A Tumoural Angiogenic Gateway Blocker, BP-1B Represses the HIF-1α Nuclear Translocation and Its Target Gene Activation against Neoplastic Progression
2.1. Introduction

Hypoxia-inducible factor-1 (HIF-1) is a heterodimeric transcription factor that regulates oxygen homeostasis in mammalian cells and it is highly involved in the pathology of a number of diseases associated with hypoxia including cancer. HIF-1 consists of a hypoxia-inducible oxygen sensitive unstable α-subunit and a constitutively expressed stable β-subunit. Under normoxia, the α-subunit is constitutively targeted for degradation by post-translational modification by proline hydroxylase (PHD) and by von Hippel-Lindau (VHL) ubiquitin ligase complexes. When oxygen becomes low, inhibition of PHD/VHL mediated ubiquitin ligase pathway leads to HIF-1α protein accumulation and stabilization [Fong, 2009; Wang et al., 1995; Kaelin Jr, 2005; Madanecki et al., 2013]

Tumour hypoxia is a well characterized critical factor for HIF-1α stabilization which facilitates nuclear translocation of HIF-1α, a hallmark event of HIF-1 transcriptional activation [Hong et al., 2004; Triantafyllou et al., 2008; Maroni et al., 2015; Chun et al., 2000]. Transcriptional activation of HIF-1 is a complex multistep process which involves HIF-1α stabilization, phosphorylation by p42/44 MAPK, nuclear localization, dimerization with HIF-1β, binding of HIF-1 complex to hypoxia-response elements of target genes and enrollment of co-factors. Also, HIF-1α nuclear localization and transcriptional activation is tightly regulated by p44/42 (Erk-1/2). The p44/42 pathways control HIF-1α post-translationally through phosphorylating one threonine (Thr796) and two serine residues (Ser641 and Ser643). This direct phosphorylation of HIF-1α by p44/42 is important for nuclear translocation and recruitment of co-activators which is a hallmark event for HIF-1α transcriptional activation [Lauzier et al., 2007; Richard et al., 1999]. Inhibition of any step in this process will eventually lead to the transcriptional inactivation of HIF-1 [Fong, 2009; Hong et al., 2004; Park et al., 2004; Semenza, 2003]. Therefore, HIF-1 transcription complex induces the expression of a battery of genes such as Vascular Endothelial Growth Factor-A (VEGF-A), fms related tyrosine kinase 1 (Flt-1), Angiopoietin-1 (Ang-1), Matrix Metallo Proteases (MMP-2 and MMP-9) which promotes the tumour angiogenesis, invasion and metastasis [Park et al., 2004; Semenza, 2003; Fong, 2008; Mole et al., 2009; Schodel et al., 2011].

Most of the solid tumour including head and neck, lung, breast, ovarian, liver renal carcinomas and so on expresses the hypoxia phenomenon (HIF-1α), as significant regions of the tumours are located at a great distance from the supporting
blood vessels (Table 2.1) [Hong et al., 2004; Folkman, 1972]. Overexpression of HIF-1α is associated with a malignant phenotype and poor prognosis [Gordan et al., 2007; Maxwell et al., 2007]. Hence, identification of small molecules that affect nuclear import of HIF-1α and its transcriptional activity is an important part of the pharmacological approach for treatment of many cancer types [Gordan et al., 2004; Maxwell et al., 2005].

Benzophenones are a group of compounds with more than 300 plus biologically active members, which have great structural diversity but share a common phenol-carbonyl-phenol skeleton. Arrays of natural benzophenones are found to be significantly cytotoxic against human neoplastic cell lines [Wu et al., 2014]. In addition, the strategy to synthesize benzophenones derivative has attracted wide-ranging consideration due to their potential pharmacological activity. In recent years, our group has identified a number of novel benzophenone-1 derivatives as potent anticancer candidate [Prabhakar et al., 2006a; Vijay Avin et al., 2014a; Prashanth et al., 2014; Gurupadaswamy et al., 2014; Al-Ghorbani et al., 2016, 2017]. Furthermore efforts have been made to enrich pharmacological potency of benzophenone-1 by combining BP-1 pharmacophore with benzimidazole moiety. One such pharmacophore, Benzophenone-1B (BP-1B) or [IUPAC name: 4-(1H-benzimidazol-2-ylmethoxy)-3,5-dimethylphenyl]-4-methoxyphenyl]methanone with one methoxy and two methyl groups on the benzophenone ring (Fig. 2.1) was found to be effective against murine ascites carcinoma targeting neovasculature [Ranganatha et al., 2013]. In continuation, BP-1B is targeted for tumour angiogenesis since biological therapies that target physiological processes required for malignant tumour growth is an effective strategy to prevent the cancer. In the present investigation, the cellular biochemical modulation underlying inhibition of tumour angiogenesis was investigated on various angiogenic assay systems and tumour models. Here we report that BP-1B effectively represses the nuclear localization and transcriptional activation of HIF-1α.
Figure 2.1: Structure of [4-(1H-benzimidazol-2-ylmethoxy)-3,5-dimethylphenyl]-(4-methoxyphenyl) methanone (BP-1B)

Table 2.1: List of cancer cells overexpressing the HIF-1α

<table>
<thead>
<tr>
<th>SL. No.</th>
<th>Cancer types</th>
<th>HIF-1α expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Squamous cell carcinoma (SCCs)</td>
<td>++++</td>
</tr>
<tr>
<td>2.</td>
<td>Lung cancer</td>
<td>++++</td>
</tr>
<tr>
<td>3.</td>
<td>Breast cancer</td>
<td>++++</td>
</tr>
<tr>
<td>4.</td>
<td>Renal cancer</td>
<td>++++</td>
</tr>
<tr>
<td>5.</td>
<td>Cervical cancer</td>
<td>++++</td>
</tr>
<tr>
<td>6.</td>
<td>Lymphoma</td>
<td>++++</td>
</tr>
<tr>
<td>7.</td>
<td>Liver cancer</td>
<td>++++</td>
</tr>
<tr>
<td>8.</td>
<td>Ovarian cancer</td>
<td>+++</td>
</tr>
<tr>
<td>9.</td>
<td>Prostate cancer</td>
<td>+++</td>
</tr>
<tr>
<td>10.</td>
<td>Pancreatic cancer</td>
<td>+++</td>
</tr>
<tr>
<td>11.</td>
<td>Rectal cancer</td>
<td>++</td>
</tr>
<tr>
<td>12.</td>
<td>Brain tumour</td>
<td>++</td>
</tr>
<tr>
<td>13.</td>
<td>Colon cancer</td>
<td>+</td>
</tr>
</tbody>
</table>

HIF-1α staining by immunohistochemistry (IHC): + indicates the 25-50% of specimen, ++ 50-75% of specimen, +++ more than 75% of specimen, ++++ more than 85% of specimen.
2.2. Materials and Methods

2.2.1. Materials

The human cancer cell lines, lung adenocarcinoma (A549, NCI-H23), breast adenocarcinoma (MCF-7, MDA-MB-231), hepatocellular carcinoma (HepG2, Hep3B), renal carcinoma (ACHN, A498) and normal fibroblast cells (NIH-3T3) from National Centre for Cell Science (NCCS), Pune, India. Cervical carcinomas (CaSki, SiHa and HeLa) and squamous cell carcinoma (SCC-9, A388) from National Centre for Biological Science (NCBS), Bengaluru, India and Murine Dalton’s Ascites Lymphoma (DLA) cells, a kind gift from Dr. Sathees C. Raghavan, Indian Institute of Science (IISc), Bengaluru, India. Human Umbilical Vascular Endothelial Cells (HUVEC) from American Type Cell Culture (ATCC), USA. Sodium bicarbonate (NaHCO₃), extracellular matrix gel (ECM or matrigel), hydron polymer (poly-hydroxyethyl-methacrylate (poly HEMA), Sucralfate solution, gelatin type A porcine skin, cobalt chloride (CoCl₂). 3-(4,5-dimethylthiazole-2-yl )-2,5-diphenyltetrazolium bromide (MTT), trypan blue, crystal violet, PMSF and anti-mouse IgG, Endothelial cell growth media (EGM), Dimethyl Sulphoxide (DMSO) & protease inhibitor cocktail were obtained from Sigma Aldrich, USA. Anti-rabbit IgG and anti-mouse IgG, Genei, Bengaluru, India. Dulbecco’s Modified Eagle Media (DMEM), Penicillin-Streptomycin, Amphotericin B (antimycotic solution), Trypsin-EDTA solution, Fetal Bovine Serum (FBS) from Invitrogen (Gibco), USA. Immunostaining kit from Leica Biosystems, New Castle Upon Tyne, UK. Anti-CD-31, anti-HIF-1α, anti-flt-1, anti-VEGF-A, anti-ang-1, anti-MMP-2 and anti-MMP -9 from Santacruz Biotechnology, USA. Anti-β–actin and anti–laminB from BD Bioscience, USA. Alexa fluor 488-anti-IgG from Thermo Fisher Scientific, Waltham, MA, USA, Anti-p42/44 and phospho p42/44 from Cell Signaling Technology, Danvers, USA. 5-bromo-4-chloro-3-indolylphosphate (BCIP)/nitro-blue tetrazolium (NBT), p-Nitrophenylphosphate (pNPP) substrate solution from Chromous Bio, Bengaluru, India. Barbitone, sodium-β-glycerolphosphate, Amino napthol sulphonic acid (ANSA), Creatinine, Picric acid, Diacetylmonoxime from E-Merck, Bengaluru, India. All the other chemicals used were of analytical grade from Himedia, Mumbai, India. T-flasks, Culture dishes, 96 well plates, 24 well plates, Transwell plates, Micropipettes, Ampoules from Corning, USA and Eppendorf, Germany. All the assays were performed at minimum three independent times and analyzed.
2.2.1.1. Emergence of active Benzophenone-1-Benzimidazole (BP-1B) hybrid

Benzophenones are well known for their potent pharmacological activity including antitumour potentials. Based on structure activity relationship, structuring benzophenone (BP-1) as parental skeleton various active analogs were synthesized by incorporating benzimidazole moiety, methyl and methoxy groups as anticancer agents with the assistance of chemist collaborators of our research group “Dr. Shaukath Ara Khanum & groups, Department of Chemistry, Yuvaraja’s College, University of Mysore, Mysuru, Karnataka”. The analogs were preliminarily screened for cytotoxic potency against murine mammary ascites carcinoma (EAC) and BP-1B emerged as lead active molecules with potent anti-angiogenic activity (Table 2.2 and Table 2.3) [Ranganatha & Prabhu et al., 2013]. The cytotoxic molecule BP-1B used for current antiangiogenic/anticancer studies to delineate the molecular mechanism of BP-1B on tumour angiogenesis.

Table 2.2: The emergence of BP-1B as a potent anti-cancer drug based on structure activity relationship (SAR).

<table>
<thead>
<tr>
<th>Molecular Structure</th>
<th>Functional Groups</th>
<th>Cytotoxicity (IC₅₀ value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzophenone-1</td>
<td>-</td>
<td>62.5 µM</td>
</tr>
<tr>
<td>Benzophenone-1-Benzimidazole</td>
<td>Benzimidazole</td>
<td>23.8 µM</td>
</tr>
<tr>
<td>Benzophenone-1-Benzimidazole</td>
<td>Two methyl groups</td>
<td>14.4 µM</td>
</tr>
<tr>
<td>Benzophenone-1-Benzimidazole</td>
<td>One methoxy group</td>
<td>4.5 µM</td>
</tr>
</tbody>
</table>
Table 2.3: The characteristics of BP-1B cytotoxic molecule

<table>
<thead>
<tr>
<th>Properties</th>
<th>Descriptions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Empirical Formula</td>
<td>C$<em>{24}$H$</em>{22}$N$_2$O$_3$</td>
</tr>
<tr>
<td>IUPAC Name</td>
<td>4-(1H-benzimidazol-2-ylmethoxy)-3,5-dimethylphenyl</td>
</tr>
<tr>
<td>Molecular Weight</td>
<td>387</td>
</tr>
<tr>
<td>Solubility</td>
<td>DMSO</td>
</tr>
<tr>
<td>Active Groups</td>
<td>Two methyl groups and one methoxy groups</td>
</tr>
</tbody>
</table>

2.2.2. The effect of BP-1B on cancer proliferation under *in-vitro* condition

2.2.2.1. *In-vitro* animal cell culture

a) Aseptic technique and cell culture practice

All the experiments were carried out strictly under aseptic conditions in class-II biosafety cabinet (Thermo scientific, USA). The working place of the biosafety cabinet was thoroughly sprinkled and swabbed with 70% ethanol. The surface of all the vessels and other instruments used for the study were also cleaned with alcohol. The equipments taken into the cabinet during cell culture procedures (media bottles, pipette tip boxes, pipette aids) were wiped with 70% ethanol. The chamber was sterilized with integral UV rays continuously for 1h before and after any cell culture procedure. The contaminated gloves or hands by touching anything outside the cabinet (especially face and hair) were re-sanitized with 70% ethanol and discarded after handling contaminated cultures.

b) Selection of cell lines

For the current investigation various types of cancer cell lines of different origin were selected. The human cancer cells SCC-9, A388, A549, NCI-H23, MCF-7, MDA-MB-231, HepG2, Hep3B, ACHN, A498, Hela, CaSki, SiHa, human normal fibroblast cells (NIH-3T3) and mouse cancer cells DLA were selected for cell based screening of BP-1B as an anti-cancer drug. All the human cells were stored and maintained aseptically under liquid nitrogen vapor and DLA cells were maintained in peritoneum of mice by serial transplantation and cultured in DMEM at the time of *in-vitro* drug treatment.
c) **Culture media preparation**

For the current research DMEM supplemented with Penicillin (50-100 U/ml) -Streptomycin (50–100 µg/ml), Amphotericin B (0.25–2.5 µg/ml), 10% heat inactivated fetal bovine serum (FBS), and 0.37% of sodium bicarbonate was used. For media preparation, the powdered DMEM was dissolved completely in sterilized double distilled water (10% less) and sodium bicarbonate, penicillin-streptomycin, Amphotericin were added and pH (0.2 less to desire) was adjusted as per the manufacturer’s recommendation. The prepared media then subjected to filter sterilization using 0.22 micron cellulose membrane filter (Milipore, USA) by suction pressure through vacuum pump (Tarsons, India). After filter sterilization, 10% FBS was added to media under aseptic condition and used for cell culture.

d) **Culturing of cells**

The ampoules containing various cells were removed from liquid nitrogen and submerged in a water bath at 37°C temperature for thawing and transferred to class II safety cabinet. The cells were added into pre-warmed growth medium slowly to dilute out the DMSO in T-flask or cell culture dishes. The cells were incubated for 2 h in CO₂ incubator (Thermo scientific, USA) at 37°C in an atmosphere of 95% air and 5% CO₂, with 98% humidity and media was replaced with fresh media. The cells were monitored microscopically in phase contrast inverted microscope (Olympus, Japan) and sub-cultured for experimental process.

