Regulation of HIF-1α (Hypoxia-Inducible Factor-1 alpha) through C-Transactivation Domain (C-TAD) in Cellular Hypoxia

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Mammary gland carcinoma is one of the most frequently diagnosed cancer across the globe with a high incidence (24.50%) and mortality rate (15.50%) in women. According to reports, there will be an estimated 2.50 million new cases of mammary gland carcinoma worldwide in the coming years, and 7,68,646 people will die from mammary gland cancer by 2025. Mammary gland carcinoma is a type of solid tumor. Initially, small lumps are formed in the mammary gland which are asymptomatic. However, when the tumor size increases, it becomes symptomatic and forms a large tumor core, making it less sensitive against chemotherapy and radiotherapy with a poor prognosis. According to previous studies, solid tumors are challenging to treat owing to the development of hypoxic conditions. Hypoxia is a low oxygen condition in the cellular vasculature of the tissues. Several studies have suggested that hypoxic environment promotes cancer growth by stabilizing the hypoxia-inducible factor (HIF-1α). HIF-1α is the primary component that triggers the hypoxic signaling in solid tumors under the hypoxic environment. It becomes transcriptionally upsurged in a hypoxic environment to develop favourable conditions for the progression and development of solid tumors via the expression of hypoxic genes.

Factor inhibiting HIF-1α (FIH-1) is an enzyme that belongs to the oxygenase-dependent family, and its activity depends on O_2, 2-oxoglutarate (2-OG), iron, and ascorbate. As an oxygen sensor, FIH-1 regulates HIF-1α transcriptional activity by inducing hydroxylation at the asparagine residue (N/803) of the C-transactivation domain (C-TAD) in O_2 dependent manner. The hydroxylated HIF-1α is incompetent to produce transcriptional activity due to the impedance interaction between the hydroxylated C-TAD and CREB-binding protein (CBP). While FIH-1 activity gets limited in the low O_2 environment, it is unable to hydroxylate HIF-1α, which causes HIF-1α transcriptional activation in solid tumors. The activity of HIF-1α and associated genes that promote tumor development can be regulated by the FIH-1. Additionally, research has shown that the ferritin-heavy chain activates FIH-1, which in turn decreases the

expression of HIF-1α, carbonic anhydrase 9 (CA9), glucose transporter-1 (GLUT-1), and vascular endothelial growth factor (VEGF) 13. Moreover, studies have demonstrated that genes related to HIF-1α, such as erythropoietin (EPO), VEGF, and phosphoglycerate kinase-1 (PGK-1), are less likely to be expressed when FIH-1 is stimulated with bortezomib, amphotericin, and YC-1. Furthermore, decreasing FIH-1 levels encourages the development of cancer. In our previous studies, we postulated that chemical activation of FIH-1 would be a potential target to combat cancer. Thus, this study was designed to assess the role of chemical activation of FIH-1 in mammary gland carcinoma.

A library of 67,609 chemical compounds was virtually screened against FIH-1 based on Lipinski's rule from the ZINC database. The BBAP-8 and BBAP-9 were screened as potential FIH-1 activators based on the excellent docking scores and favorable ADMET profiles. Further, their *in-vitro* cytotoxicity and apoptotic activity were scrutinized against MCF-7 cells and *in-vivo* activity against 7,12-dimethylbenz[a]anthracene (DMBA) induced mammary gland carcinoma in Wistar rats. The *in-vitro* study was divided into four groups and evaluated BBAP-8 and BBAP-9 cytotoxicity and apoptotic activity against MCF-7 cells when scrutinized through DAPI, AO/EB, and JC-1 staining. The *in-vitro* studies revealed, BBAP-8 and BBAP-9 FIH-1 activators with significant potential to induce apoptosis. Consequently, we extend our work to evaluate the *in-vivo* efficacy of BBAP-8 and BBAP-9 against DMBA-induced mammary gland carcinoma. The animals were divided into 6 groups in both studies and treated with low and high doses of BBAP-8 and BBAP-9. After the completion of the experimental study design ECG and HRV of animals were recorded. Afterward, animals were anesthetized and sacrificed under the standard protocol. The blood and mammary gland tissue samples were collected and preserved at -20 ºC to carry out western blotting, qRT-PCR and *in vivo* anti-oxidant biochemical like TBARS, Protein carbonyl, SOD, Catalase, GSH, NO, and H_2S. The collected blood was centrifuged and the serum was carefully separated and used for serum

metabolomic analysis. For the evaluation of BBAP-8 as an anti-neovascularization effect, the mammary gland tissue was stretched onto the glass slide to perform carmine staining. Mammary tissues were preserved in formalin (10%) to assess the surface morphology using H&E staining SEM analysis. The results of both the studies have evidenced the mammary gland inhibitory potential of the BBAP-8 and BBAP-9 and discussed summarized in the preceding section separately.

