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BP-1C Targets Inflammatory Hypoxic Tumor Micro Environment to Counteract ROS Mediated Angiogenesis in Lung Cancer

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ABSTRACT: Lung Cancer is a prime and defied malignancy tumor with multiple hall marks of cancer. Establishing the treatment strategy for lung cancer is extremely challenging due to aggressive adaptation of cancerous cell to TME. The ROS, inflammation and hypoxia are the major key parameters which are interconnected and interdependent in lung cancer responsible for angiogenesis. Understanding the relationship between these TME parameters could be the advantageous to develop the therapeutic targets. Previously we have reported that, BP-1C induces apoptosis and anti-angiogenesis mediated antitumor properties in both ascites and solid tumor model. Further investigation has identified JAK2/STAT3 signaling mediated regression of lung cancer. Current research postulates that, BP-1C targets pathological features of Lung TME, particularly ROS and hypoxia to counteract lung cancer. The *in-vitro* study results revealed that, BP-1C reduces hypoxia as verified by EF5, a hypoxic marker and reduced ROS and lipid per oxidation assay with increased Glutathione. Further, reduced pro-inflammatory cytokines such IL-6, IL-1β and TNF-α, as well as angiogenic players such as VEGF, MMP-2 & 9 were evident. The results correlates with earlier findings of BP-1C medicated affect, such as induction of apoptosis and anti-angiogenesis. The current investigation establishes molecular cause of these events induced by BP-1C. The research outcome states that, BP-1C could be developed as an effective target specific molecule to counteract Lung cancer.

Keywords: Lung cancer, ROS, Inflammation, Hypoxia, BP-1C, Angiogenesis.

INTRODUCTION

Lung Cancer is a prime and defied malignancy tumor all over the world with high number of mortality due to its complexity (Dela Cruz *et al.,* 2011). The lung cancer tumor micro environment is tightly associated with diseases progression and rapidity, in which inflammatory micro environment plays a remarkable role over the disease occurrence and development (Whiteside, 2008). Prevailing hypoxia and elevated Reactive Oxygen Species (ROS) in lung cancer TME, activates multiple signaling pathways which contributes overall prognosis of the disease. Several studies signify the strong association between lung cancer pathology, hypoxia and ROS (Tan *et al.,* 2021). Understanding the relationship between these pathological conditions in lung cancer progression could be the advantageous to identify the more therapeutic targets for lung cancer treatment.

Hypoxic microenvironment arises due to consumption of excessive oxygen by growing tumor cells and insufficient oxygen supply by dysfunctional tumor vessels. Hypoxic nature is a predominant feature of tumors and a crucial constituent of the TME, as it influences numerous aspects of tumor biology, resistance to apoptosis, angiogenesis, metastasis, immune evasion as well as drug resistance (Muz *et al.,* 2015). One of the Key important transcription factor produced in hypoxic TME is HIF-1α which is over expressed in many cancer condition including lung cancer, which is strongly associated with the up regulation of a variety of angiogenic factors such as VEGF, Angiopoietins, MMP's and thereby contributing poor prognosis (Al-Ostoot *et al.,* 2021).

On the other hand, ROS generated through metabolism influence numerous biological processes. Excessive ROS formation usually leads to diseases, including cancer, owing to its detrimental effects on biological macromolecules. ROS levels are increased in cancers and cancer cells fine-tune ROS concentrations via ROS inducers and scavengers to benefit their proliferation and metastasis (Liou and Storz 2010). ROS contributes to overall disease progression through over expression of many proinflammatory cytokines such as IL-6, IL-1β, and TNF-α by activating numerous signaling pathways (Pawluk *et al.,* 2020). These processes ultimately initiate establishment of tumors through abnormal angiogenesis, which is eventually regulated by hypoxia and its regulators. Intriguingly, hypoxic system regulates ROS production, which in turn modulates the stability or activity of HIF, thereby prognosis of the disease (Tafani *et al.,* 2016). Thus, modulation of ROS or ROS-interconnected hypoxic signaling has been considered as a promising

therapeutic strategy for cancer. Many research investigations have developed various therapeutic approaches to target ROS and hypoxia.