In case of DLA cells, the cells were harvested from peritoneum of mice and washed with the phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.47 mM KH₂PO₄, pH-7.4). The cells were counted using neubaur haemocytometer chamber and exactly 1x10⁶ cells were inoculated on the 60 mm culture dish containing sterilized DMEM media under aseptic condition. The cultured cells were used to study the BP-1B cytotoxic and antiproliferative effects.

2.2.2.2. *In-vitro* BP-1B treatment

The cells SCC-9, A388, A549, NCI-H23, MCF-7, MDA-MB-231, HepG2, Hep3B, ACHN, A498, Hela, CaSki, SiHa, NIH-3T3 and DLA were grown in DMEM medium, supplemented with 10% FBS in 96 well plates and incubated at 37°C in CO₂ incubator. The cells were serum starved for 2 h and treated with varying concentration of BP-1B (0, 2, 5, 10, 25, 50, 100 µM in DMSO) for 48 h and
cytotoxic efficacy of BP-1B was investigated by Trypan blue dye exclusion, MTT assay and Lactate dehydrogenase (LDH) release assay as described below.

2.2.2.3. Determination of BP-1B cytotoxic effect by cytotoxic studies

a) Trypan blue dye exclusion assay

The effects of cytotoxic molecule BP-1B on various types of cancer cells were determined by Trypan blue dye exclusion assay. The cultured cells were treated with BP-1B for 48 h in triplicates (n = 3). After incubation period, the cells were detached by 0.5 % trypsin-EDTA solution, harvested by centrifugation at 3000 rpm for 5 min and diluted in 1 ml of phosphate buffered saline (PBS). Trypan blue dye solution (0.4%) 10 µl was added to each sample and cells were re-suspended well. About 20 µl of cell suspensions were carefully and continuously filled in the haemocytometer chamber and viable cells were counted in four 1 x 1 mm squares of each chamber and the average number of cells per square determined [Grailler et al., 1988]. Total viable cells were counted by following formula,

\[
\text{Total viable cells} = \frac{A + B + C + D}{4} \times \text{Dilution factor} \times 10^4 \text{ cells /ml}
\]

Values were plotted in MS excel version 8.1 and graphed and the inhibitory concentration-50 (IC_{50}) values were estimated.

b) MTT assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution (5 mg/ml) was prepared by dissolving MTT in sterile PBS (pH 7.5) and filtered through 0.22 micron membrane filter to sterilize and remove the little trash of insoluble residues. To the 100 µl of cell suspension in 96 well culture plates 1/10th volume of MTT was added to the various cells treated with BP-1B and incubated for 4 h in a humidified incubator at 37°C. After the incubation period, 200 µl of DMSO was added to each well and re-suspended methodically to dissolve the insoluble blue formazan crystals. The plates were read on the microtitre plate-ELISA reader (Robonics, India) at 570 nm. This experiment was performed in triplicates (n = 3) and values were obtained. The readings were recorded and the IC_{50} values of BP-1B against each cancer cell lines were calculated [Gerlier & Thomasset, 1986].
c) **Lactate Dehydrogenase (LDH) release assay**

The various cancer cells were cultured and exposed to increasing concentration of BP-1B for 48 h. After incubation, the culture media along with the cells were centrifuged at $4^\circ\text{C}$ for 5 min at 3000 rpm. The supernatant was removed and stored in another plate as it was in older plate. About 800 µl of 1% β-NADH solution prepared in buffer-A (81.3 mM Tris base, 203.3 mM NaCl, pH - 7.2) was mixed with 70 µl of supernatant. The reaction was started by adding 170 µl of 9.76 mM Pyruvate solution in buffer-A. The cells (pellet) were lysed using 0.1% Triton-X 100 in 100 µl of PBS. About 70 µl of cell lysate was mixed with 800 µl of 1% β-NADH solution in buffer A and the reaction started by adding 170 µl of pyruvate solution. The reaction was measured at 490 nm using an ELISA reader. The percentage of LDH release was calculated [Mosmann, 1983] as follows

$$\text{LDH release} = \frac{\text{LDH release in media}}{(\text{LDH release in media} + \text{intracellular LDH release})} \times 100$$

2.2.2.4. **Colony formation assay**

Colony formation assay is an apposite system for validation of prolonged cytotoxic potentiality of cytotoxic molecules [Franken et al., 2006].

- **Cell preparation:** The SCC-9 and A549 cells cultured in T-flasks were trypsinized with 0.5% trypsin for 5 min and harvested thoroughly, washed with PBS thrice. The cell concentration was determined by counting the cells by haemocytometer. The desired seeding concentration (400 ± 10 cells/dish) was fixed and then seeded into dishes.

- **Treatment and clonogenesis:** The cells were cultured for 2 h in CO$_2$ incubator at $37^\circ\text{C}$ to allow them to attach with dishes. The cells were exposed with BP-1B at 0, 3, 5, 10 µM ($n = 3$) of concentration for 6 h at CO$_2$ incubator and rinsed with fresh medium twice. The cells were cultured further in DMEM containing 10% FBS for 12 days to form the colonies. Then media was discarded and colonies were fixed with adding 1 ml of methanol to each well, stained with crystal violet (0.4 g/L) for 15 min at room temperature. The plates were rinsed three times with tap water. The 50 cells per colony was counted by phase contrast inverted microscope.
(Olympus, Japan) and photographed with Canon power shot Sx500 IS camera. The colony formation inhibition was calculated by the formula

\[
\text{Colony inhibition} = \frac{\text{Number of colonies counted}}{\text{Number of cells plated}} \times 100
\]

2.2.3. BP-1B efficacy on in-vitro tumour hypoxic conditions.

2.2.3.1. Hypoxic conditions, in-vitro treatment

To examine the BP-1B efficacy on tumour microenvironment events, various oxygen conditions were used.

a) BP-1B treatment under normoxic condition: The SCC-9, A549 and MCF-7 cells were cultured in 60 mm dishes containing DMEM and grouped into four (\(n = 3/\text{Group}\)). The cells were treated with BP-1B at 0 \(\mu\text{M}\) (DMSO, Group-A), 3 \(\mu\text{M}\) (Group-B), 5 \(\mu\text{M}\) (Group-C) and 10 \(\mu\text{M}\) (Group-D) and incubated for 48 h at 5\% CO\(_2\), 98\% humidity in CO\(_2\) incubator which served as control for hypoxic and pseudohypoxic condition for each experimental setup. Then cells were subjected to lysate preparations.

b) BP-1B treatment under hypoxic condition: The SCC-9, A549 and MCF-7 cells were cultured in 60 mm dishes containing DMEM and grouped into four (\(n = 3/\text{Group}\)). The cells were treated with BP-1B at 0 \(\mu\text{M}\) (DMSO, Group-A), 3 \(\mu\text{M}\) (Group-B), 5 \(\mu\text{M}\) (Group-C) and 10 \(\mu\text{M}\) (Group-D) and incubated for 24 h in a hypoxia chamber with a mixed gas of 1\% O\(_2\), 5\% CO\(_2\) and 94\% N\(_2\) in CO\(_2\) incubator. After incubation, the cells were subjected to lysate preparations.

c) BP-1B treatment under pseudohypoxic condition: The chemical Cobalt Chloride (CoCl\(_2\)) is a well known to induce the pseudohypoxic nature in in-vitro. It acts as a hypoxic mimetic agent by upregulating intracellular hypoxia inducible factors (HIFs). For current investigation, CoCl\(_2\) was used to evaluate the BP-1B effect on HIF-1\(\alpha\) in tumourigenesis. The SCC-9, A549 and MCF-7 cells were cultured in 60 mm dishes containing DMEM with 10\% FBS and categorized into five groups as follows,
The culture dishes were incubated for 48 h with 5% CO\textsubscript{2}, 98% humidity in CO\textsubscript{2} incubator. After incubation, the conditioned media was collected and stored for VEGF-A quantification by ELISA and MMP-2 and MMP-9 activity by gelatin zymogram. The cells from all types were subjected to lysate preparations.

2.2.3.2. Lysate preparations and quantification

a) Cytosolic and Nuclear Fractions:

The SCC-9, A549 and MCF-7 cells treated with BP-1B under pseudo-hypoxic and hypoxic conditions were harvested thoroughly by scraping the cells with sterile cell scraper under ice-cold condition. The harvested cells were suspended in 400 µl of Solution-A (10 mM Potassium chloride (KCl), 10 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 0.5 mM Dithiothreitol (DTT), 0.5 mM Phenylmethylsulfonyl fluoride (PMSF), 1.5 mM Magnesium chloride (MgCl\textsubscript{2}), 0.05% Nonidet P-40 (NP40) and Protease inhibitor cocktail). The cell suspensions were lysed with 10 strokes by using homogenizer under ice-cold conditions. Then, lysed cell suspensions were transferred to 1ml microfuge tubes and centrifuged for 4\textdegree{}C for 1 min at 14,000 rpm. Supernatant containing cytosolic fractions were collected in fresh microfuge tubes, quantified and stored at -20\textdegree{}C freezer until further use. About 200µl of Solution B (5 mM HEPES, 0.5 mM DTT, 0.5 mM PMSF, 1.5 mM MgCl\textsubscript{2}, 0.2 mM EDTA, 26% Glycerol (v/v) and Protease inhibitor cocktail) was added to each residual pellets, re-suspended thoroughly. The suspensions were lysed again with homogenizer for 10 strokes and kept for 30 min in ice. The lysed cell suspensions were centrifuged at 14,000 rpm, 4\textdegree{}C for 1 min and supernatant containing nuclear fractions were collected in fresh microfuge tubes, quantified and stored at -20\textdegree{}C freezer [Xu et al., 2013].

<table>
<thead>
<tr>
<th>Group (n = 3/group)</th>
<th>CoCl\textsubscript{2} (100 µM)</th>
<th>BP-1B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group – 1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Group – 2</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Group – 3</td>
<td>+</td>
<td>3 µM</td>
</tr>
<tr>
<td>Group – 4</td>
<td>+</td>
<td>5 µM</td>
</tr>
<tr>
<td>Group – 5</td>
<td>+</td>
<td>10 µM</td>
</tr>
</tbody>
</table>
b) Whole cell lysate preparation:

The SCC-9, A549 and MCF-7 cells treated with BP-1B under normoxic/hypoxic/pseudo-hypoxic conditions were harvested thoroughly by scraping the cells with sterile cell scraper under ice-cold condition. The harvested cells were suspended in 400 µl of pre-chilled Radioimmunoprecipitation assay (RIPA) buffer (100 mM Tris buffer pH- 7.5, 140 mM sodium chloride (NaCl), 0.1% Sodium dodecyl sulfate (SDS), 5 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.5 mM PMSF and Protease inhibitor cocktail). The suspensions were lysed with homogenizer for 10 strokes, vortexed for 30 sec and kept on ice for 30 min. Around 2 U/ml of DNase I and RNase were added to suspensions. The lysed cell suspensions were centrifuged at 10,000 rpm, 4°C for 30 min and supernatant containing whole cell lysates were collected in fresh microfuge tubes, quantified and stored at -20°C freezer.

c) Quantification:

The cytosolic fractions, nuclear fractions, whole cell lysates and conditioned media from all treated and untreated groups were quantified in Nanodrop-Biospectrophotometry (Eppendorf, Germany) by reading at 280 nm.

2.2.3.3. Cancer cell migration assay

Cancer cell migration assay was performed to assess the anti-migration ability of BP-1B on A549 cells [Cory, 2011; Liang et al., 2007].

i. The appropriate number of A549 cells (1x10⁶ cells/ml) was cultured in DMEM in 24-well plates to form monolayer (100% confluence).

ii. The monolayer cells were scratched uniformly to form 5 mm size wound by using a microtip. Carefully, the cell debris was removed and the media was aspirated. Slowly, fresh DMEM was added into the wells

iii. The wells were divided into five groups and the cells were treated with BP-1B as shown below

<table>
<thead>
<tr>
<th>Group (n = 5/group)</th>
<th>CoCl₂(100 ìM)</th>
<th>BP-1B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group – I</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Group – II</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Group – III</td>
<td>+</td>
<td>3 ìM</td>
</tr>
<tr>
<td>Group – IV</td>
<td>+</td>
<td>5 ìM</td>
</tr>
<tr>
<td>Group – V</td>
<td>+</td>
<td>10 ìM</td>
</tr>
</tbody>
</table>
Then incubated at $37^\circ$C in CO$_2$ incubator for 48 h.

iv. After 48 h of incubation, the cells were fixed with 2 ml of methanol and acetic acid (1:1) mixture and stained with crystal violet (0.4 g/L) for 1 h at room temperature.

v. After staining, wells were washed with tap water thrice and the wound closure and final growth were observed under phase contrast inverted microscope and photographed at identical locations with Canon power shot Sx500 IS camera.

vi. The effect of BP-1B on A549 cell migration was analyzed by comparing final gap width to initial gap width by ImageJ software.

2.2.3.4. Matrigel transwell invasion assay

Anti-invasive efficacy of BP-1B was validated against the A549 cells by following the protocol reported by Tolboom & Huizinga [Tolboom & Huizinga, 2007].

- **Transwell insert preparation:** The ECM gel was thawed and liquefied by maintaining $4^\circ$C for 12 h. The 24 well plate containing transwell insert and ECM liquid maintained ice were kept under aspetic condition (Biosafety cabinet). The transwell insert containing filter membranes (5 µm size pores) were coated with ECM liquid by a pipette having pre-chilled microtips. The transwell inserts were placed 24 well plates and incubated at $37^\circ$C for 1 h in CO$_2$ incubator to allow the ECM liquid to solidify.

- **Cell Maintenance:** The A549 cells cultured in T-flask or dishes was detached by enzymatic process using 0.5% trypsin- EDTA solution. The cells were pelleted by centrifugation for 5 min at 3000 rpm and the existing media was aspirated completely. The cells were washed with PBS thrice and suspended in serum free DMEM.

- **Invasion chamber system:** Serum-free medium containing $5 \times 10^4$ of A549 cells (200 µl) were added on the top of the ECM gel coated transwell inserts in 24 well plates and incubated for at $37^\circ$C for 10 min in CO$_2$ incubator to allow the cells to settle down. Very carefully 700 µl of the complete DMEM containing 10% FBS were added into the bottom of the lower chamber in the 24-well plate using a pipette without making any bubbles.
• **BP-1B treatment:** The well with transwell inserts were alienated into five insert types as below and treated accordingly.

<table>
<thead>
<tr>
<th>Inserts (n = 5/type)</th>
<th>CoCl(_2) (100 µM)</th>
<th>BP-1B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insert type – 1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Insert type – 2</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Insert type – 3</td>
<td>+</td>
<td>3 µM</td>
</tr>
<tr>
<td>Insert type – 4</td>
<td>+</td>
<td>5 µM</td>
</tr>
<tr>
<td>Insert type – 5</td>
<td>+</td>
<td>10 µM</td>
</tr>
</tbody>
</table>

Then cells were incubated at 37\(^0\)C with 5% CO\(_2\) and 98% humidity for 24 h in CO\(_2\) incubator. After incubation, the transwell inserts were removed from the plate. The media was carefully removed and the remaining cells that have not migrated from the top of the membrane were carefully removed by using a wet cotton-tipped applicator.