In the BBAP-8 study, the *in-silico* screening represented significant potential to activate FIH-1, when scrutinized through 2-OG dependent assay and consequently subjected to further evaluation exhibited significant cytotoxic potential against MCF-7 cells in comparison to TAM. The cellular nuclear apoptotic morphological changes were determined using DAPI staining. BBAP-8 treated MCF-7 cells, were evident for CC, NF, decrease in nucleus size, and formation of AB; indicating apoptosis-like phenomenon. Subsequently, apoptotic changes associated with cell membrane were scrutinized through dual AO/EB staining. AO stains live cells whereas EB penetrates in the cells in early and late phases of apoptosis. BBAP-8 treated cells showed apoptotic morphological changes, including MB, and formation of AB, along with early and late apoptotic cells. Early and late apoptotic cells appeared condensed, and frequently fragmented orange to red nuclei, suggesting the apoptotic potential of BBAP-8. Further, the effect of BBAP-8 on MMP was validated using JC-1 dye. In control cells, the MMP was greater, and JC-1 dye form J-aggregates (dimeric form) into the mitochondrial matrix, which emits red fluorescence. Although cells have decreased MMP, JC-1 is unable to accumulate in the mitochondrial matrix and remains in its monomeric form, which emits green fluorescence. Accordingly, the shift in the red-to-green fluorescence ratio in the treated groups determines the change in MMP, and the decrease in MMP provides evidence of early apoptosis. In this study, green fluorescence was observed in the BBAP-8 treated groups, compared with the control group which indicated the loss of MMP, thus causing apoptosis. *In-vitro* studies

revealed, BBAP-8 as a prospective FIH-1 activator with significant potential to induce apoptosis. Consequently, we extend our work to evaluate the *in-vivo* efficacy of BBAP-8 against DMBA induced mammary gland carcinoma.

DMBA is a potent carcinogen and used to induced mammary gland carcinoma in experimental animals. Autonomic dysfunction is highly prevalent in clinical cases with advanced cancer and is associated with low survival. Thus, we considered it worth to evaluate the effect of BBAP-8 on ECG and HRV parameters. DMBA treatment was evident for autonomic dysfunction, as visualized through decrease HF and LF/HF ratio in comparison to normal control. The BBAP-8 dose-dependently restored the autonomic dysfunction. The cellular proliferation and surface architecture of mammary gland tissue were examined through carmine staining followed by H&E staining. The carmine staining of the DMBA treated animals was evident for the increase in LO, AB, and TEB. In line with the carmine staining, H&E staining revealed degradation of mammary ducts, LEC, MEC, atypical ductal hyperplasia, lobule enlargement, and AD invasion to neighbouring cells. The findings from the carmine staining and H&E staining demarcated significant tumor growth in DMBA treated animals. BBAP-8 treatment reduced the number of LO, AB, and TEB, suggesting the anti-proliferative potential of BBAP-8 at both doses. In line with the carmine stain, H&E staining of mammary gland tissue revealed reasonable restoration of histological architecture towards normal after BBAP-8 treatment.

The study reveals a significant association between oxidative stress and cancer progression in mammary glands. Oxidative stress and antioxidant systems are altered during the development of mammary gland carcinoma, with variations in these markers strongly predicted. Malondialdehyde (MDA) and protein carbonyl (PC) levels increased in the DMBA-treated group, but were restored to normal levels in the control group after treatment with BBAP-8. BBAP-8 therapy decreased TBAR's and PC levels while restoring antioxidant defense. Nitric oxide (NO) plays a crucial role in mammary gland carcinogenesis, with increased levels in

DMBA-induced mammary gland cancer. The study found that DMBA-treated rats showed higher NO levels than the control group, while BBAP-8 therapy reduced these levels. H_2S levels are also linked to cancer initiation and promotion, and BBAP-8 therapy reduced these levels in rats compared to DMBA-treated rats. The solid tumor microenvironment contains lactate, an oncometabolite. Angiogenesis, metastasis, and glycolysis may promote cancer growth signaling, according to research. BBAP-8 reduced lactate concentrations compared to DMBA-treated rats when scrutinized using a glycolysis kit, suggesting it may be a promising mammary gland carcinoma treatment.