The polyphenolic class of compounds Coumarins are known to be numerous medical beneficiary (Wu *et al.,* 2020). Evidences based results showed the Coumarins potentiality exhibit multi-targeting task with various pharmacological potency. Further, modification pattern of the coumarins with benzophenones enhances its capability in multiple mode of action (Musa *et al.,* 2018). Our group previously proven the potency of the Benzophenone conjugated Coumarin derivative against neoplasia. The antitumor pharmacophore "N-{2-(2-(2-bromobenzoyl)-4-methylphenoxy)-acetyl}-hydrazine methanone Coumarin" or BP-1C, exhibited potent cytotoxic effect in multiple cancer cell lines and specified study in lung cancer shows that BP-1C target the $JAK2^{Tyr1007/8}$ phosphorylation in A549 cells for the inhibition of the lung cancer progression (Vijay Avin *et al.,* 2014; Sherapura *et al.,* 2022). With this pharmacological background and to further unravel BP-1C induced multi pharmacological target such as apoptosis and angiogenesis, the current investigation aims to target the hypoxic TME to counteract ROS mediated lung cancer progression. The results upholds the multimode efficacy of the BP-1C in contradiction of lung cancer progression by significantly reducing the ROS dependent cytokine production and its associated hypoxic tumor angiogenesis.

MATERIALS AND METHODS

Lung cancer cell line A549 were procured from ATCC, Virginia, and USA. Human VEGF-A, IL-6, TNF-α and IL-1β ELISA kit, Lipid peroxidation assay kit were obtained from Sigma Aldrich, USA. High glucose DMEM, antibiotic-antimycotic, Trypsin-EDTA & FBS, EF5 staining kit &ROS measurement kit from Invitrogen (Gibco) Grand Island, NY, USA.All the other chemicals used were of analytical grade from Sigma and Hi-Media source. The photographs were taken using Canon EOS Rebel T5. The fluorescence *in-vitro* images of experiments were taken in EVOS FL cell imaging from Thermo Scientific, USA.

1. Cell culture and *In-vitro* BP-1C treatment: To examine the BP-1C efficacy on the hypoxic mechanism, A549 cells were cultured under hypoxic condition and treated at different concentrations (0, 3 and 5 µM). The exposed cells were subjected to various experiments for further analysis.

2. IF: Immunofluorescence analyses for EF5 expression was performed on A549 cells grown on cover slips according to the manufacturer's instructions. In brief, experimental cells were treated, fixed, permeabilized and incubated with EF5 primary antibody at 4^0C overnight, followed by respective fluorescence tagged secondary antibody incubation. Nuclei were stained using DAPI. Images were taken and fluorescent intensity was measured and analyzed.

3. Gelatin Zymogram: Gelatin Zymogram was performed using earlier reported protocol to measure the secretion of MMP-2&9. In brief, secreted gelatinases from conditioned culture media separated on SDS-PAGE gels containing gelatin, stained with CBBR-250 and de-stained. The transparent bands of MMPs were recorded and quantified using Bio-Rad gel documentation TM XR+ Imaging System (Sherapura *et al.,* 2022).

4. ELISA: The levels of ROS dependent inflammatory cytokines such as VEGF, IL-6, TNF-α and IL-1β secretion in *in-vitro* system were quantified by ELISA. Briefly, 100µL of conditioned media from the control and BP-1C treated group were coated using coating buffer at 4°C, incubated with primary followed by their respective secondary antibodies and substrate treatment. The cytokines measurement was quantified by measuring the absorbance at 405nm (Al-Ostoot *et al.,* 2021).