• **The cell fixation and staining:** About 500 µl of methanol was added into each well of 24 well plates and then lower chambers containing invaded cells were fixed by immersing it in methanol in 24 well plate for 10 min at room temperature. The cells were stained with crystal violet (0.4 g/L) and washed with distilled water twice. Invaded cells were counted in Olympus phase contrast inverted microscope and photographed with Canon power shot Sx500 IS camera.

### 2.2.4. \textit{In-vitro} anti-angiogenic potency of BP-1B on recombinant VEGF\(_{165}\) induced angiogenesis

#### 2.2.4.1. Endothelial cell migration assay

1. The HUVECs were cultured in endothelial cell growth media (EGM) with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin and 100 µg/ml streptomycin at 37\(^0\)C, 5% CO\(_2\) with 98% humidity in CO\(_2\) incubator.

3. The harvested appropriate number of HUVECs (2.5x10\(^5\) cells/ml) were cultured in EGM in a 96-well plate for 100% confluence in 24 h.

4. The HUVEC monolayer cells were scratched uniformly to form 1 mm size wound by using a microtip.

5. Carefully, the cell debris was removed and the media was aspirated. Slowly, EGM media was added against the well wall to cover the bottom of the well.
and initial growth at day 0 was photographed with Canon power shot Sx500 IS camera.

6. The wells were divided into five groups and treated with recombinant VEGF<sub>165</sub> (rVEGF<sub>165</sub>) and BP-1B as below,

<table>
<thead>
<tr>
<th>Group (n = 5/Group)</th>
<th>rVEGF&lt;sub&gt;165&lt;/sub&gt; (30 ng/ml)</th>
<th>BP-1B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group – A</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Group – B</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Group – C</td>
<td>+</td>
<td>3 µM</td>
</tr>
<tr>
<td>Group – D</td>
<td>+</td>
<td>5 µM</td>
</tr>
<tr>
<td>Group – E</td>
<td>+</td>
<td>10 µM</td>
</tr>
</tbody>
</table>

Then cells were incubated at 37ºC in CO<sub>2</sub> incubator for 48 h.

7. After incubation, the wound closure and final growth were observed under phase contrast inverted microscope and photographed at identical locations.

8. The effect of BP-1B on HUVEC cell migration was analyzed by comparing final gap width to initial gap width by ImageJ software.

2.2.4.2. Endothelial cell capillary tube formation assay

Anti-tube forming efficiency of BP-1B was unveiled by performing endothelial cell capillary tube formation assay as mentioned below,

- **Endothelial cell culture:** The endothelial cells (HUVECs) were cultured in endothelial cell growth media (EGM) supplemented with 10% FBS in CO<sub>2</sub> incubator.

- **ECM gel Pre-preparation:** Extracellular matrix membrane (ECM or Matrigel) was liquefied by thawing at 4ºC for overnight and aliquoted into 500 µl in vials under ice bucket using pre-chilled microtips and stored at -20ºC.

- **Preparation of culture plate:** The vials containing ECM gel was thawed at 4ºC for 12 h and transferred to bio-safety cabinet and maintained under 4ºC by keeping the aliquots on ice. The 96 well culture plate was coated with 100 µl of ECM liquid by a pipette having pre-chilled microtips and plate was incubated for 1 h at 37ºC for gelling process.

- **Seeding of ECs:** The HUVECs were harvested in a 15 ml conical tube by centrifugation at 3000 rpm for 5 min and re-suspended cells in culture media.
Around 100µl of HUVECs (2.5x10^5 cells/ml) suspended in EGM medium was added to each well coated with ECM gel.

- **BP-1B treatment**: The wells containing HUVECs were divided into five groups and treated as follows,

<table>
<thead>
<tr>
<th>Groups (n = 5/Group)</th>
<th>rVEGF&lt;sub&gt;165&lt;/sub&gt; (30 ng/ml)</th>
<th>BP-1B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group – A</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Group – B</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Group – C</td>
<td>+</td>
<td>3 µM</td>
</tr>
<tr>
<td>Group – D</td>
<td>+</td>
<td>5 µM</td>
</tr>
<tr>
<td>Group – E</td>
<td>+</td>
<td>10 µM</td>
</tr>
</tbody>
</table>

Then, incubated at 37°C in CO₂ incubator for 24 h for tube formation.

- **Vascular network quantification**: Inhibition of the tube formation was measured by validating number of tubes; number of loops, number of branch nodes, length of tubes under a phase-contrast microscope and photographed. Results were analyzed by by ImageJ software.

### 2.2.4.3. In-vitro (ex-vivo) Chorioallantoic membrane (CAM) assay

The preliminary angiomodulatory effectiveness of BP-1B was investigated by *ex-vivo* CAM assay [Olfa et al., 2005].

- Fertilized hen’s eggs were procured from local market of Shivamogga, Karnataka, India. All the eggs were wiped with 70% alcohol prior to incubation. Then the fertilized eggs were incubated at 37°C in a humidified incubator.

- The condiment cups were sterilized by autoclave and transferred to biosafety cabinet. The sterilized condiment cups were wrapped with cling PVC wrap and sprayed with 70% ethanol and swabbed with cotton.

- On 4<sup>th</sup> day of incubation, a window was made on each eggs with sterile scalpel and the entire egg contents were poured on condiment cups wrapped with PVC wrap separately. These preparations (*ex-vivo* CAMs) were closed with sterile petridish and re-incubated for 24 h.

- After incubation, *ex-vivo* CAMs were separated into five systems and treated with rVEGF<sub>165</sub> and BP-1B as below,
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<table>
<thead>
<tr>
<th>Systems (n = 6/system)</th>
<th>rVEGF165 (10 ng/CAM)</th>
<th>BP-1B</th>
</tr>
</thead>
<tbody>
<tr>
<td>System 1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>System 2</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>System 3</td>
<td>+</td>
<td>3 µg/CAM</td>
</tr>
<tr>
<td>System 4</td>
<td>+</td>
<td>5 µg/CAM</td>
</tr>
<tr>
<td>System 5</td>
<td>+</td>
<td>10 µg/CAM</td>
</tr>
</tbody>
</table>

After treatment, all ex-vivo CAM systems were re-incubated for 48 h at humidified incubator.

- Angiogenesis modulations on ex-vivo CAMs of treated and untreated were photographed and analyzed. Vessel length was calculated by ImageJ software.

2.2.4.4. Rat aortic ring assay

Rat aortic ring assay was performed by following the protocol reported earlier with minor modification to validate the angiopreventive activity of BP-1B [Kruger et al., 2000].

- **Separation of aorta**: The thoracic aorta was surgically separated from Swiss albino rats weighing 150–155 g under sterile conditions and immediately washed with sterile physiological phosphate buffered saline (PBS) thrice and serum free DMEM once. Fibroadipose tissue and fat bodies around the vessel was accurately removed by fine micro-dissecting forceps and scissors.

- **Culture of aortic rings**: The aorta was chopped into 1 mm wide tiny rings. The rings were washed with PBS three times and DMEM containing 100 U/ml of penicillin, 100 µg/ml of streptomycin, and 0.25 µg/ml of Amphotericin B. The wells of 96 well plates were filled with 100 µl of ECM gel under 4°C and gelled by keeping at 37°C for 30 min. The rings were embedded in each well (1 ring/ well) by using ECM gel.

- **Treatment with BP-1B**: The wells with aortic rings were categorized into five groups treated with rVEGF165 and BP-1B as mentioned below. Then, the rings were cultured in DMEM of 20% FBS for 6 days at 37°C in humidified CO₂ incubator.
<table>
<thead>
<tr>
<th>Group (n = 5/Group)</th>
<th>rVEGF₁₆₅ (30 ng/ml)</th>
<th>BP-1B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group – A</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Group – B</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Group – C</td>
<td>+</td>
<td>3 µM</td>
</tr>
<tr>
<td>Group – D</td>
<td>+</td>
<td>5 µM</td>
</tr>
<tr>
<td>Group – E</td>
<td>+</td>
<td>10 µM</td>
</tr>
</tbody>
</table>

- **Angiomodulation:** After the indicated time period, endothelial sprouting from aortic rings were photographed with Canon power shot Sx500 IS camera connected to the microscope (Olympus, magnification 20x). Measurement of the area covered by blood vessels (in mm²) was performed using the software ImageJ. The distance from the ring to the edge of the cell front (in millimeters), reflecting cell proliferation, migration and tube formation was assessed by the same software and the concentration dependant effect of BP-1B on endothelial sprouting was documented.

### 2.2.5. Animals and ethics

The animal models used for the study includes healthy Swiss albino and BALB/c mice weighing (25–30 g) and Male Swiss albino rat (150–155 g). All the animals were grouped and housed in polyacrylic cages with not more than ten animals per cage with adequate food and water supply and maintained under secured conditions (27 ± 6°C) with 12 ± 2 h dark/light cycle. The standard food pellets procured from Krish Scientist’s Shopee, Bengaluru, India and feed to animals during course of experimentations. All procedures for animal experimentation were carried out in accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines and approved by the Institutional Animal Ethics Committee, National College of Pharmacy, Shimoga, Karnataka, India (NCP/IAEC/CL/101/05/2012-13) and S.J.M. College of Pharmacy, Chitradurga, Karnataka, India (SJMCP/IAEC/09/2015-16).

### 2.2.6. In-vivo efficacy of BP-1B on recombinant VEGF₁₆₅ induced neoangiogenesis

#### 2.2.6.1. Rat corneal micropocket assay

The rat corneal micro pocket assay was performed to evaluate the effect of BP-1B [Zhao et al., 2009]. A normal cornea is clear of vasculature. Although, blood
vessels can be stimulated to grow and continue to exist in the cornea when potent angiogenic factors are administered. This distinctiveness has made the cornea pocket assay one of the most used systems for angiogenesis investigations. The cornea consists several layers of cells. It is therefore possible to implant a pellet containing the angiogenic/antiangiogenic factor of interest in the cornea to examine its angiogenic/antiangiogenic effect [Rogers et al., 2007].

**a) Pellet preparation:**

The poly-HEMA Hydron polymer powder was dissolved in absolute ethanol (12% w/v) for preparation of casting solution at 37°C with uninterrupted stirring over night. Sucralfate solution was prepared by dissolving it in sterile PBS at 100 mg/ml concentration. An equivalent volume of hydron casting solution and sucralfate solution were combined and the aliquot of this mixture was pipetted onto teflon sheet and cut into 2 x 2 mm size. The hydron pellets were grouped into five pellet types and coated with rVEGF\textsubscript{165} and BP-1B as described below.

<table>
<thead>
<tr>
<th>Pellets (n = 5/type)</th>
<th>rVEGF\textsubscript{165} (10 ng/ml)</th>
<th>BP-1B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pellet type – A</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pellet type – B</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Pellet type – C</td>
<td>+</td>
<td>3 µg/pellet</td>
</tr>
<tr>
<td>Pellet type – D</td>
<td>+</td>
<td>5 µg/pellet</td>
</tr>
<tr>
<td>Pellet type – E</td>
<td>+</td>
<td>10 µg/pellet</td>
</tr>
</tbody>
</table>

Then, permitted to air dry under a laminar flow chamber at room temperature for 1 h. The pellets were incubated for overnight at 4°C. The entire procedures were executed under sterile state.

**b) Pellet implantation:**

Rats were anesthetized with intraperitoneal injection of the mixture of ketamine (75 mg/kg) and xylazine (5 mg/kg). A drop of topical anesthetic Tetracaine was applied on the cornea of rat eyes and placed under a surgical microscope. A 1.5 mm external slit was made at the cornea and a micropocket was formed with a von Graefe cataract knife. The prepared sterile Hydron pellet was rehydrated with a drop of sterile PBS and a pellet was inserted slowly with a pair of forceps into the pocket. The cornea was applied with ophthalmic ointment Tobramycin after pellet implantation. Newly formed blood vessels number and
length were quantified after the five days of the implantation in PBS and rVEGF_{165} and BP-1B treated groups and photographed using Canon power shot Sx500 IS camera. Corneal angiogenesis and remodeling by histopathology was photographed and corneal MVD was quantified.

2.2.6.2. Matrigel plug angiogenesis assay

Matrigel plug angiogenesis assay has been performed for the validation of physiological anti-angiogenic efficacy of BP-1B [Park et al., 2001].

a) Matrigel implantation: The matrigel mix solution was prepared by combining ECM gel, rVEGF_{165}, DMSO and different concentration of BP-1B molecule. The healthy BALB/c mice were grouped into five. Mice were injected subcutaneously (s.c) with 400 µl of ECM gel containing rVEGF_{165} and BP-1B as mentioned below.

<table>
<thead>
<tr>
<th>Group (n = 6/Group)</th>
<th>rVEGF_{165} (30 ng/ml)</th>
<th>BP-1B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group – 1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Group – 2</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Group – 3</td>
<td>+</td>
<td>3 µM</td>
</tr>
<tr>
<td>Group – 4</td>
<td>+</td>
<td>5 µM</td>
</tr>
<tr>
<td>Group – 5</td>
<td>+</td>
<td>10 µM</td>
</tr>
</tbody>
</table>

b) Matrigel angiogenesis:

The matrigel implants were allowed to form neoangiogenesis in mice for 10 days. After 10 days, neovascularization on matrigel plugs were analyzed by removing the matrigel explants from experimental animals surgically. The vascular response to BP-1B on matrigel angiogenesis was observed and photographed with Canon power shot Sx500 IS camera.

c) Quantification of haemoglobin:

The haemoglobin content in each plugs were determined by Drabkin’s method [Park et al., 2001]. The plugs from each group were quantified by weighing them into preweighed tube of 1ml distilled water and homogenized on ice for 10 min. The samples were centrifuged for 5 min at 10,000 rpm in a microcentrifuge (Eppendorf, Germany) and the aqueous phase was collected for haemoglobin measurement. Around 50µl of supernatant were mixed with 950 µl Drabkin’s reagent and incubated at room temperature for 30 min and then 100 µl of these mixtures
were transferred to a 96-well plates. Absorbance was read with an ELISA reader (Robonics, India) at 540 nm. Haemoglobin level in each plugs were determined by comparison with a standard curve. The relative haemoglobin level was calculated by rVEGF\textsubscript{165} versus BP-1B. Haemoglobin concentration is a reflection of the number of blood vessels in the plugs.

