Modulating Prolyl Hydroxylase (PHD_2) activity to alter Glycolytic Pathway and Fatty acid Synthase Expression in Tumor Cells

SUMMARY

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SUBMITTED BY

Manjari Singh

Enrollment No. – 177/11

SUPERVISOR

Dr. Gaurav Kaithwas

Associate Professor

DEPARTMENT OF PHARMACEUTICAL SCIENCES

SCHOOL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES

BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY

(A CENTRAL UNIVERSITY)

VIDYA VIHAR, RAIBARELI ROAD, LUCKNOW-226025 (U.P.), INDIA

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As per our hypothesis, the present study was undertaken for the screening of possible and potential PHD-2 activators for the curtailment of overexpressed HIF-1α and FASN in mammary gland carcinoma.

In literature, three PHD-2 activators were known but their potential effect upon HIF-1α and FASN was not elaborated. On the basis of 50% structure similarity, we screened a library of 28000 compounds using Zinc database and Asinex database through Maestro version 9.3 (Schrödinger). After screening the compounds from various filters and CDRUG server, we found only two compounds (BBAP-1 and BBAP-2) which were effective. The *in silico* toxicity profile of both the compounds was estimated using TEST. The *in vitro* PHD-2 assay also confirmed the activation of PHD-2 using 2-OG in the reaction mixture.

After getting confirmed from the *in silico* results, we elaborate the effect of BBAP-1 and BBAP-2 against ER-MCF-7 cells. For the cytotoxity estimation of the compounds, MTT assay was performed which revealed that the IC50 value of BBAP-1 and BBAP-2 was 15.29μM and 16.61μM respectively in comparison to control and tamoxifen treated cells. The results revealed that the compounds have better efficacy in comparison to the well known drug tamoxifen. The apoptosis was evaluated using AO/EtBr staining of the cells. It was well known that AO is used for the staining of viable and non-viable cells and it produces green fluorescence when attached with DNA whereas, EtBr stains only dead cells and produced red fluorescence. In the present study, the apoptotic markers were clearly seen after BBAP-1 and BBAP-2 treatment. Subsequently, the mitochondrial depolarization was measured using JC-1 stating of ER+MCF-7 cells. JC-1 is a cationic dye and exhibits as potential dependent accumulation in mitochondria. This accumulation is indicated by fluorescence emission shift from green (~525nm) to orange (~590nm). Mitochondrial depolarization is indicated by an increase in

orange/green fluorescence intensity ratio. BBAP-1 and BBAP-2 were increased the orange/green ratio and produced mitochondrial depolarization. Subsequently, the cell cycle phase distribution was analysed using flow cytometry. PI is a fluorogenic dye and it binds with nucleic acids so that fluorescence emission is proportional to DNA content of the cell. After treatment with BBAP-1 and BBAP-2, the DNA content was high in G0/G1 phase and the cell cycle was arrested in G2/M phase.

Afterwards, the efficacy of BBAP-1 was evaluated against MNU induced mammary gland carcinoma model. It was a well known fact that autonomic dysfunction is associated with breast cancer. In breast cancer patients, the heart rate is increased with decreased in HRV parameters and the similar observations were found after ECG and HRV analysis of the animals treated with BBAP-1. The morphology of the mammary gland tissue was evaluated using carmine staining, H&E staining and SEM analysis. It is a well known fact that ABs, lobules and DF score are the markers of cell proliferation and angiogenesis. In the present study the increased levels of ABs, lobules and DF score were decreased after BBAP-1 treatment. The cell organelles like adipocytes, duct, LCT, DCT, CEC, MEC and lymphocytes were distorted after MNU treatment and BBAP-1 treatment restored all the cell organelles about to normal. The surface architecture of tissues was evaluated using SEM and the results revealed that BBAP-1 treatment reduced the formation of microvessels and intra-arterial cushion. The role of BBAP-1 in antioxidant defence mechanism was evaluated using antioxidants markers like TBARs, SOD, catalase, GSH and PC. BBAP-1 treatment has a pronounced effect upon antioxidant markers. After that we evaluated the markers of apoptosis using caspase 3 and caspase 8 assay. Low dose of BBAP-1 was reduced the level of caspase 3 and caspse 8 in serum samples. The participation of mitochondrial death apoptosis pathway was measured using the immunoblotting and qRT-PCR assay. The protein

expression of fold change gene expression in Bcl-2 family of proteins (Bcl-2, Bcl-xl, BAX, VDAC, Apaf-1, procaspase 9 and cytochrome c) revealed that BBAP-1 produced mitochondrial death apoptosis. The activation of PHD-2 was further screened through immunoblotting and qRT-PCR study of hypoxic pathway. The results revealed that BBAP-1 upregulated the expression and gene fold change of PHD-2 along with decreased protein expression and fold change of HIF-1α, FASN, SREBP-1c, NFκBp65 and UCHL-1.

It was conclude that from the line of evidences derived from the present work that the exogenous chemical activation of PHD-2 can favourably regulate the cellular proliferation through mitochondrial mediated apoptosis. In the same line, BBAP-1 and BBAP-2 are recorded to be the chemical activators of PHD-2 with subsequent down-regulating expression of HIF-1α and FASN. The present study explicitly endorses the *in vivo* efficacy of BBAP-1 against mammary gland carcinoma and commands the need for clinical validation for exploring its future prospect as an anticancer agent.