4.0. General Summary
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4.1. Hypoxia is a key player in tumour angiogenesis

Hypoxic microenvironment, a characteristic feature of locally advanced solid tumours, has emerged as an essential factor of tumour physiology because it can promote tumour initiation, progression and resistance to therapy [Carnero et al., 2015; Semenza, 2016]. Beyond its role in neovascularization as a mechanism for tumour adaptation to nutrient and oxygen deprivation, hypoxia has been related to elongated life span and immortality, changes in metabolism, stem cell proliferative deregulation and inflammation, among other tumour hallmarks [De Bock et al., 2011, Carnero et al., 2015]. During hypoxia, oxygen deliverance is abridged because of abnormalities in the tumour vascularization, including swollen capillaries characterized by permeable and sluggish blood stream [Hida et al., 2011; Persano et al., 2007]. HIF-1α is key molecule overexpressed by hypoxia which plays an important role in tumour adaptation to hypoxic microenvironment. Overexpression and stabilization of HIF-1α by tumour hypoxia leading to nuclear accumulation of HIF-1α, which induces the expression of battery of angiogenic genes including VEGF, Flt-1, MMPs, Ang-1 which are predominantly involved in sprouting neo-vasculature [Goh et al., 2007; Langenkamp & Molema, 2009]. Targeting HIF-1α stabilization and nuclear translocation by small molecule is a prime strategy in the development of anti-angiogenic drugs.

4.2. Benzophenone derivative is a valid drug for cancer therapeutics

Benzophenones are a group of compounds with more than 300 plus pharmacologically effective members, which have vast structural diversity but share a common phenol-carbonyl-phenol skeleton [Wu et al., 2014]. Hence, the strategy to synthesize benzophenones derivative has fascinated wide-ranging consideration due to their potential pharmacological activity. Recently, we have identified a number of novel benzophenone-1 derivatives as effective antitumour candidate [Prabhakar et al., 2006a; Vijay Avin et al., 2014a; Gurupadaswamy et al., 2014; Al-Ghorbani et al., 2016, 2017, Prabhakar et al., 2006b]. The efforts have been made to enhance the pharmacological potency of benzophenone-1 by combining BP-1 pharmacophore with other biologically active moieties such as benzimidazole and thiazole moiety [Prashanth & Prabhu et al., 2014; Ranganatha & Prabhu et al., 2013]. Such active pharmacophores BP-1B and BP-1T emerged as effective drug
against angiogenesis with ~7.5 fold enriched activity compared to parental molecule. The biochemical modulation under the BP-1B and BP-1T mediated anti-angiogenic mechanism was evaluated on non-tumourigenic and tumourigenic angiogenesis by in-vitro, in-vivo and in-silico models.

4.3. BP-1B inhibits angiogenesis by blocking nuclear import of HIF-1α

Molecular mechanism of BP-1B on tumour angiogenesis was analyzed by cytotoxic, in-vitro & in-vivo anti-angiogenic, in-vitro & in-vivo anti-tumour and in-silico studies. The cytotoxic experimental evidences postulate that BP-1B exhibits the tumour specific cytotoxic actions against various cancer types 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 with prolonged action. VEGF being a predominant player in induction of angiogenesis, is used in reliable non-tumour angiogenesis assay models. Moreover BP-1B efficiently counteracts rVEGF165 endothelial cell capillary formation in non-tumour angiogenic assay systems such as HUVECs migration & tube formation, rat aortic ring, ex-vivo CAM, rat corneal angiogenesis assays. In addition, BP-1B showed the effectual angiopreventive effect on tumour angiogenic systems such as peritoneal angiogenesis, CAM- A549 xenograft models. BP-1B exhibited molecular signaling was analyzed by immunoblot & immunocytocchemistry studies. Results reveal that BP-1B arrests nuclear translocation of HIF-1α devoid of p42/44 pathway under CoCl2 induced hypoxic conditions in various cancer cells. The insufficient nuclear entry of HIF-1α by BP-1B abrogated HIF-1α dependent activation of VEGF-A, Flt-1, MMP-2, MMP-9 and Ang-1 angiogenic factors as assessed by immuoblot, ELISA and zymogram. The inhibition of MMP-2 and MMP-9 resulted in retarded cancer cell migration and invasions as validated by migration and invasion assays. In-silico approach reveals that BP-1B interacts with Thr796 of HIF-1α by H-bond distance 2.94 Å and by hydrophobic bonding with Gln10, Tyr14, Asp15 and Val18 of HIF-1α thereby inactivates the HIF-1α activation. The in-vitro results were revalidated in the reliable in-vivo solid tumour model. The administration of BP-1B diminished tumour parameters such as tumour growth, tumour volume and extended the survivability of the animals in both murine ascitic lymphoma (DLA) and murine solid lymphoma (DLS) models. The intratumoural gene alterations were studied by
immunoblot, zymogram and IHC. The results unveiled that BP-1B counteracted the VEGF-A, Flt-1, MMP-2, MMP-9 and Ang-1 expression by blocking nuclear entry of HIF-1α in DLS tumour model. As consequence BP-1B restrained the microvascular density in DLS tumour as assessed by histopathology and CD-31 immunostain. Taken together, BP-1B impairs angiogenesis by blocking nuclear localization of HIF-1α and by counteracting HIF-1 dependant angiogenic gene activations.