2.2.7. Tumour models and treatment

2.2.7.1. CAM Xenograft model

*In-vivo* chicken chorioallantoic membrane (CAM)-A549 xenograft model was performed to evaluate tumour angiogenesis [Sys et al., 2013].

a) CAM pre-preparation

The Gririraja breed fertilized hen’s eggs were procured from local market of Shivamogga, Karnataka, India. All the eggs were wiped with 70% alcohol prior to incubation. Then the fertilized eggs were incubated at 37\(^\circ\)C in a humidified incubator (Rotex, India).

b) A549 cell culture

The A549 cells were cultured in DMEM and harvested by trypsinization followed by centrifugation at 3000 rpm for 5 min and washed with PBS thrice and re-suspended cells in serum free culture media.

c) Cell suspension preparation

The ECM gel was liquefied by thawing at 4\(^\circ\)C for 12 h. Cell suspensions were prepared by combining 100 µl of A549 (1×10\(^6\)) cells, 100 µl of ECM liquid, rVEGF\textsubscript{165} and BP-1B as shown below,

<table>
<thead>
<tr>
<th>Groups (n = 6/Group)</th>
<th>Cell suspension (1×10(^6)cells + ECM)</th>
<th>rVEGF\textsubscript{165} (10 ng/cell suspension)</th>
<th>BP-1B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group – I</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Group – II</td>
<td>+</td>
<td>+</td>
<td>3 µM</td>
</tr>
<tr>
<td>Group – III</td>
<td>+</td>
<td>+</td>
<td>5 µM</td>
</tr>
<tr>
<td>Group – IV</td>
<td>+</td>
<td>+</td>
<td>10 µM</td>
</tr>
</tbody>
</table>

On 8\(^{th}\) day of fertilized eggs incubation, window were made on each egg’s shell carefully with sterile scalpel and 200 µl cell suspensions from all groups were placed directly by pipette with pre-chilled microtips on the CAMs carefully without disturbing the CAMs. All the windows of eggs were sealed with tapes and re-incubated for another 6 days.
d) Tumour inhibition

After incubation, A549-xenograft tumours were isolated from CAMs aseptically and photographed. The tumour parameters such as size, volume were recorded and the part of tumour mass of xenografts were used for cell fractionation process (refer section 2.2.3.2.a) and microvessel density (MVD) assessment by Haematoxyline & Eosin (H & E) stain.

2.2.7.2. Determination of the Lethal Dose 50 (LD₅₀) of BP-1B

For the assessment of LD₅₀ of BP-1B, ‘staircase’ method (Shetty & Alwar, 2007) was used. Healthy Swiss albino male mice weighing 27-30g were employed and were separated into six groups (n = 6, each). BP-1B was dissolved in DMSO and administered to animals at increasing concentrations of 100, 200, 500, 1000, 2000, 3000 mg/kg body weight (b.w) by intraperitoneal (i.p) injections. The mice were then noticed constantly for 4 h for general behavioural, neurological, autonomic profiles and then each 30 min for the next 4 h and lastly for death after 24 h [Botham, 2004]. The maximum non lethal and the minimum lethal doses were thus determined. Based on lethal dose (3000 mg/kg), 1/30th, 1/20th and 1/15th of this LD₅₀ dose (1000 mg/kg) such as 30, 50 and 70 mg/kg was selected as the therapeutic dose for the assessment of in-vivo antiangiogenic and anti-cancer activity of BP-1B molecule.

2.2.7.3. Development of Dalton’s Lymphoma Ascites (DLA) model and treatment

a) Cell transplantation and tumour development

The Swiss albino female mice weighing 27-30 g were acclimated for 10 day by maintaining five animals per group. The Dalton’s lymphoma ascites cell lines is a T-cell lymphoma which form the ascites tumour in mice and usually maintained in peritoneum of mice by serial transplantation. For DLA tumour model development, Swiss albino mice bearing DLA tumour were sacrificed and the DLA cells were harvested by incising peritoneum by sterile scissors under aseptic conditions. The harvested cells were centrifuged by 3000 rpm at room temperature for 5 min. Contaminating RBC’s in DLA cells (if any) were removed by lysing with 0.4% (w/v) ammonium chloride solution. Then cells were washed with thrice with sterile PBS to remove ammonium chloride completely by centrifugation. The cell
pellet was suspended in PBS and cells were counted by haemocytometer. Approximately $5 \times 10^6$ cells/mouse (500 µl of cell suspension) were injected dorsa of the mice intraperitoneally (i.p). The cells were allowed to grow in mice peritoneum to form an ascites tumour with massive abdominal swelling. The animals were observed for the increase in the body weight in daily basis [Al-Ghorbani et al., 2016].

b) BP-1B administration

After 4th day of DLA tumour development, the mice divided in to five groups

<table>
<thead>
<tr>
<th>Name of the group (n = 5/group)</th>
<th>Dalton’s Ascites tumour (DLA)</th>
<th>BP-1B (mg /kg body weight (i.p))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group-A</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Group-B</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Group-C</td>
<td>+</td>
<td>30</td>
</tr>
<tr>
<td>Group-D</td>
<td>+</td>
<td>50</td>
</tr>
<tr>
<td>Group-E</td>
<td>+</td>
<td>70</td>
</tr>
</tbody>
</table>

The tumour bearing animals were treated with BP-1B as described above for three doses (i,p) on every alternate days.

c) Tumour growth

After treatment, Tumour growth was monitored carefully by weighing the body weight of tumour bearing animals. Finally, tumour growth rate of each group was determined by subtracting initial body weight from final day body weight. The tumour growth inhibition was calculated by following formula,

$$\text{Growth (%)} = \frac{\text{Treated tumour growth (g)}}{\text{Control tumour growth (g)}} \times 100$$

d) Ascites volume

After 3rd dose or on 10th day of tumour implantation, DLA bearing mice of all groups were sacrificed, DLA cells along with ascites fluid were harvested and ascites volume was recorded.
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e) **Cell density by trypan blue dye exclusion assay**

The DLA cells pelleted from the above step were washed with PBS thrice by centrifugations. The pelleted DLA cells were re-suspended in PBS and placed on ice. About 10 µl of 0.4% trypan blue dye was added to each sample and cells were counted using haemocytometer. Total numbers of viable cells were counted by entering number of viable cells present in four squares of haemocytometer using following formula,

\[
\text{Total viable cells} = \frac{A + B + C + D}{4} \times \text{Dilution factor} \times 10^4 \text{ cells /ml}
\]

2.2.7.3.1. Peritoneal angiogenesis

The peritoneum of mice from BP-1B treated and untreated groups opened surgically with sterile scissors and forceps. The inner lining of the peritoneal cavity was examined for the neo-vascular sprouting from main vessels and photographed using Canon power shot Sx500 IS camera. The portion of peritoneum tissues from each group of mice was incised and fixed with 10% formalin for H & E staining and CD-31 Immunohistochemistry for MVD quantifications.

2.2.7.3.2. Survivability

DLA tumour was developed by above described procedure. After onset of tumour establishment, ascitic lymphomas (DLA) treated with BP-1B (0, 30, 50 & 70 mg/kg (b.w), n = 10 each group) (i.p) for three doses on every alternate days. After 3\textsuperscript{rd} dose, mice were allowed to maximum survival and survivability were noticed and documented in the form of Kaplan - Meier survivability curve.

2.2.7.4. Solid tumour model development and treatment

a) **Dalton’s Solid lymphoma (DLS) development**

The Swiss albino female mice weighing 27-30 g were acclimated for 10 day by maintaining five animals per group. For Dalton’s solid lymphoma (DLS) tumour model development, Swiss albino mice bearing DLA tumour (donor mice) were sacrificed and the DLA cells were harvested without contamination of RBC by incising peritoneum by sterile scissors under aseptic conditions. Then cells with ascites were suspended with sterile PBS in 1:1 range. Around 400 µl of DLA cells...
(5x10⁶ cells/thigh) from suspension were re-injected into right thigh region of the mice subcutaneously (s.c) to develop DL solid tumour (DLS). Then, tumour volume was measured in mice using vernier calipers in daily basis and recorded [Hegde et al., 2012; Vijay Avin et al., 2014a].

b) BP-1B administration

After onset of DLS tumour development or 100 mm³ of tumour size, the mice divided in to five groups

<table>
<thead>
<tr>
<th>Name of the group (n = 5/group)</th>
<th>Solid Dalton’s Lymphoma (DLS)</th>
<th>BP-1B (mg /kg body weight (i.p))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group-A</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Group-B</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Group-C</td>
<td>+</td>
<td>30</td>
</tr>
<tr>
<td>Group-D</td>
<td>+</td>
<td>50</td>
</tr>
<tr>
<td>Group-E</td>
<td>+</td>
<td>70</td>
</tr>
</tbody>
</table>

The tumour bearing animals were treated with BP-1B as described above for six doses on every alternate days. Tumour growth was monitored regularly by vernier caliper.

c) Tumour Size

After 35th day, mice were sacrificed from all groups by cervical dislocation and then DLS tumours were separated surgically by using sterile scissors, scalpels and forceps. Tumour volume of isolated DLS tumours were measured in weighing balance (Sartorius, Germany). Tumour size of DLS tumours from all groups were compared and photographed.

d) MVD Assessment

The portion of DLS tumour tissue from all groups were fixed with 10% formalin and processed for MVD quantification by H & E stain and CD-31 immunostain.

e) Lysate preparation

The cytosolic fraction and nuclear fractions, whole cell lysates were prepared from DLS tumours of BP-1B treated and untreated groups and quantified as described in the section 2.2.3.1.
f) Serum Collection

For the collection of serum, the blood was collected from all treated and untreated groups and allowed to clot for 30-40 min at 37°C. Serum was separated from the clot and any residual insoluble materials removed by centrifugation at 4°C for 10 min at 10,000 rpm and used for quantification of alkaline phosphates (ALP), creatinine, urea and VEGF-A [Prasanna et al., 2008].

2.2.7.4.1. Acute toxicity assessment

a) Quantification of RBC and WBC in blood

To collect blood samples, the jugular vein of mice from normal, BP-1B untreated and treated groups were incised with sterile scalpel and blood was collected separately in fresh tubes containing 0.3% EDTA, The blood samples were used for the counting of erythrocyte and leukocyte levels with haemocytometer using Leishman’s stain.

b) Estimation of ALP

i. The serum collected from all the groups were subjected for the presence of alkaline phosphatase [Peake et al., 1988].

ii. Standard curve for ALP was prepared by range of 10-50 KA Unit/dL (70- 350 U/L) through phenol working standard solution.

iii. The serum was treated with barbitone buffer (5mM Barbitone, pH- 7.4) initially and the substrate sodium-β-glycerol-phosphatase was added to each samples and then incubated for 37°C for 30 min.

iv. After incubation, the reaction was terminated by adding terminating solution (20% trichloroacetic acid (TCA)). The reaction mixtures were centrifuged for 10 min at 6000 rpm in room temperature and the supernatant was collected in fresh microfuge tubes (Eppendorf, Germany).

v. The supernatant was mixed with 2.5% ammonium molybdate, followed by 20-50 μl of amino napthol sulphonic acid (ANSA). The solution was mixed by gentle shaking and allowed to stand for 10 min at room temperature and the contents were measured at 680 nm in Bio-spectrophotometer (Eppendorf, Germany).
c) Estimation of Creatinine

i. The levels of creatinine was quantified by using the serum from normal, BP-1B treated and untreated animals according to modified Jaffe’s method [Husdan & Rapoport, 1968].

ii. The creatinine standard (1 mg/ml) solution was prepared in distilled water used as the standard.

iii. Working standard creatinine was added in microtitre plate in the 0 - 1 mg/dL range. The standard solution of varying concentrations and serum samples were mixed with 1N NaOH and 1% picric acid.

iv. The reaction mixtures were mixed with vortexing for 1 min and incubated at room temperature for 15 min.

v. The reaction mixtures were measured at 500 nm in Bio-spectrophotometer (Eppendorf, Germany). The creatinine was measured through the standard curve by taking concentration of urea along X-axis and absorbance along Y axis.

d) Estimation of Urea

a) The rate of urea was quantified by using the serum of experimental animals from BP-1B treated and untreated groups according to DAM (Diacetylmonoxime) method [Beale et al., 1961].

b) Reaction mixture (2% DAM) was prepared by dissolving 2 gm of DAM in 100 ml of 2% acetic acid.

c) Working standard urea was added in microtitre plate in the range of 0 - 100mg /dL. The standard solution of varying concentrations and serum was mixed with 0.4 ml of DAM and Acid mixture (25 ml of concentrated H₂SO₄ and 75 ml of 85% orthophosphoric acid and 70 ml of distilled water).

d) All the samples were kept in boiling water bath exactly for 20 min and immediately cooled under running tap water for 5 min and all the contents were mixed with vortexer.

e) The purple coloured complex was measured at 480 nm against blank. The concentration of the urea in the serum sample was measured using standard graph.
2.2.7.4.2. Survivability

DLS tumour was developed and after onset of tumour establishment, mice were treated with BP-1B (0, 30, 50, 70 mg/kg body weight (b.w), n = 10 each group) intraperitoneally (i.p) for six doses on every alternate days. After 6th dose, mice were allowed to maximum survival and survivability were noticed and documented in the form of Kaplan - Meier survivability curve.

2.2.7.5. Haematoxylin and Eosin (H & E) stain for MVD assessment

The H & E staining was performed to quantify the microvessel density (MVD) in rat corneal tissues, peritoneal tissues and DLS tissues of BP-1B treated and untreated groups and to analyze the tissue architecture by following the below mentioned procedure.

a) Preparation of Microsections;

The formalin (10%) fixed tissues were treated with alcohol (70 %, 80% and 95%) for 2 h each wash. The tissues were then treated with xylene (80% and 95%) for 1 h each to clear the alcohol and tissues were immersed in molten paraffin wax in an embedding oven for infiltration and impregnation for 1 h. The infiltrated tissues embedded in mould containing warm liquid paraffin. The sections were taken at 5 µm thickness by using the technique of microtomy, attached to microscopic slides using Mayer’s glycerol-albumin mixture. Prior to staining, the slides were placed in xylene (90%, 80% and 70 %) for 5 min each to remove the paraffin from the sections, then hydrated through decreasing grades of alcohol and finally in distilled water.

b) Staining:

The staining was done by the staining with haematoxylin for 30 min, rinsed with tap water and the slides were dipped in of 0.5 % HCl for few seconds. The sections were dipped in 0.2% ammonia water and washed with tap water. The sections were placed in 95% alcohol and stained with eosin for 1 min. The slides were placed for 2-5 min with 80 %, 95 %, absolute alcohol and lastly with xylene serially. The sections were mounted by DPX with a cover slip and examined under EVOS FL cell imaging system (Thermo Scientific, USA) for the evaluation of MVD and tissue architecture.
2.2.8. Evaluation of molecular mechanism of BP-1B against the tumour angiogenesis

2.2.8.1. Immunohistochemistry (IHC)

The peritoneum tissues of ascites tumour (DLA) bearing mice and solid tumour tissues of treated and untreated with BP-1B (0, 30, 50, 70 mg/kg b.w) were subjected to sectioning process and 5 µm thickness sections were prepared by microtome [Zheng et al., 2013]. The CD-31 immunostain was performed for peritoneal sections and DLS sections. The HIF-1α, VEGF-A, MMP-2 and MMP-9 immunostains were performed for DLS sections using anti-HIF-1α, anti-VEGF-A, anti-MMP-2 and anti-MMP-9 monoclonal antibodies (1:100 dilution) and the NovolinkTM secondary antibody kit as per the manufacturers instruction (Leica Biosystem, UK) as described below.