The metabolomics has emerged as a revolutionary method in cancer biology; to effectively elaborate metabolites during cancer progression. Consequently, we considered it worth to study complex metabolic molecules through 1H-NMR. The metabolomic analysis revealed that DBMA treatment upregulated the serum lactate levels. The upsurge in serum lactate can be attributed to repeated glycolysis (Warburg Phenomenon); in the want of oxygen deficiency. Previously increase in lactate has been very well corroborated with increase in serum glucose and vice-versa, due to the energy requirements of fastly growing cancer cells. Similar, increase in glucose alongwith lactate was observed in DMBA treated group. Concomitant, treatment with BBAP-8, more favourably regulated the lactate levels in comparison to glucose. This can be attributed to the fact that BBAP-8 activated FIH-1 to regulate the transcriptional activation of HIF-1α. The overall analysis of the metabolites through 2D-PLSDA plot revealed the overall shifting of metabolites towards control after BBAP-8 treatments. Hypoxic signals work as a machinery for the synthesis, signaling, and reprogramming of markers that are linked to cancer progression. Accordingly, authors preferred to scrutinize the expression of various markers associated with glycolysis, angiogenesis, metastasis, and apoptosis through western blotting and qRT-PCR. FIH-1 has been reported to negatively regulate the hypoxic markers in cancer, in which decreased FIH-1 expression prompted HIF-1α mediated genes such as GLUT-1,

VEGF, and Twist-1 in the DMBA treated group. In contrast, BBAP-8 treatment had significant opposite effects in dose-dependent way. This study affirms that BBAP-8 activates FIH-1 and subsequently decreases the expression of GLUT-1, VEGF, and Twist-1.

Pro- and anti-apoptotic proteins play important roles in mitochondrial apoptosis. The effect of BBAP-8 on apoptotic proteins was also evaluated. BBAP-8 increased the expression of pro-apoptotic BAX and decreased the expression of anti-apoptotic BCL-2 in comparison to the DMBA-treated group. BBAP-8 treatment elevated the levels of caspase-8 and 3, signifying apoptosis. The qRT-PCR analysis of the expression of the genomics contributors verified the efficacy of BBAP-8 treatment by mirroring immunoblotting findings.

In the BBAP-9 study, BBAP-9 activated FIH-1 when scrutinized against *in-silico* study. BBAP-9 has potential cytotoxic effect against MCF-7 cells and decreased their viability even at low concentration compared to TAM. Thereafter, the cytological changes were evaluated using DAPI and AO/EB staining methods which revealed that the BBAP-9 has good apoptotic potential. In DAPI staining, the BBAP-9-treated cells showed nuclear morphological changes including CC and NF of the nucleus; indicating apoptotic changes. Afterward, the apoptotic morphological changes were evaluated using AO/EB staining. The BBAP-9 treated cells showed apoptotic morphological features such as early and late apoptotic cells, which appeared yellow and orange colour, respectively. The percentage of healthy cells was significantly reduced by BBAP-9 treatments compared to the control cells. The oral administration of BBAP-9 exhibited significantly increased percentage of early and late apoptotic cells; which suggests the apoptotic potential of BBAP-9. Moreover, the impact of BBAP-9 on MMP was further confirmed using JC-1 staining, because depolarisation of MMP is a hallmark of apoptosis. The MMP was higher in control cells, where JC-1 dye accumulates within the matrix of the mitochondria in the form of J aggregates and generates red fluorescence. While, JC-1 is unable to accumulate within the matrix and remains in monomeric form when cells have lower

MMP and display green fluorescence. From the findings, the alteration in MMP in the BBAP-9 treated groups is determined by the change in the red-to-green fluorescence ratio, and the declined MMP proves that apoptosis has occurred.

Based on *in-vitro* findings, BBAP-9 was observed as a FIH-1 activator, and cytotoxic potential with the ability to trigger apoptosis. Subsequently, authors expand their research to assess BBAP-9 *in-vivo* efficacy against DMBA-induced mammary gland carcinoma. DMBA induced mammary gland carcinoma model is closely mimics to human mammary cancer.