4.4. **BP-1T inhibits angiogenesis by degrading of HIF-1α through the activation of p53/MDM2 proteasomal pathway**

To find the molecular mechanism underlying cytotoxic/antiangiogenic effects of BP-1T on tumour angiogenesis, various assays and model systems were employed. Cytotoxic studies showing that BP-1T exhibits potent cytotoxicity with prolonged activity against A549, MCF-7 and SCC-9 cells. The rVEGF_{165} stimulated neoangiogenesis in *ex-vivo* & *in-vivo* CAM assays, rat corneal micropocket & aortic ring assay models was considerably abridged by BP-1T inferring its potentiality of anti-angiogenic action under non-tumour condition. Also, BP-1T distinctly reticence the human functional endothelial cell migration, adjacent cell association and capillary tube forming competence of HUVECs with irregular/apoptotic cell morphology. Further BP-1T anti-angiogenic efficacy was revalidated in tumour induced angiogenic models such as A549-CAM-xenograft and DLA induced peritoneal angiogenesis. Evidences showed that BP-1T effectively regressed the tumour development by repressing neovessel formation both in xenograft and peritoneal angiogenesis as analyzed by histopathology and CD-31 endothelial marker stain. The expression of CoCl₂ induced HIF-1α was inhibited by BP-1T in various p53 (WT) expressing cancer cells, including A549, MCF-7 and DLA but not in mutant p53 expressing SCC-9 cells. The involvement of p53 in HIF-1α inhibition was analyzed by silencing p53 in A549 cells. Result demonstrated the inability of BP-1T in HIF-1α inhibition which validates the role p53 in BP-1T mediated action. Proteosome inhibition assay using MG132 further validated the results. In the absence of MG132, BP-1T induces the proteasomal degradation of HIF-1α through the mediation of p53/MDM2 but is reversible in the presence of MG132. Mechanistically, BP-1T mediates the HIF-1α proteasomal degradation by activating p53/MDM2 pathway and thereby down regulated HIF-1α dependent angiogenic
genes such as VEGF-A, Flt-1, MMP-2 &-9 under hypoxic condition of in-vitro as validated by immunoblot, zymogram and ELISA. The inhibition of MMP-2 and MMP-9 resulted in abolition of migration and invasion of A549 cells as observed by migration and invasion assays. The molecular interaction between BP-1T and MDM2 studied by in-silico approach reveals that BP-1T interacts with Ser17 of p53 binding site of MDM2 by H-bond with the bond distance 2.76 Å. It also formed hydrophobic interaction with 11 amino acid residues – Phe55, Gly58, Met62, Leu54, Gln72, Tyr67, Ile61, His96, Gly16, Val93 and Gln59 which are chief members in p53 interacting domain (Fig.3.7A & B). This interaction blocks p53 from its degradation and facilitates the p53 cellular accumulation which allows p53/MDM2-mediated polyubiquitylation and degradation of HIF-1α. Since p53 has a critical role in apoptosis, induction of p53 by BP-1T lead to considerable apoptotic cell death in p53 WT expressing cells but not in p53 silenced or mutant cells as verified by FACS. BP-1T effect revalidated in in-vivo DLS tumour model postulate that BP-1T potentially reduced the tumour development by abridging MVD as evaluated by CD-31 IHC. The BP-1T exhibited biochemical modulation was analyzed by immunoblot, IHC, zymogram and ELISA. Under in-vivo solid tumour hypoxia, BP-1T degrades the HIF-1α proteasomally by upregulating p53/MDM2 expressions which consequently resulted in decreased expression of HIF-1 dependant activation of angiogenic factors. Together results conclude that BP-1T acts on HIF-1α degradation through p53/MDM2 proteasome pathway.

Taken together, experimental evidences summarize that BP-1B and BP-1T plays an effective role against tumour angiogenesis and it could be developed as potent anti-angiogenic drug for cancer therapeutics in near future.