- **Deparaffinisation:** The slides containing tissue sections were heated at 60°C for 1 h in hot air oven. Then, the slides were deparaffinised by washing with xylene (90%, 80% and 70%) for 6 min each and with isopropyl alcohol (90%, 80% and 70%) for 5 min and then washed with running tap water

- **Antigen retrieval:** Heat-induced epitope retrieval method was used for antigen retrieval. The Slides were boiled in antigen retrieval buffer (Sodium citrate buffer, pH - 6.0) in a pressure cooker until full pressure was reached and maintained for another 2 min.

- **Blocking:** Tissue sections were blocked with peroxide blocking solution for 5 min and then for protein block for 30 min.

- **Primary Antibody incubation:** The slides were then incubated with desired primary antibody and kept for 1 h at room temperature.

- **Secondary antibody incubation:** After washing with the phosphate buffered saline tween 20 (PBST), the slides were incubated with NovoLink™ Polymer anti- mouse IgG secondary antibody and incubated for 30 min.

- **Staining:** The sections were developed by 3, 3 – diaminobenzidizine (DAB) solution for 5 min and washed with tap water and then counter stained with haematoxyline for 5 min and rinsed with water twice.

- **Observation:** All the slides were dehydrated, cleaned and mounted using DPX, observed and photographed under EVOS FL cell imaging system.
Chapter II: The effect of BP-1B on tumour angiogenesis

(Thermo Scientific, USA). The IHC sections were independently evaluated by two pathologists and photographed.

- **MVD assessment:** CD-31 microvascular density (MVD) was assessed by ImageJ software.

### 2.2.8.2. SDS-PAGE and Immunoblots

#### a) Sodium dodecyle sulphate – Polyacrylamide gel electrophoresis (SDS-PAGE)

Equal concentration of protein was resolved on gel using Bio-rad protein mini vertical electrophoresis system (Bio-rad, USA) as described below according to the method of Laemmli [Laemmli, 1970].

- **Plate setup:** To prepare 1 mm thickness gel two glass plates (8 x 8 cm) containing 1 mm thickness inbuilt spacer were clamped together. The combined plates were fixed strongly in plate holders to avoid the leakage of gel solutions.

- **Resolving gel:** Resolving gel percentage varies depending upon the molecular weight of protein of interest described in Table 2.4. Based on the molecular weight, Resolving solutions were prepared by mixing acrylamide-bisacrylamide solution (30% acrylamide and 0.8% N-N-bisacrylamide in distilled water), distilled water, Tris buffer-A (1.5 M Tris-HCl, pH 8.8), 10% SDS, 10% ammonium per sulphate (APS) and N,N,N',N'-tetramethylethylene diamine (TEMED). The contents were poured immediately into gel plate setup up to 3/4th level and left it for 15 min for gelling process at room temperature.

- **Stacking gel:** Stacking gel solution (4.5%) was prepared by mixing 0.75 ml of 30% acrylamide –bisacrylamide solution, 2.95 ml distilled water, 1.25 ml Tris buffer B (0.5 M Tris-HCl, pH- 6.8), 50 µl 10% SDS, 50 µl 10% APS and 10 µl TEMED. The stacking solution was poured over resolving gel and immediately, plastic comb was inserted into the stacking
phase to make the well for loading proteins samples. It was allowed for
gelling process for 15 min at room temperature.

**Table 2.4. Separating gel preparation**

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume (10 ml resolving gel solution)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8% gel</td>
</tr>
<tr>
<td>H₂O</td>
<td>4.6ml</td>
</tr>
<tr>
<td>30% acrylamide/bisacrylamide solution</td>
<td>2.7ml</td>
</tr>
<tr>
<td>1.5M Tris-HCl pH- 8.8</td>
<td>2.5ml</td>
</tr>
<tr>
<td>10% APS</td>
<td>100μl</td>
</tr>
<tr>
<td>10% SDS</td>
<td>100μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>10μl</td>
</tr>
</tbody>
</table>

- **Loading well:** Once gel polymerized, comb was removed from the gel very
carefully without forming any air bubbles and blockage. In case of any
bubbles, the wells were cleaned with a piece of filter paper.

- **Sample preparation:** About 30 µg of cytosolic/nuclear fractions or whole
  cell lysates or conditioned media from BP-1B treated and untreated groups
  under *in-vitro* studies (refer section 2.2.3.2) and *in-vivo* studies (refer section
  2.2.7.4.d) were mixed with 6X-sample buffer (250 mM Tris-HCl pH-7.0,
  50% Glycerol, 9% SDS, 0.03% Bromophenol blue, 5% β- mercaptoethanol
  in distilled water).

- **Electrophoresis:** These protein mixes was boiled for 5 min and centrifuged
  for 30 sec at 3000 rpm. Then, electrophoresis chambers were filled with the
  running buffer (12.4 mM Tris-HCl, 192 mM glycine, 0.1% SDS, pH 8.0).
The protein samples were loaded in the gels and electrophoresis was done at
100 V for 2-3 h.
b) **Immunoblot analysis**

The proteins samples from cytosolic/ nuclear fractions and whole cell lysates of BP-1B treated and untreated groups under *in-vitro* and *in-vivo* conditions were resolved in 8-12% SDS-PAGE based on the molecular weight of protein of interest. The gels were subjected to western blot protocol [Bair et al., 2009] with desired primary and secondary antibodies as described below

- **Wet transfer:** The gels containing resolved proteins subjected to wet transfer process by sandwiching the gels and nitrocellulose membrane (*Invitrogen*, USA) through rough and smooth pads from negative to positive charge. The plate containing sandwich was placed in the western blot module (*Bio-rad*, USA) filled with pre-chilled running buffer (25 mM Tris base, 190 mM Glycine, 20% Methanol, pH adjusted to 8.0) in the direction of negative to positive charge. Then proteins were transferred to membranes at 100 V for 1h at 4°C with continuous stirring.

- **Blocking:** The membranes were removed from transfer apparatus and blocked immediately using blocking buffer (3% BSA in 10 mM Tris base pH - 7.5, 100 mM NaCl, 0.1% Tween 20) for 1 h in gel rocker at room temperature.

- **Incubation with primary antibody:** Blocking buffer was aspirated completely and the membrane was then incubated in the blocking buffer containing desired dilution of primary antibody as recommended by the manufacturer (Table 2.5) for overnight at 4°C.

- **Incubation with secondary antibody:** After probing with primary antibody the membranes were rinsed with washing buffer (TBST of 10 mM Tris base pH - 7.5, 100 mM NaCl, 0.1% Tween 20) for 10 min at three times. The membranes were re-probed with anti-IgG secondary antibody with desired dilution as per the manufacturer recommendation (Table. 2.5).
Table 2.5: List of primary/secondary antibodies with working dilution

<table>
<thead>
<tr>
<th>SL No</th>
<th>Primary/Secondary Antibody</th>
<th>Working dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Anti-HIF-1α (Primary Ab)</td>
<td>1:500</td>
</tr>
<tr>
<td>2</td>
<td>Anti-VEGF-A (Primary Ab)</td>
<td>1:1000</td>
</tr>
<tr>
<td>3</td>
<td>Anti-Flt-1 (Primary Ab)</td>
<td>1:1000</td>
</tr>
<tr>
<td>4</td>
<td>Anti-MMP-2 (Primary Ab)</td>
<td>1:500</td>
</tr>
<tr>
<td>5</td>
<td>Anti-MMP-9 (Primary Ab)</td>
<td>1:500</td>
</tr>
<tr>
<td>6</td>
<td>Anti-Ang-1 (Primary Ab)</td>
<td>1:500</td>
</tr>
<tr>
<td>7</td>
<td>Anti-Lamin B (Primary Ab)</td>
<td>1:2000</td>
</tr>
<tr>
<td>8</td>
<td>Anti-β-actin (Primary Ab)</td>
<td>1:5000</td>
</tr>
<tr>
<td>9</td>
<td>Anti-mouse IgG-ALP (Secondary Ab)</td>
<td>1:10,000</td>
</tr>
<tr>
<td>10</td>
<td>Anti-rabbit IgG-ALP (Secondary Ab)</td>
<td>1:10,000</td>
</tr>
</tbody>
</table>

- **Substrate incubation:** The secondary antibody solutions were removed thoroughly from membranes and rinsed with washing buffer thrice for 10 min each. The membrane was developed with the BCIP-NBT chromogen at 25°C for 10 min in dark. The reaction was terminated by washing the membrane with distilled water for 10 min.

- **Result documentation:** The results were recorded using Bio-rad Gel Documentation™ XR+ Imaging System (Bio-rad, USA). The protein expression patterns were assessed by comparing treated with control by using ImageJ software.

2.2.8.2. Immunofluorescence

Immunostaining was performed to determine the nuclear accumulation of HIF-1α at three independent times [Lyberopoulou et al., 2007; Zheng et al., 2013].

- **Cell culture:** Poly L-lysine coated cover slips were placed in 12-well plates. The A549 and DLA cells were cultured on the cover slips at a density of ~10,000/cm² for 12 h in CO₂ incubator.

- **BP-1B exposure:** The cover slips containing SCC-9 and DLA cells were exposed with BP-1B at 0 μM and 10 μM concentrations in the presence of 100 μM of CoCl₂ for 48 h in CO₂ incubator.

- **Fixation and Permeabilization:** After treatment, the cells were rinsed with PBS thrice for complete removal of media. Then cells were fixed in
coverslips by fixative solution (4% paraformaldehyde in PBS) for 30 min at room temperature and washed with PBS gently for 5 min twice. The cells were permeabilized with permeabilizing solution (0.5% Triton X-100 in PBS) for 30 min at 37°C and rinsed again three times with PBS.

- **Blocking:** The permeabilized cells were then blocked with blocking buffer (3% BSA in PBS) for 1 h at 37°C for avoiding non-specific binding of primary antibodies.

- **Incubation with Primary antibody:** The blocking buffer was removed from coverslips completely. Primary antibody mix (1:500 of anti-HIF-1α: blocking buffer) (200 µl) was added on the each coverslips slowly and incubated for overnight at 4°C.

- **Incubation with secondary antibody:** Primary antibody mix was removed entirely and washed with PBS three times for 10 min with gentle shaking. Then, coverslips were incubated with secondary antibody mix (1: 10000 of Alexa fluor 488-labeled anti-IgG: blocking buffer) for 1 h in dark at room temperature.

- **Imaging:** Secondary antibody mix was decanted thoroughly from coverslips and washed with PBS three times for 5 min with gentle shaking. The clean glass slides coated with 20 µl of antifading agent (Thermo Scientific, USA). The cover slip rinsed with distilled water and the cover slips with the cells facing down were mounted on the glass slides. The cover slips were sealed with polish nail. The HIF-1α nuclear localizations were observed and imaged under EVOS FL cell imaging system (Thermo Scientific, USA).

### 2.2.8.3. VEGF-ELISA

The *in-vitro* and *in-vivo* secretion of VEGF levels were quantified by ELISA [Prabhakar et al., 2006b] through the following the protocol described below and experiments were performed three independent times.

- **Sample preparation:** The SCC-9 cells were cultured and treated with BP-1B in the presence of CoCl₂ as described in section 2.2.3.1. After treatment, conditioned media was collected from each group for quantification of secreted VEGF-A. The serum from BP-1B treated and untreated animals were collected for quantification of serum VEGF-A as described in section 2.2.7.4.d.
Coating: About 100 µl of serum and conditioned media were coated separately on 96 well microtiter ELISA plates with coating buffer (0.05 M sodium bicarbonate, pH - 9.6) and incubated at 4°C for 16 h.

Blocking: The plates were washed three times with washing buffer or PBST (0.05 M PBS pH-7.4, 0.1% Tween 20) for 10 min each. Plates were incubated for 1 h at room temperature with blocking buffer (5% skimmed milk powder in PBST) at room temperature.

Primary antibody binding: Blocking buffer was decanted thoroughly from the plates by multichannel pipette (Eppendorf, Germany). Plates were washed three times with PBST for 10 min each. Primary antibody mix (1:1000 of anti-VEGF-A: blocking buffer, pH 7.4) 100 µl was added to each well and incubated for 2 h at 37°C.

Secondary antibody binding: Plates were washed with PBST thrice and incubated with 100µl of secondary antibody mix (1:10000 of anti-IgG conjugated with ALP: blocking buffer, pH 7.4) for 2 h at 37°C.

VEGF-A quantification: Plates were washed with PBST thrice for 10 min each and incubated with 100 µl of pNPP substrate solution. Reaction was terminated with 50 µl of 0.1 N NaOH and absorbance was read at 405 nm in ELISA reader (Robonics, India).

2.2.8.4. Gelatin Zymogram

Gelatin zymogram is an appropriate assay system to quantify gelatinase activity of MMP-2 and MMP-9 [Chen et al., 2006; Jayasooriya et al., 2013].

Cell culture: A549 cells were cultured in serum free DMEM supplemented with ampicillin, streptomycin and amphotericin B at CO₂ incubator and treated with BP-1B under pseudohypoxic condition as described in the section 2.2.3.1.c. Conditioned media was collected and centrifuged for 5000 rpm for 5 min for the removal of debris.

MMP isolation from conditioned media: In a microfuge tube, 1ml of conditioned media was mixed with 30 µl of the (50% gelatin–agarose bead suspension in TBS-B (50 mM Tris-HCl pH -7.5, 150 mM NaCl, 5 mM CaCl₂ and 0.02% Brij-35) and rotated for 1 h at 4°C for MMP binding. The tubes were centrifuged in a microcentrifuge (Eppendorf, Germany) at 13,000 rpm for 2 min and carefully aspirated the supernatant. The beads containing the
bound MMPs washed with 1 ml of cold TBS-B twice for 5 min. About 20 µl of 1X sample buffer (250 mM Tris-HCl pH - 6.8, 10% Glycerol, 8% SDS, and 0.01% Bromophenol blue) was added to the beads to elute the bound enzymes. The samples were centrifuged at 10000 rpm for 2 min and eluents were used directly in SDS-PAGE.

- **Whole cell lysate preparation from DLS:** The whole cell lysates were prepared from DLS tumour tissue of treated with or without BP-1B as described in section 2.2.7.4.e) and 30 µg of lysates were mixed with 4X sample buffer (250 mM Tris-HCl, pH - 6.8, 40% Glycerol, 8% SDS and 0.01% Bromophenol blue).

- **Gelatin gel:** The 8% SDS-PAGE gel containing 0.1% (w/v) gelatin type A porcine skin was prepared as described in the section 2.2.8.2.

- **SDS-PAGE electrophoresis:** Conditioned media and whole cell lysates samples were loaded carefully in pre-prepared 8% SDS-PAGE gel containing 0.1% (w/v) gelatin. The gel was resolved in running buffer (12.4 mM Tris-HCl, 192 mM Glycine, 0.1% SDS, pH - 8.0) at 100 V for 1 h.