5. Intracellular ROS detection assay: Intracellular ROS detection studies were performed by using a ROS detection kit as per the manufacturer's protocol. In brief, after treatment completion A549 cells were incubated with serum-free medium containing chloromethyl derivative of fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA), namely CM-H_2DCFDA (25 µM). Samples were then measured immediately with using microreader. Intracellular ROS reduction was analyzed through fluorescent microscope (Lin *et al*., 2018).

6. Quantification of Lipid Peroxidation inhibition: Lipid peroxidation inhibition in BP-1C treated A549 cell protein extracts were quantified by measuring the concentration of TBARS by a spectrofluorometric assay using a TBARS assay kit. Quantification was achieved by parallel measurements of a standard curve of known malondialdehyde (MDA) concentrations; and results were plotted as nM/mg of total protein concentration (Lin *et al*., 2018).

7. Glutathione (GSH) Measurement: To evaluate the BP-1C induced antioxidant effect on A549 cells, intracellular GSH was determined by colorimetric assay as per the earlier protocol. Briefly, after treatment completion cells were washed, scraped and centrifuged at 1100 rpm for 3min at 25°C, cell pellet re-suspended in ice-cold metaphosphoric acid (MPA) and immediately homogenized, centrifuged at 3000 g, 4°C for 10 min. Later, 4- Chloro-1-Methyl-7-Triflurомethyl-Quinolinium Methylsulfate and 30% sodium hydroxide reagents were added and incubated for 10 min at room temperature in dark. The absorbance was measured at 400 nm, and total glutathione content was plotted with a standard curve (De Simone *et al.,* 2013).

8. Statistical Analysis: All experiments were repeated three independent times and statistically analyzed through one-way ANOVA followed by Brown–Forsythe test. The results were expressed as mean ± standard deviation (SD). The results are expressed as mean ± standard deviation (SD). Statistical significance was set at $*p<0.05$ and $**p<0.01$. All data analyses were performed using Graph pad prism version 8.0.

RESULT AND DISCUSSION

1. BP-1C reduces the hypoxic TME establishment: The A549 cancer cell were treated with BP-1C (0, 3

and 5 µM) for 48 hr under hypoxic condition and cellular hypoxia measured through EF5 IF staining: Fluorescent Microscopic image of A549 cells showing reduced EF5 with reduced fluorescence intensity graph.

Lung cancer TME is prevailing with hypoxia and ROS. The connection between hypoxia and ROS is well established. The key hypoxic TME is associated with the pathological changes for aggression of the lung cancer progression (Tafani *et al.,* 2016) and intracellular hypoxia could be measured with the EF5 hypoxic marker (Evans *et al.,* 2007). The BP-1C reduces prevailing hypoxia TME in A549 cells in a concentration dependent manner as measured through EF5 marker by fluorescent intensity. The fluorescent intensity reduced upto 63.28 % and 81.59 % indicating BP-1C target.

