chloride, a chemical inducer of hypoxia-inducible factor-1α in U251 human glioblastoma cell line. J Saudi Chem Soc 2010;14:197-201.
19. Muz B, de la Puente P, Azab F, Azab AK. The role of hypoxia in cancer progression, angiogenesis, metastasis, and resistance to therapy. Hypoxia (Auckl) 2015;3:83-92.
20. Vaupel P, Mayer A. Hypoxia in cancer: Significance and impact on clinical outcome. Cancer Metastasis Rev 2007;26:225-39.
21. Semenza GL. Targeting HIF-1 for cancer therapy. Nat Rev Cancer 2003;3:721-32.
22. Semenza GL. Regulation of oxygen homeostasis by hypoxia-inducible factor 1. Physiology (Bethesda) 2009;24:97-106.
23. Jain RK. Barriers to drug delivery in solid tumors. Sci Am 1994;271:58-65.
24. Tsuzuki Y, Fukumura D, Oosthuyse B, Koike C, Carmeliet P, Jain RK. Vascular endothelial growth factor (VEGF) modulation by targeting hypoxia-inducible factor-1alpha–> hypoxia response element–> VEGF cascade differentially regulates vascular response and growth rate in tumors. Cancer Res 2000;60:6248-52.
25. Apt D, Watts RM, Suske G, Bernard HU. High Sp1/Sp3 ratios in epithelial cells during epithelial differentiation and cellular transformation correlate with the activation of the HPV-16 promoter. Virology 1996;224:281-91.
26. Li J, Zhang C, Jiang H, Cheng J. Andrographolide inhibits hypoxia-inducible factor-1 through phosphatidylinositol 3-kinase/AKT pathway and suppresses breast cancer growth. Onco Targets Ther 2015;8:427-35.
27. Ahmada MS, Ahmad S, Arshad M, Afzald M. *Andrographis paniculata* a miracle herbs for cancer treatment: *In vivo* and *in vitro* studies against aflatoxin B1 toxicity. Egypt J Med Hum Genet 2014;15:163-71.
28. Shi L, Zhang G, Zheng Z, Lu B, Ji L. Andrographolide reduced VEGFA expression in hepatoma cancer cells by inactivating HIF-1α: The involvement of JNK and MTA1/HDCA. Chem Biol Interact 2017;273:228-36.