- **Renaturation and incubation:** The gels were washed with 2% of Triton X-100 (renaturation buffer) for 1 h at room temperature, then incubated at 37°C in incubation buffer (50 mM Tris-HCl, 200 mM NaCl and 5 mM CaCl₂) for 18 h.

- **Staining:** The gel was stained with staining solution (0.1% of Coomassie brilliant blue R250 in 1:5:4 mix of glacial acetic acid, methanol and water) for 1 h at room temperature.

- **De-staining:** The gel was subjected to de-staining process by soaking it in de-staining solution (glacial acetic acid, methanol and water (1:5:4)) for 1 h with at least three changes in gel rocker.

- **Documentation:** Inhibition of gelatin lysis zones were analyzed and documented using Bio-rad Gel Documentation™ XR+ Imaging System (Bio-rad, USA).

### 2.2.9. Molecular interaction of BP-1B with HIF-1α by a molecular algorithmic (in-silico) studies:

An entirely Java based in-house developed drug discovery informatics system OSIRIS was used to perform ADMET based calculations. It provides
reusable cheminformatics functionality and was used to predict the toxicity risks and overall drug score via in-silico [Sander et al., 2009]. The structure of BP-1B cytotoxic molecule and the standards were drawn in ChemBioDraw tool (ChemBioOffice Ultra 14.0 suite) assigned with proper 2D orientation and structure of each was checked for structural drawing error. Energy of molecule was minimized using ChemBio3D (ChemBioOffice Ultra 14.0 suite). The energy minimized ligand molecule BP-1B was then used as input for AutoDockVina, in order to carry out the docking simulation [Trott & Olson, 2010]. The protein databank (PDB) file with the PDB ID ‘1L3E.pdb’ was used as receptor molecule, which constituted of NMR structure of the C-TAD HIF-1α [Freedman et al., 2002]. Further SPDBV DeepView was used to automatically rebuild the missing side chains in receptor. The Graphical User Interface program ‘MGL Tools’ was used to set the grid box for docking simulations. In the present study, the grid center was set on C-TAD of HIF-1α and a cubic grid box was set. The grid box volume was set to 18, 18, and 18 Åunits for x, y and z dimensions respectively, and the grid center was set to 6.81, 12.029 and -15.317 for x, y and z center respectively. AutoGrid 4.0 Program supplied with Auto-Dock tools was used to produce grid maps [Morris et al., 1998]. The docking algorithm provided with AutoDockVina was used to search for the best docked conformation between ligand and protein. The AutoDock docking algorithm was run on Corei7 Intel processor CPU with 16 GB DDR3l RAM. AutoDockVina was compiled and run under Windows 8.0 professional operating system. LigPlot+ [Laskowski and Swindells, 2011] and PyMol [DeLano, 2002] was used to deduce the pictorial representation of interaction between the BP-1B and the HIF-1α protein.

2.2.10. Statistical analysis

All the in-vitro experiments performed at minimum three independent times and all the in-vivo experiments were performed using each groups having five experimental animals. Values were expressed as mean ± standard deviation (SD). Statistical significance was evaluated by one-way analysis of variance (ANOVA) followed by 2-tailed Student’s t-test. MS excel 8.1 version software was used for data analysis and Statistical significant values were expressed as *p < 0.05 and **p < 0.01 in the figures.
2.3. Results

2.3.1. BP-1B exposes tumour-specific cytotoxic action against various cancer types.

BP-1B is a benzophenone-benzimazole pharmacophore which has effective anti-proliferative potentiality against murine cancer cell under *in-vitro* and *in-vivo* through anti-angiogenic effect [Ranganatha & Prabhu et al., 2013]. In this present research study, anti-proliferative investigations of BP-1B were extended to various cancer cells of different origin by cytoxic and clonogenic assays.

i) **Cell based action of BP-1B**

In the current study, efficacy of BP-1B was investigated against SCC-9, A388, A549, NCI-H23, MCF-7, MDA-MB-231, Hela, CaSki, SiHa, ACHN, A498, HepG2, Hep3B and DLA cells by performing trypan blue dye exclusion, MTT and LDH release assays. Results postulate that BP-1B showed a considerable higher to moderate cytotoxicity against varied tumour cells as compared with normal cells and there was a significant deviation of sensitivity to BP-1B among cells used (Table 2.6). The sensitivity to BP-1B on human cancer cells was nearly in the following order: squamous cell carcinoma > lung adenocarcinoma > murine lymphoma > breast carcinoma > cervical carcinoma > renal carcinoma > hepatocellular carcinoma and no sensitivity to normal cells.

ii) **BP-1B with prolonged effect**

Following the potential cytotoxicity of BP-1B, prolonged activity was assessed by performing colony formation assay using SCC-9 and A549 cells. Results reveal that BP-1B exhibited the long-term effect against the clonogenesis of SCC-9 cells with 70.3 %, 81.4% & 94.3% and A549 cells 62.6 %, 78.5% & 90.4% at three different concentrations (Figure 2.2 A - D).
Table 2.6. IC$_{50}$ value of BP-1B against various human and mouse cancer cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>BP-1B IC$_{50}$ Value (µM)</th>
<th>5-FU IC$_{50}$ Value (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MTT</td>
<td>Trypan blue</td>
</tr>
<tr>
<td>Squamous Cell Carcinoma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SCC-9</td>
<td>4.3±0.5**</td>
<td>4.2±0.4**</td>
</tr>
<tr>
<td>A388</td>
<td>4.7±0.9</td>
<td>4.3±0.8</td>
</tr>
<tr>
<td>Lung cell adenocarcinoma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A549</td>
<td>5.1±0.7*</td>
<td>5.4±0.6*</td>
</tr>
<tr>
<td>NCI-H23</td>
<td>5.6±0.4**</td>
<td>5.7±0.4**</td>
</tr>
<tr>
<td>Breast adenocarcinoma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCF-7</td>
<td>6.8±0.6*</td>
<td>6.9±0.7*</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>7.3±0.6*</td>
<td>7.1±0.9</td>
</tr>
<tr>
<td>Cervical carcinoma</td>
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<td></td>
</tr>
<tr>
<td>Hela</td>
<td>7.7±0.6*</td>
<td>7.6±0.7*</td>
</tr>
<tr>
<td>CaSki</td>
<td>8.1±0.7*</td>
<td>8.3±0.7*</td>
</tr>
<tr>
<td>SiHa</td>
<td>12.8±1.6</td>
<td>13.4±1.6</td>
</tr>
<tr>
<td>Renal carcinoma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACHN</td>
<td>14.1±0.9</td>
<td>14.7±1.2</td>
</tr>
<tr>
<td>A498</td>
<td>17.8±1.6*</td>
<td>16.8±1.4</td>
</tr>
<tr>
<td>Liver carcinoma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HepG2</td>
<td>26.4±1.3</td>
<td>28.7±1.7</td>
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<tr>
<td>Hep3B</td>
<td>26.8±1.5</td>
<td>27.6±1.4</td>
</tr>
<tr>
<td>Ascites lymphoma (Murine)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DLA</td>
<td>6.6±0.5**</td>
<td>6.5±0.4**</td>
</tr>
<tr>
<td>Normal cells</td>
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<td></td>
</tr>
<tr>
<td>NIH-3T3</td>
<td>94.3±3.5</td>
<td>94.8±3.1</td>
</tr>
</tbody>
</table>

5-Fluorouracil is used as a positive control. Cytotoxicity was measured by MTT, Trypan blue and LDH release assays against each cell lines in three independent (n = 3). DMSO is used as a vehicle control which showed very negligible cytotoxicity. Values are indicated in mean ± SD and statistically significant values are expressed as *p<0.05 and **p<0.01.
Figure 2.2: BP-1B exhibits the prolonged anti-clonogenic potency against SCC-9 and A549 cells.

(A) Concentration dependant suppression of SCC-9 clonogenesis. (B) Concentration dependant A549 clonogenic inhibition. (C) Graphical representation of percentage of SCC-9 colony formation inhibition. (D) Graphical representation of percentage of A549 colony formation inhibition. (Note: Statistically significant values expressed as *p < 0.05 and **p < 0.01).
2.3.2. BP-1B abridges the rVEGF_{165} induced endothelial cell migration and neovessel formation in non-tumourigenic condition.

VEGF is one of the most critical and specific angiogenic factor of vascular endothelial cells for regulating angiogenesis [Fong, 2009; Wang et al., 1995]. In our examination, rVEGF_{165} was used to induce the sprouting angiogenesis in reliable model systems such as endothelial cell migration & tube formation assays, ex-vivo chorioallantoic membrane assay and rat aortic ring assay to examine the BP-1B angiopreventive efficacy under non-tumourigenic conditions.

i) **HUVEC tube formation inhibition**

The antiangiogenic potential of BP-1B in HUVEC was determined using an in-vitro migration and capillary tube formation assay. In the absence of BP-1B, HUVECs migrated rapidly toward the wounded area and covered it (Fig. 2.3A). However, cell migration was retarded markedly with 46-68% by BP-1B at different concentration (Fig. 2.3A & E). Further, we assessed the efficacy of BP-1B on HUVECs tube formation. HUVECs are capable of forming tubes via linking to adjacent cells in ECM gel but BP-1B treatment restricted the capillary tube formation and tube length compared to rVEGF_{165} treated and untreated (Fig. 2.3B). Tube length and branch numbers were significantly reduced up to 62.4%, 72.1% and 82.3% by BP-1B at three ranges of concentrations (Fig. 2.3F).

ii) **Regression of EC sprouting in aortic rings and CAMs**

Further, BP-1B mediated endothelial sprouting inhibition was re-assessed in rVEGF_{165} induced rat aortic rings and in-vitro CAM models. The results reveal that BP-1B effectually regressed the newly formed endothelial tubes from aortic rings and vascular density from 63.4% to 79.7% when compared with rVEGF_{165} alone treated (Fig. 2.3C & G). In-vitro sprouting neoangiogenesis on CAM results strengthened BP-1B effect and showed the noticeable inhibition with 46.8%, 62.6% and 72.4% on varying concentrations (Fig. 2.3D & H).
**Figure 2.3:** BP-1B inhibits the endothelial cell migration and neovessel formation under *in-vitro* condition in concentration dependant manner.

(A) Inhibition of HUVEC cell migration. (B) Decrease of tube forming efficiency of HUVECs. (C) Reticence of sprouting neovaculature from aortic rings. (D) Reduction of neovessel formation in *in-vitro* CAM. (E) Percentage of HUVEC cell migration inhibition. (F) Percentage of tube formation inhibition. (G) Percentage of aortic ring sprouting angiogenesis inhibition. (H) Percentage of CAM neovascularization inhibition. (Note: Statistically significant values expressed as *p < 0.05 and **p < 0.01*).
2.3.3. BP-1B confines the physiological neovascularization process under non-tumourigenic condition.

Following in-vitro strong antiangiogenic effect of BP-1B, angiopreventive potency was validated on in-vivo angiogenic models such as rat corneal micropocket angiogenic assay and matrigel plug angiogenesis assay under non-tumourigenic conditions.

i) Inhibition of corneal angiogenesis

Rat corneal micropocket assay is gold standard and extensively employed assay system to validate the angiogenic modulatory effect of pharmacophores. Corneal neovascularization was stimulated with rVEGF_{165} and treated with BP-1B at 3, 5, 10 µg/eye concentrations. Results inferred that sprouting corneal neoangiogenesis towards rVEGF_{165} implant was inhibited by BP-1B remarkably with 12 ± 3, 8 ± 3 and 6 ± 1.5 MVD/eye in concentration dependant manner, compared to 34 ± 5 MVD/eye of rVEGF_{165} treated (Fig. 2.4A & D). The corneal angiogenesis inhibition by BP-1B improved the corneal gesture and tissue architecture which is clear from histopathology (H & E stain) (Fig. 2.4B).

ii) Reticence of neovessel formation in matrigel implants

Physiological angiopreventive effectiveness of BP-1B re-evaluated in matrigel plug angiogenesis models. Matrigel or ECM gel is well known to exhibit the physiological environment. ECM gel containing rVEGF_{165} and BP-1B at different concentrations were injected into mice subcutaneously and left for 10 days to form angiogenesis. The results demonstrated that BP-1B noticeably lessened the rVEGF_{165} induced vasculogenesis in matrigel plugs but BP-1B untreated implant exhibited extensive vasculature. Haemoglobin level quantified from matrigel implants showed BP-1B reduced haemoglobin level with 1.5, 5.3 & 7.4 folds compared to that of control in concentration dependant manner (Fig. 2.4C & E). The decrease of haemoglobin content is associated with reduced angiogenesis and microvascular density (MVD).
**Figure 2.4:** BP-1B counteracts the non-tumourigenic neoangiogenesis in varying concentration *in-vivo*.

(A) Repression of microvascular sprouting in cornea. (B) Inhibition of vascular density and improvement of corneal gesture. (C) Inhibition of neovasculogenesis in matrigel. (D) Graphical representation of total MVD count in cornea. (E) Pictographical demonstration of haemoglobin content in matrigel implants. (Note: Statistically significant values expressed as *p < 0.05 and **p < 0.01)
2.3.4. **Acute toxicity studies.**

Animals were maintained in the dark and light environment and fed with appropriate feed pellets and water for acclimatization. Then, mice were administered with BP-1B at 100-3000 mg/kg (b.w) intraperitoneally for determination of lethal dose. The mice which administered 3000 mg/kg body weight of BP-1B showed abnormal aggressive behavior and died within 24 h. The animals administered with 2000 mg/kg body weight of BP-1B showed abnormal behavior with less food and water consumption. The animals injected with 1000 mg/kg body weight of BP-1B was found to be normal and therefore around 1/30th, 1/20th and 1/15th of this dose i.e. 30 mg, 50 mg and 70 mg/kg body weight were selected as therapeutic dose for further analysis.

2.3.5. **BP-1B reticence the pathological neovascularization *in-vivo* under tumourigenic condition.**

i) **BP-1B mediated inhibition of ascites tumour development**

To investigate the potentiality of BP-1B on tumour growth and peritoneal angiogenesis, Dalton’s ascites lymphoma (DLA) model developed and treated with BP-1B. The DLA tumour is known for secreting ascitic fluids which is the direct nutritional source for tumour cell growth and a rapid increase in the secretion of this fluid is an extremely essential characteristics for tumour survival. Therefore reducing ascites secretion is one of the essential parameters in reticence of the tumour growth. The results inferred that BP-1B considerably regressed the tumour progression and cell density with 1.2, 2.3 & 5.2 fold reduction in concentration dependant manner (Fig. 2.5A & B). Administration of BP-1B significantly reduced the ascitic fluid secretion compared to the control in dose dependant manner (Fig. 2.5C). An anticancer drug is considered reliable if it can prolong the life span of the animal. Interestingly, BP-1B treatment have prolonged the life span of animals from the 10th day to >40th day, with two - three fold increase in survivability (Fig. 2.5D).

ii) **Decreased tumour induced angiogenesis**

DLA is known for stimulating local inflammatory reaction and angiogenesis in peritoneum [Vijay Avin et al., 2014a; Thirusangu et al., 2017]. Treatment of BP-1B significantly decreased the tumour angiogenesis at 30 mg, 50 mg and 70 mg/kg bw concentration and it is visibly clear from peritoneal morphology...
Histopathological examination of peritoneum revealed that BP-1B remarkably diminished the microvascular density with 2.03, 3.01 and 4.38 fold of MVD/peritoneum compared to untreated at increasing concentrations (Fig. 2.6B & D). The CD-31 endothelial cell specific staining of peritoneum sections exhibited a reduced CD-31-MVD total counts by up to 1.96, 3.08 and 3.82 folds in BP-1B treated sections corresponding to the untreated (Fig. 2.6C & E).