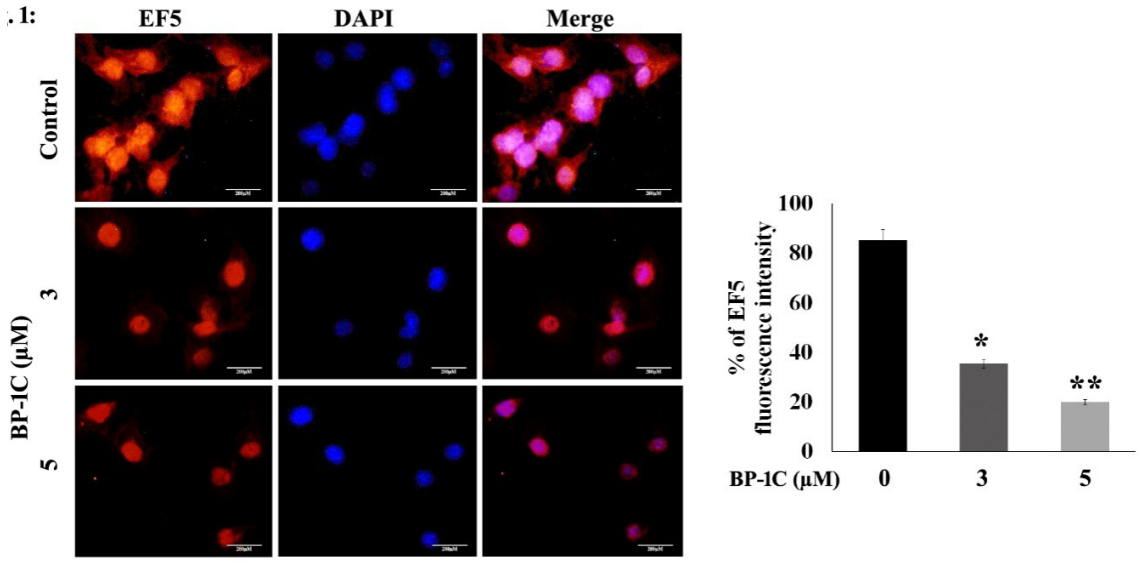

Fig. 1. BP-1C reduces hypoxic TME: The A549 cells were treated with BP-1C (0, 3 and 5 µM) for 48 hr under hypoxic condition and cellular hypoxia measured through EF5 IF staining: Fluorescent Microscopic image of A549 cells showing reduced EF5 with reduced fluorescence intensity graph.

2. BP-1C diminishes ROS: The BP-1C treated (0, 3 and 5 µM) A549 cell were measured for intracellular ROS. A) Fluorescent microscopic images showing reduced ROS generation in A549 cells with respective DCF % inhibition graph. B) Graphical representation of increased anti-oxidant glutathione. C) Graphical representation of lipid peroxidation inhibition.

Establishment of the inflammatory tumor micro environment was interceded by the ROS and its mediators. The oxidative stress with the TME causes the enormous ROS production. The BP-1C action against intracellular ROS was assessed through the ROS detection DCF assay. The results interpreted that BP-1C effectively reduced the intracellular ROS generated through the tumor prone microenvironment up to 77.57 % and 88.35% in dose dependent manner (Fig. 2A). The Glutathione, one of the major ROS inhibitory anti-oxidants helpful in the glutathione mediated DNA damage and apoptosis of the cancerous cells (Kwon *et al.,* 2019). The total amount of reduced glutathione by glutathione reductase enzyme was quantified by colorimetric assay. The result inferred the potency of BP-1C in increasing the glutathione concentration within the cells with the increase range of 43.25 % and 83.27 % (Fig. 2B). Further, ROS created by the oxidative stress can react with multiple lipid membrane fatty acids group and causes the lipid peroxidation, which intern obviously affects the DNA modification and sub cellular inflammatory cytokines secretion (Bhattacharyya *et al.,* 2014). The BP-1C promisingly inhibited the lipid peroxidation up to 1.56 and 3.57 folds (Fig. 1C). Overall BP-1C effectively inhibited the ROS and its mediators and showing its potency in anti-inflammatory activity.

3. BP-1C reduced the ROS dependent pro-inflammatory cytokines secretion: The A549 cells were treated with BP-1C and conditioned media was subjected for cytokines measurement through ELISA: A-C) Graphical representation of reduced expression of the pro-inflammatory cytokines IL-6, TNFα and IL-1β. D) Reduced expression of pro-angiogenic VEGF-A secretion. E) Reduced expression of MMP2&9.

Tumor micro environment rich in ROS are prone to the aggressive activation and secretion of several pro-inflammatory cytokines such as IL-6, TNFα and IL-1β. The secreted pro-inflammatory cytokines help the tumor cells for immune escape and activates multiple cellular signaling molecules (Pawluk *et al.,* 2020). The BP-1C inhibitory action against the ROS dependent pro-inflammatory cytokine was promising as assessed through the ELISA. The BP-1C reduced these pro-inflammatory cytokines secretion in dose dependent manner up to 2.5 & 5.5 folds for IL-6, 2.31 & 5.5 folds for TNFα and 2.4 & 6.28 folds for IL-1β.