Previous evidence suggests that cancer and chemotherapy-related autonomic dysfunction is very common and linked to increased cardiac-associated mortality in cancer patients. Therefore, we believed it was worthwhile to assess BBAP-9 impact on ECG and HRV parameters. The DMBA-treated group was visible for autonomic dysfunction via the reduction in HR and increase in HRV parameters compared to the control group. The results are in corroboration with the previous reports. The autonomic dysfunction was dose-dependently eliminated/restored by concomitant administration of BBAP-9.

Thereafter, the efficacy of BBAP-9 was examined to investigate the small proliferative lesions, cellular morphology, and surface architecture of mammary gland tissue using carmine staining, histopathology, and SEM. HIF-1α stimulates the formation of small blood vessels in solid tumors to fulfil the requirements of nutrients and oxygen to the fastly dividing cancerous cells. In this study, excessive cellular proliferation was observed after DMBA treatment as represented through the increase in the number of LO, AB, TEB, and DF scores, which is in corroboration with the antecedent reports. BBAP-9 treatment decreased the number of LO, AB, TEB, and DF scores in dose-dependent manner, suggesting the anti-proliferative potential of BBAP-9. After that to validate the results of carmine staining, the histopathological changes and SEM evaluation were also scrutinized. The deterioration of md, lec, mec, ah, and, ad invasion in surrounding cells have been described in the histology of mammary gland tissues

in DMBA treated animals. In the current study, DMBA-treated animals exhibited these aberrant effects, and BBAP-9 therapy significantly curtailed towards normal control. The SEM analysis of DMBA-treated animals represented scattered cellular architecture with distorted mr and deformed mv pattern with pitted epithelial surface. The BBAP-9 showed reasonable restoration of mr and mv. BBAP-9 treatments regularized the cellular proliferation, surface architecture, and membrane ruffles. Based on the above discussed microscopical parameters authors would like to pertinent the protective effect of BBAP-9 treatment against mammary gland carcinoma. Reactive oxygen species (ROS) are produced primarily by oxidative stress, which is also linked to the development of mammary gland carcinoma and the suppression of antioxidant enzymes that fight cancer. According to the studies, oxidative stress and the antioxidant system are altered during the development of mammary gland carcinoma. This study demonstrated the substantial association between them and the progression of cancer, which is strongly evaluated by variations in the oxidative stress markers. It is widely known that cancer triggers peroxidation of the lipids and proteins in mammary gland tissues. A persistent lipid peroxidation biomarker is malondialdehyde (MDA), and protein carbonyl (PC) is a protein peroxidation biomarker. In the current study, the MDA and PC levels in the DMBA-treated group increased, and these levels were subsequently restored to normal by the administration of BBAP-9. The BBAP-9 therapy decreased TBAR's and PC levels while balancing the antioxidant defence offered by SOD, catalase, and GSH. Lactate is an oncometabolite that is observed in the tumor microenvironment of solid tumors. Studies suggest that it initiates cancer growth signaling by promoting angiogenesis, metastasis, and glycolysis. The BBAP-9 therapy effectively decreased the level of lactate compared to DMBA treated animals which suggests BBAP-9 has a promising potential candidate in cancer therapeutics against mammary gland carcinoma.

All in all, the authors would like to conclude that the current study is the first to investigate the potential of FIH-1 activators as a new therapeutic intervention for mammary gland carcinoma. Based on BBAP-8 research findings, this study concludes that BBAP-8 activates FIH-1 and has the potential cytotoxicity and apoptotic activity against MCF-7 cells with chemotherapeutic efficacy against DMBA-induced mammary gland carcinoma. Moreover, BBAP-9 research findings demonstrate that BBAP-9 has potential cytotoxic activity and induced apoptosis significantly in MCF-7 cells which was confirmed using DAPI, AO/EB, and JC-1 stainings. Moreover, BBAP-9 has chemotherapeutic efficacy on DMBA-induced mammary gland carcinoma in animals and this can be attributed to its propensity to enhance antioxidant profiles and also inhibit cellular proliferation, improve surface architecture, soothing membrane ruffles, and decrease the lactate effects. Thus, we postulated that BBAP-9 might have a novel FIH-1 activator in mammary gland carcinoma. To demonstrate BBAP-9 efficacy as an anticancer drug, more research is needed to explore the underlying molecular mechanisms.