**Figure 2.5:** BP-1B confines murine ascitic lymphoma development *in-vivo*. 
(A) Dose dependent gradual reduction of tumour growth. (B) Reduction of cell density. (C) Dose dependant decrease of ascites fluid secretions. (D) Kaplan-Meier graphical representation of concentration dependant extended survivability. (Note: Statistically significant values expressed as *p < 0.05 and **p < 0.01).
Figure 2.6: BP-1B impairs the tumour angiogenesis in DLA tumourigenesis.

(A) Regression of tumour angiogenesis in peritoneums. (B) Reduction of vascular density (by H & E analysis). (C) Inhibition of CD-31 endothelial marker stain of neovessels. (D) Peritoneal MVD count by H & E stain. (E) Total CD-31 count of peritoneum. (Note: Statistically significant values expressed as *p < 0.05 and **p < 0.01).
2.3.6. BP-1B attenuates the nuclear translocation of HIF-1α.

   i) **HIF-1α nuclear import inhibition in-vitro**

   To address the angiopreventive mechanism of BP-1B, HIF-1α was overexpressed by treating cancer cells with CoCl$_2$ or hypoxia, since HIF-1α is a key transcription factor for many angiogenic signaling factors. CoCl$_2$ is known to induce pseudohypoxic condition by blocking activity of PHD and induces the expression of HIFs [Fan et al., 2014]. To assess the effects of BP-1B on HIF-1α expression under hypoxic conditions, SCC-9, A549 and MCF-7 cells treated with BP-1B and consequently cell fractionation & immunoblots were carried out. Results indicated that BP-1B has not altered the HIF-1α protein level expressions (Fig. 2.7A & B) but it has significantly inhibited the nuclear translocation of HIF-1α which is evident from cellular protein fractionation studies (Fig. 2.7C - E). Since HIF-1α phosphorylation by p42/44 is essential for nuclear accumulation, BP-1B showed no significant alteration in phospho-p42/44 and it is clear that blocking HIF-1α nuclear import by BP-1B is devoid of p42/44 expressional alterations (Fig 2.7F & G). The results were reassessed in low oxygen tension where BP-1B blocked the nuclear accumulation of HIF-1α in SCC-9 and A549 cells (Fig. 2.8A & B). Further, immunofluorescence analysis was performed and it showed that BP-1B blocks the nuclear localization of HIF-1α in SCC-9 and DLA cells but untreated cells exposed the HIF-1α stain in nuclei (Fig. 2.9A & B) which synchronizes to the immunoblot studies.

   ii) **HIF-1α nuclear import inhibition in-vivo**

   HIF-1α nuclear entry blocking by BP-1B was revalidated on A549-CAM xenograft model and results depicted that BP-1B remarkably reduced A549 xenograft tumour volume with 1.42, 2.24 and 3.26 fold when equalized to that of control at different concentrations (Fig. 2.9C & D). Cell fractionation study of A549-xenograft demonstrate that BP-1B potentially inhibits nuclear import of HIF-1α (Fig. 2.9E) which resulted in reduction of angiogenesis with 8 ± 1, 5 ± 0.7 and 3 ± 0.3 MVD/High per field (HPF) at different concentration compared to 35 ± 1.5 MVD/HPF of rVEGF$_{165}$ treated (Fig. 2.9F & G). Together, the experimental evidences postulate that BP-1B attenuate the nuclear import of HIF-1α.
Figure 2.7: BP-1B attenuates nuclear translocation of HIF-1α in various cancer cells in varying concentrations under CoCl₂ pseudohypoxic condition.
(A) HIF-1α protein expression not altered in SCC-9 cells. (B) HIF-1α protein expression not altered in MCF-7 cells. (C) Inhibition of nuclear translocation of HIF-1α in SCC-9 cells. (D) Inhibition of nuclear translocation of HIF-1α in A549 cells. (E) Inhibition of nuclear translocation of HIF-1α in MCF-7 cells. (F) No significant changes in phospho p42/44 in SCC-9 cells. (G) No significant changes in phospho p42/44 in A549 cells.

(Note: Pictographs are representing the relative normalized expression of respective immunoblots and statistically significant values expressed as *p < 0.05 and **p < 0.01)

Figure 2.8: BP-1B attenuates nuclear translocation of HIF-1α in various cancer cells in varying concentrations under hypoxic condition.
(A) Inhibition of nuclear accumulation of HIF-1α in SCC-9 cells. (B) Inhibition of nuclear accumulation of HIF-1α in A549 cells.

(Note: Pictographs are representing the relative normalized expression of respective immunoblots and statistically significant values expressed as *p < 0.05 and **p < 0.01).
Figure 2.9: BP-1B blocks nuclear import of HIF-1α in SCC-9 and DLA in-vitro and in A549-xenograft in-vivo.

(A) HIF-1α nuclear import inhibition in SCC-9 cells (by immunofluorescence). (B) HIF-1α nuclear localization Inhibition in DLA cells (by immunofluorescence). (C) Reduction of A549-xenograft tumour size. (D) Graphical representation of decrease of tumour volume. (E) Restriction of nuclear import of HIF-1α in cells of xenograft. (F) Reduction of vascular density (H & E stain) and (G) Graphical representation of MVD/HPF decrease in A549-xenograft. (Note: Pictographs are representing the relative normalized expression of respective immunoblots and statistically significant values expressed as *p < 0.05 and **p < 0.01).
2.3.7. BP-1B interacts with C-TAD domain of HIF-1α by binding with threonine amino acid.

The HIF-1α nuclear localization and transcriptional activation is tightly regulated by p44/42 (Erk-1/2). The p44/42 pathways controls HIF-1α post-translationally through the phosphorylating one threonine (Thr796) and two serine residues (Ser641 and Ser643). Since BP-1B blocks the nuclear entry of HIF-1α in devoid of p44/42, the molecular interaction between HIF-1α and BP-1B was evaluated by a bioinformatics approach. The result uncovered that BP-1B strongly interacts with Thr796 of HIF-1α in C-TAD region (Thr12 of C-TAD domain) with H bond distance 2.94 Å (in green colour), where phosphorylation by p42/44 promote the nuclear accumulation and transcriptional activation of HIF-1α. It also formed hydrophobic interaction with four amino acid residues Gln10, Tyr14, Asp15 and Val18 of HIF-1α [Fig. 2.10A-C].
Figure 2.10: BP-1B interacts with C-TAD domain of HIF-1α by binding with threonine amino acid through H – bond.

(A) The 2D-view of interaction between BP-1B and C-TAD of HIF-1α. (B) The molecular bonding of BP-1B with HIF-1α. (C) The 3D-view of molecular interaction of C-TAD of HIF-1α and BP-1B
2.3.8. BP-1B counteracts the HIF-1α targeted angiogenic factors expressions at in-vitro level.

i) Downregulation of HIF-1α dependant angiogenic factors

Following HIF-1α nuclear import arrest by BP-1B, HIF-1α target genes such as VEGF-A, Flt-1, MMP-2, MMP-9 and Ang-1 expressions were addressed by immunoblot. Under normoxia, the exposure of BP-1B on SCC-9 cells has not altered the HIF-1α dependant proteins such as VEGF-A, Flt-1, MMP-2, MMP-9 and Ang-1 as studied by immunoblots (Fig. 2.11A & B). But insufficient nuclear accumulation of HIF-1α by BP-1B exhibited down regulation of HIF-1α dependant angiogenic factors including VEGF-A, Flt-1, MMP-2, MMP-9 and Ang-1 under hypoxic conditions in concentration dependant manner as verified by immunoblot. (Fig. 2.11C & D).

ii) Inhibition of secreted angiogenic factors

Cancer cells handle the secreted growth factors as tumour promoting mediators for aggressive proliferation and development. Furthermore efforts have made to measure the level of HIF-1α mediated secreted factors by ELISA and gelatin zymogram. VEGF-ELISA results demonstrated that BP-1B counteracted the secreted level of VEGF-A noticeably with 380 ± 24, 228 ± 12 and 185 ± 10 equated to 762 ± 42 of control (Fig. 2.12A), which synchronizing to immunoblot result. Gelatin zymogram results depicted that BP-1B abridged the secreted MMP-2 and MMP-9 which resulted in reduced gelatin lysis in zymogram when equalized to that of untreated (Fig. 2.12B).

iii) Diminishment of migration and invasion of cancer cells

Cancer migration and invasion assays were performed to confirm anti-migration and anti-invasive effect of BP-1B on invasive A549 cancer cells. Restrained MMP-2 and MMP-9 by BP-1B impaired the migration with 58.6%, 71.8% & 83.2% when equalizing to control (Fig. 2.12C & E) and invasion with 63.2%, 78.3% & 86.7 % (Fig. 2.12D & F) in concentration dependant manner. These collections of results conclude that the inhibition of downstream angiogenic genes is due to decrease of nuclear import of HIF-1α.
Figure 2.11: BP-1B down regulates the HIF-1 dependant angiogenic gene activation in-vitro.

(A) No alteration in expression of VEGF-A, Flt-1, MMP-2, MMP-9 and Ang-1 under normoxic condition. (B) Pictographical representation of relative normalized intensity of VEGF-A, Flt-1, MMP-2 MMP-9 and Ang-1 expressions. (C) Inhibition of expression of VEGF-A, Flt-1, MMP-2, MMP-9 and Ang-1 under CoCl₂ induced pseudohypoxic condition. (D) Pictographical representation of relative normalized intensity of VEGF-A, Flt-1, MMP-2 MMP-9 and Ang-1 expressions. (Note: Statistically significant values expressed as *p < 0.05 and **p < 0.01).
Figure 2.12: BP-1B represses the secretion of VEGF-A, MMP-2 and MMP-9 and acts against cancer cell migration and invasion in vitro. (A) Gradual decrease of secreted VEGF-A by ELISA. (B) Inhibition of gelatinase activity of MMP-2 and MMP-9 by zymogram. (C) Graphical representation of gelatinase activity of MMP-2 and MMP-9. (D) A549 cells migration inhibition. (E) Prohibition of A549 cell invasion. (F) Bar graphical representation of percentage of migration inhibition. (G) Bar graphical representation of respective percentage invasion inhibition. (Note: Statistically significant values expressed as *p < 0.05 and **p < 0.01).
2.3.9. **BP-1B restrains the neoplastic growth of Solid tumour.**

i) **Regression of DLS proliferation**

The pathophysiological response of BP-1B was investigated in the solid tumour (DLS) model. The solid tumour model system is the most reliable, representative of histological types of cancer and tumour angiogenesis, which thereby provides a rapid action of drug delivery [Powis & Kirkpatrick, 2004; Brown & Giaccia, 1998; Eklund et al., 2013]. The treatment of BP-1B on DLS at 30 mg, 50 mg & 70 mg/kg bw concentration, noticeably reduced tumour establishment as measured by vernier caliper (Fig. 2.13A) and gradually inhibited tumour growth in dose dependant manner (Fig. 2.13B). Anatomical appearance of thigh containing tumour postulate that BP-1B regressed DLS tumour size visibly (Fig. 2.13C) with 1.91, 3.15 & 4.42 fold gram of tumour when corresponded to that of untreated (Fig. 2.13F).

ii) **Reticence of DLS tumour angiogenesis**

Histopathology of DLS tumour explained that BP-1B made a noteworthy modification in microvascular density (Fig. 2.13D) with 24 ± 1.5, 13 ± 3.2 & 10 ± 1.1 MVD/HPF compared to 37 ± 4.0 MVD/HPF of untreated tumour (Fig. 2.13G). CD-31 immunostain of DLS demonstrate that tumour neovascular density gradually decreased by BP-1B (Fig. 2.13E) by reducing MVD/HPF to 42 ± 3.1, 21 ± 3.2 & 12 ± 0.8 in concentration dependant manner, corresponded to 62 ± 4.2 MVD/HPF of untreated (Fig. 2.13H).

iii) **Prolonged survivability**

The regression of DLS progression and tumour angiogenesis by BP-1B made significant alterations in life span of mice bearing DLS tumour. The survivability of animals were extended up to 98th, 103rd and >120th days on varying concentrations of BP-1B when most of the untreated animals died before 40th day of tumour development (Fig. 2.13I).
Figure 2.13: BP-1B antagonizes the tumourigenesis of DLS by targeting tumour angiogenesis on varying concentrations.
(A) Dose dependant inhibition of tumour volume. (B) Percentage of tumour inhibition. (C) Inhibition of tumour size. (D) Histopathological analysis tumour vascular density inhibition. (E) Inhibition of CD-31 microvascular stain. (F) Graphical representation of tumour volume. (G) Pictographical illustration of MVD count by H & E stain. (H) Pictographical demonstration of CD-31 count. (I) Kaplan-Meier curve illustration of survivability. (Note: Statistically significant values expressed as *p < 0.05 and **p < 0.01).
2.3.10. BP-1B exhibits no toxicological effect in solid tumour bearing animals.

The toxicological parameters were analyzed after treatment with BP-1B on DLS by separating liver and spleen and by histopathology. The morphological appearance of the livers and spleens of treated groups have not exhibited any sign of toxicity and its histopathology were comparable with those of normal animals (Fig. 2.14A & C). Additionally, BP-1B reduced infiltration of tumour cells with not as much of histological injure in the respective organs, which is a proof for BP-1B treatment is not cytotoxic to animals (Fig. 2.14B-D).

In addition, BP-1B in spite of being a most effective anti-tumour molecule, had more restricted or no undesirable side effect as confirmed by serum and haematological parameters from mice bearing DLS tumour. The level of ALP, creatinine and urea in BP-1B treated were not significantly altered compared to normal and control. Haematological profile postulated that BP-1B not modulated the rate of RBC and WBC in blood corresponded to control and normal mice. The results are summarized in Table 2.7.