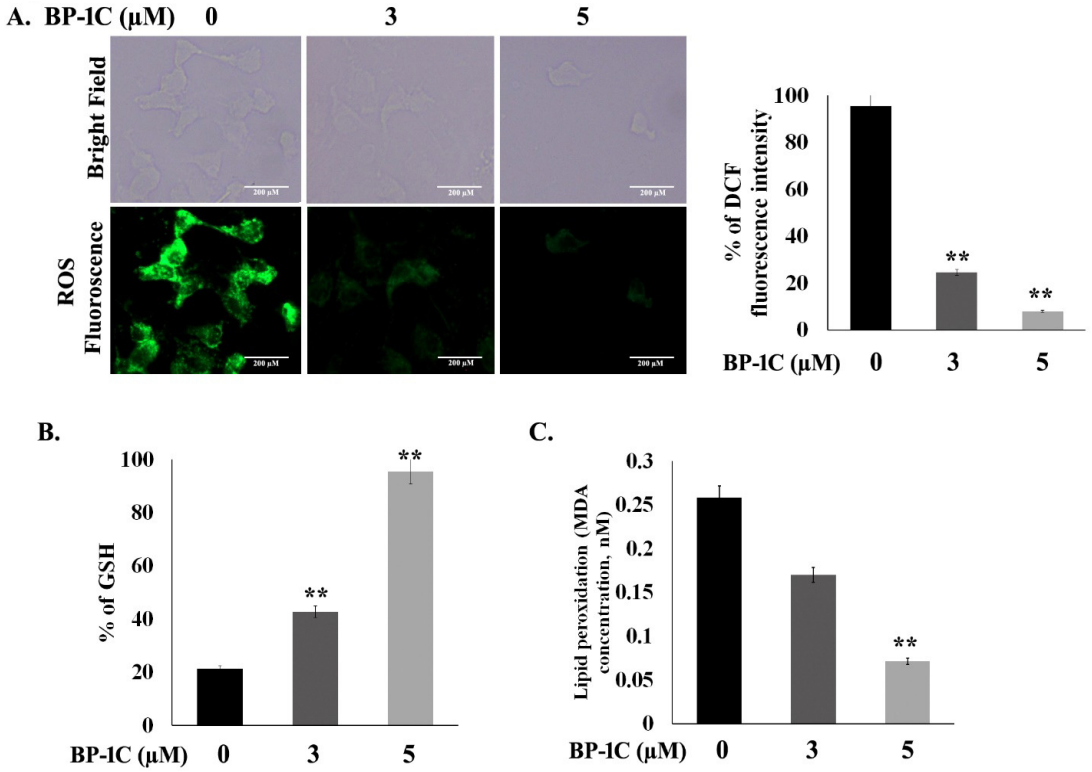

Fig. 2. BP-1C modulates ROS. The BP-1C treated (0, 3 and 5 µM) A549 cell were measured for intracellular ROS. A) Fluorescent microscopic images showing reduced ROS generation in A549 cells with respective DCF % inhibition graph. B) Graphical representation of increased anti-oxidant glutathione. C) Graphical representation of lipid peroxidation inhibition.

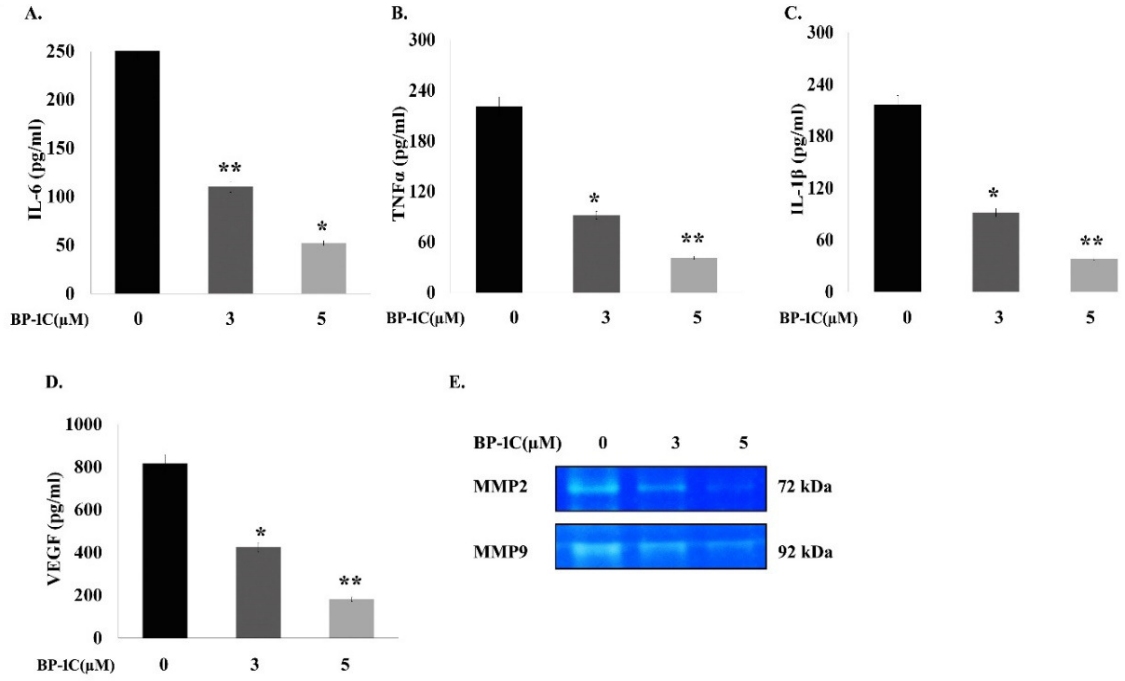

Fig. 3. BP-1C abrogates the ROS dependent pro-inflammatory secretion and alters angiogenic genes: The A549 cells were treated with BP-1C and conditioned media was subjected for cytokines measurement through ELISA: A-C) Graphical representation of reduced expression of the pro-inflammatory cytokines IL-6, TNFα and IL-1β. D) Reduced expression of pro-angiogenic VEGF-A secretion. E) Reduced expression of MMP2&9.

Down line evaluation of the effect of BP-1C on hypoxia responsive elements shows the altered gene expression of VEGF-A as evaluated by ELISA (Fig. 3D). Further, MMP2&9 expressions were also inhibited on concentration dependent manner as assessed through gelatin zymogram assay systems (Fig. 3E). The results correlate with BP-1C induced multi target effect.

CONCLUSIONS

The current investigation postulates the multi-mode approach of the BP-1C in exhibiting its anti-tumor property. The results authenticated the BP-1C counteract both ROS and hypoxia. The BP-1C effectively reduced the ROS and hypoxic TME and its associated angiogenic gene expression. Overall, BP-1C is a potent anti-neoplastic agent with multi-target drug for therapeutic approach of lung cancer.

FUTURE SCOPE

Targeting the multiple hallmarks of cancer is a beneficial approach in the drug development process for cancer treatment. BP-1C, is a one such compound with potentiality to target the multiple cancer hallmarks. Evidence based studies shown the BP-1C induced anti-cancer effects by counteracting ROS. In near future it can be developed as a potential small inhibitory molecule with multiple targets.

Author's contribution: AS performed all the experiments, YLR supported through the suggestions and guidance. AS and PBT designed the experiments, wrote the manuscript and monitored the entire investigation.

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Conflict of Interest. None.

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