Table 2.7: Haematological and serum profile of DL tumour bearing mice following treatment with BP-1B at three different concentration.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal</th>
<th>Control</th>
<th>BP-1B (30 mg/kg)</th>
<th>BP-1B (50 mg/kg)</th>
<th>BP-1B (70 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALP (U/L)</td>
<td>124.55±2.15</td>
<td>203.45±2.3</td>
<td>162.65±3.6</td>
<td>142.35±3.3</td>
<td>135.31±3.1</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>0.53±0.05</td>
<td>0.90±0.7</td>
<td>0.79±0.05</td>
<td>0.59±0.06</td>
<td>0.56±0.08</td>
</tr>
<tr>
<td>Urea (mg/dl)</td>
<td>32±1.78</td>
<td>89±1.2</td>
<td>45±1.9</td>
<td>41±1.4</td>
<td>36±1.2</td>
</tr>
<tr>
<td>RBC (10⁶/µl)</td>
<td>5.68±0.45</td>
<td>3.35±0.1</td>
<td>5.98±0.75</td>
<td>5.28±0.75</td>
<td>5.12±0.93</td>
</tr>
<tr>
<td>WBC (10⁶/µl)</td>
<td>3.42±1.2</td>
<td>5.88±1.5</td>
<td>3.92±0.5</td>
<td>3.91±0.7</td>
<td>3.51±0.7</td>
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</table>

Values are indicated in mean ± SD.
Figure 2.14: BP-1B exhibits the no adverse effects to secondary lymphoid organs liver and spleen in DLS tumour.

(A) Morphology of liver. (B) Histopathology of liver. (C) Morphology of spleen. (D) Histopathology of spleen
2.3.11. BP-1B impairs HIF-1 signaling by blocking nuclear import of HIF-1α in \textit{in-vivo}.

Solid tumour models are the most appropriate model system to investigate action of hypoxia inhibitor due to its hypoxic nature [Hong et al., 2004; Powis & Kirkpatrick, 2004; Brown, 2007]. The molecular events of BP-1B exhibited anti-angiogenesis in DLS tumour was elucidated by performing immunoblot, gelatin zymogram and IHC. Results demonstrates that BP-1B blocked the nuclear accumulation of HIF-1α (Fig. 2.15A & B) which resulted in inhibition of downstream target angiogenic factors such as VEGF-A, Flt-1, MMP-2 MMP-9 and Ang-1 (Fig. 2.15C & D), that is parallel to the \textit{in-vitro} studies. Also, BP-1B abolished the intracellular expression of MMP-2 and MMP-9 which is evident from gelatin lysis zone in zymogram (Fig. 2.15F & G). ELISA result depicts that BP-1B represses the secreted VEGF-A in serum of mice bearing DLS tumour (Fig. 2.15E). In addition, IHC analysis of tumour sections inferred that BP-1B markedly decreased the expression of VEGF-A, MMP-2 and MMP-9 where HIF-1α overexpressed but blocked to nuclear entry (Fig. 2.16A - D). Taken together, current experimental results summarize that BP-1B inhibits the tumour neovascularization by blocking HIF-1α nuclear import, a gate way of HIF-1 transcriptional activation and by restraining the expression of HIF-1 target genes.
Chapter II: The effect of BP-1B on tumour angiogenesis

Figure 2.15: BP-1B counteracts the critical angiogenic factors expression by impairing nuclear import of HIF-1α \textit{in-vivo}.

(A) Restriction of nuclear import of HIF-1α. (B) Graphical representation of relative normalized intensity of HIF-1α expression in cytosol and nucleus. (C) Expressional modulation of angiogenic factors VEGF-A, Flt-1, MMP-2, MMP-9 and Ang-1. (D) Pictographical representation of relative normalized intensity of VEGF-A, Flt-1, MMP-2 MMP-9 and Ang-1 expressions. (E) Serum level reduction of VEGF-A secretions. (F) Counteraction of gelatinase activity of MMP-2 & MMP-9. (G) Graphical representation of gelatinase activity of MMP-2 and MMP-9. (Note: Statistically significant values expressed as *p < 0.05 and **p < 0.01).
Figure 2.16: BP-1B decreases the intratumoural VEGF-A, MMP-2, MMP-9 expression by blocking nuclear import of HIF-1α in vivo.

(A) Intratumoural expression of VEGF-A by IHC. (B) Intratumoural expression of MMP-2 by IHC. (C) Intratumoural expression of MMP-9 by IHC. (D) No alteration in intratumoural expression of HIF-1α by IHC since BP-1B inhibits nuclear localization of HIF-1α. (Note: Statistically significant values expressed as *p < 0.05 and **p < 0.01).
2.4. Discussion

Hypoxic tumour cells are frequently defiant to radiotherapy and most chemotherapeutic molecules, thereby representing these cells extremely aggressive and invasive. Over expression of HIF-1 is related with augmented neovascularization, treatment failure and a poor prognosis [Fong, 2009; Wang et al., 1995; Kaelin Jr, 2005; Madanecki et al., 2013]. In recent years, finding and the development of novel small molecules targeting HIF-1 has been an attracting area of cancer drug development that has grown exponentially. Despite this, none of the recently reported HIF-1 inhibitors have been translated to the clinical setting [Xia et al., 2012]. Furthermore, research work on developing new molecules is warranted to identify more specific HIF-1 inhibitors, to study their mechanism of action, and to translate these developments into clinical trials.

Benzophenones, the heterocyclic moieties exists in many natural products and synthetic drugs with a broad spectrum of pharmacological activities [Wu et al., 2013, Henry Jacobs et al., 1999; Karrer et al., 2000]. More recently, we have reported that benzophenone hybrids have extensive pharmacophoral potentiality compared to benzophenone alone (Prabhakar et al., 2006a; Prashanth et al., 2014; Vijay Avin et al., 2014a; Gurupadaswamy et al., 2014; Al-Ghorbani et al., 2016). In search of the pharmacologically active novel small molecule, we found that the incorporation of benzimidazole moiety (a therapeutically active pharmacophoral ring [Wang et al., 2015] ) into benzophenone can produce pharmacologically enriched potent compounds. One such molecule BP-1B was found to inhibit the in-vivo murine mammary carcinoma by targeting angiogenesis with very negligible toxicological and secondary complication. Structurally, a methoxy group at the para position and the two methyl groups at the ortho position in benzophenone rings are important for the pharmacological activity of BP-1B against tumour progression (Fig. 2.1) [Ranganatha et al., 2013]. Also, extensive research suggests that presence of methyl and methoxy groups are responsible for various drug’s potential pharmacological behavior [Vijay Avin et al., 2014a, Shen et al., 2010; Rschin et al., 2003; Tozer et al., 2008]. In continuation, BP-1B was subjected for anti-angiogenic investigation for elucidating the molecular targets by using appropriate in-vitro and in-vivo angiogenesis strategies and tumour angiogenic models.

The angio preventive effect of BP-1B encouraged our investigation to extend cytotoxic assessment of BP-1B on various human cancer types including head and
neck, epithelial, lung, breast, cervical, renal and hepatocellular carcinomas which are highly expressive of HIFs. The exposure of BP-1B showed cell specific action against the cancer cell proliferation (Table 2.6) with prolonged activity (Fig. 2.2A - D). Hence, BP-1B may become a therapeutically active and specific candidate in the cancer treatment.

Angiogenesis, the formation of new vessels from preexisting vasculature, is an important mechanism used by tumours to promote oxygen supply, growth and metastasis [Fong, 2009; Wang et al., 1995; Kaelin Jr, 2005; Madanecki et al., 2013]. This process is promoted by various angiogenic stimulators including VEGF which is one of the most critical and specific angiogenesis factor regulating normal physiological and tumour angiogenesis [Fong, 2009; Folkman, 1972; Brown & Giaccia, 1998]. It is very clear from the results of in-vitro non- tumour angiogenic models that BP-1B has effectual potentiality to regress the rVEGF\textsubscript{165} induced endothelial cell proliferation, migration, neovessel formation and MVD (Fig. 2.3A - H). Moreover, BP-1B was capable of reproducing the angiopreventive effect in in-vivo non-tumour angiogenic models such as corneal micropocket, matrigel implant angiogenesis (Fig. 2.4A - E). Also, BP-1B demonstrated vascular regression on tumour induced peritoneal angiogenesis models with the significant reduction of MVD (Fig. 2.6A - E). The regression of peritoneal angiogenesis resulted in decreased ascetic tumour development as assessed by tumour growth, cell density, ascites secretion and prolonged lifespan (Fig. 2.5A - D).

Tumour angiogenesis is a complex interrelated multistep process. Reticence of any events in this process may lead to the disruption of neovascularization and can serve as a potential anti-tumour therapy [Fong, 2009; Wang et al., 1995; Kaelin Jr, 2005; Madanecki et al., 2013; Folkman, 1972; Brown & Giaccia, 1998]. Recently, it has been reported that the tumour microenvironment increases accumulation of angiogenic factors targeting HIF-1α synthesis, nuclear translocation and transcriptional activity for the adaptation of cellular hypoxia. Nuclear import of HIF-1α is a hallmark event in HIF-1 dependant angiogenic gene activation including VEGF-A, Flt-1, Ang-1, MMP-2 and MMP-9 which promotes tumour angiogenesis and metastasis [Fong, 2009; Wang et al., 1995; Kaelin Jr, 2005; Yeo et al., 2004; Chau et al., 2005]. Our experimental evidences showed that BP-1B does not modulate the expression of HIF-1α (Fig. 2.7A & B) but notably
attenuated the nuclear localization of HIF-1α in SCC-9, A549 and MCF-7 as verified by immunoblots (Fig. 2.7C – E and Fig. 2.8A & B ) and immunofluorescence (Fig. 2.9A & B). The exposure of BP-1B also blocked the HIF-1 α nuclear import in A549-Xenograft with decreased MVD count (Fig.2.9C - G). During hypoxia, direct phosphorylation of HIF-1α by p42/p44 (Erk-1/2) mitogen-activated protein kinases (MAPKs) is essential for nuclear translocation and recruitment of cofactors, which is a primary hallmark process for HIF-1 transcriptional activity [Lauzier et al., 2007; Richard et al., 1999]. As our experimental evidences suggests that BP-1B does not affect the expression of phospho-p42/44 (Fig. 2.7F & G), it is clearly evident that BP-1B inhibiting the nuclear import of HIF-1α is devoid of p42/44 pathway inhibition or phosphorylation. But it is more apparent from many experimental systems that BP-1B potently blocks HIF-1α nuclear entry but not through the p42/44, hence molecular interaction between BP-1B and HIF-1α was assessed by a bioinformatics approach. BP-1B strongly interacts with Thr796 of HIF-1α in C-TAD region with H bond distance 2.94 Å, where phosphorylation by p42/44 promote the nuclear accumulation and transcriptional activation of HIF-1α (Fig.2.10 A - C ). It also formed hydrophobic interaction with four amino acid residues Gln10, Tyr14, Asp15 and Val18 of HIF-1α. Under normoxia, BP-1B did not alter HIF-1α dependant genes such as VEGF-A, Flt-1, MMP-2, MMP-9 and Ang-1 (Fig. 2.11A & B) but in hypoxic conditions, inhibition of nuclear translocation of HIF-1α has down regulated the expression of VEGF-A, Flt-1, MMP-2, MMP-9 and Ang-1 (Fig. 2.11C & D and Fig. 2.12A-C). Also MMPs plays critical role in invasion of the cancer cells [Albini & Benelli, 2007].Counteraction of MMP-2 and MMP-9 expression lead to the inhibition of cancer cell migration and invasion (Fig. 2.12D - G).

Most of the solid tumour types are hypoxia in nature where they exhibit extensive intratumoural accumulation of HIF-1α (Table.2.1) [Hong et al., 2004; Folkman, 1972; Powis & Kirkpatrick, 2004; Brown & Giaccia, 1998]. As mentioned afore, HIF-1α participates in every step of angiogenesis which facilitates tumour adaptation to hypoxia. Validating in-vitro results in in-vivo tumour models is an important practice in course of developing and validating the pharmacophore of the active candidate [Eklund et al., 2013]. DL Solid (DLS) tumour model is the most appropriate model for the investigation of solid tumour hypoxia.
versus hypoxia inhibitor. The treatment of BP-1B repressed the tumour progression of DLS by inhibiting tumour angiogenesis and MVD, with remarkable extended survivability (Fig. 2.13A - I) without any symptom of adverse effect in level of RBC, WBC, Creatinine, ALP, Urea in blood (Table 2.7) and internal organs (Liver and Spleen) (Fig. 2.14A - D). BP-1B induced cellular biochemical events on DLS were assessed and it noticeably repressed the nuclear translocation of HIF-1α (Fig. 2.15A & B). The inadequate HIF-1α nuclear translocation by BP-1B suppressed the expression of several genes such as VEGF-A, Flt-1, Ang-1, MMP-2 and MMP-9 (Fig. 2.15C - G and Fig. 2.16A - D) as a consequence tumour angiogenesis was significantly inhibited (Fig. 2.13D & E). Together, mechanistically, BP-1B inhibits the angiogenic signaling factors by arresting the nuclear import of HIF-1α (Fig. 2.17).

Taken together, the current investigation conclude that (a) BP-1B has potential cell specific cytotoxic effects with prolonged activity, (b) BP-1B effectively inhibits neovascularization in both in-vitro and in-vivo level, and c) BP-1B acts as a potent HIF-1α nuclear translocation inhibitor which inhibits the tumour angiogenesis through the alteration of HIF-1 target gene activation.
Figure 2.17: Schematic representation of BP-1B action on hypoxia mediated tumour angiogenesis.
2.5. Summary

Hypoxia is an important module in all solid tumours including head & neck, oral squamous carcinoma, breast, lung, lymphoma, cervical, renal carcinomas, etc., to promote angiogenesis, invasion and metastasis. Stabilization, activation of HIF-1α and subsequent nuclear localization of HIF-1α subunits results in the activation of tumour promoting target genes such as VEGF, Flt-1, MMPs, Ang-1 which are predominantly involved in neovessel formation. Increased HIF-α and its nuclear translocation have been correlated with pronounced angiogenesis, aggressive tumour growth and poor patient prognosis leading to current interest in HIF-1α as an anticancer drug target. Benzophenone-1B ([4-(1H-benzimidazol-2-ylmethoxy)-3,5-dimethylphenyl]-(4-methoxyphenyl) methanone, or BP-1B) is a new antineoplastic agent with potential angiopreventive effects. Current investigation reports the cellular biochemical modulation underlying BP-1B cytotoxic/antiangiogenic effects. Experimental evidences postulate that BP-1B exhibits the tumour specific cytotoxic actions against various cancer types with prolonged action. Moreover BP-1B efficiently counteracts endothelial cell capillary formation in in-vitro, in-vivo non-tumour and tumour angiogenic systems. Molecular signaling studies reveal that BP-1B arrests nuclear translocation of HIF-1α devoid of p42/44 pathway under CoCl$_2$ induced hypoxic conditions in various cancer cells thereby leading to abrogated HIF-1α dependent activation of VEGF-A, Flt-1, MMP-2, MMP-9 and Ang-1 angiogenic factors resulting in retarded cell migration and invasions. The in-vitro antiangiogenic signaling modulation by BP-1B was reproducible in the reliable in-vivo solid tumour model with reduced tumour growth and MVD. Taken together, we conclude that BP-1B impairs angiogenesis by blocking nuclear localization of HIF-1α which can be translated into a potent HIF-1α inhibitor.