To Identify Possible Inducers of Prolyl Hydroxylase 2 (PHD2) and to Evaluate Them Against Mammary Gland Carcinoma

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SUMMARY

The current study was undertaken to identify possible inducers of prolyl hydroxylase-2 and evaluate their efficacy in treating mammary gland cancer. A library of compounds was downloaded from PubChem (DTP-NCI) and was screened based on structural similarity with R59949 and the furan chalcone scaffold. After screening the compounds were docked with PHD-2 and based on similarity and binding energy a list of compounds was prepared. Drug-likeliness and ADMET properties of identified hits were determined. After completing *in-silico* studies BBAP-6 and BBAP-7 were retrieved as the most suitable compounds for further evaluation.

The PHD-2 activation potential of BBAP-6 and BBAP-7 was determined using *in-vitro* PHD-2 assay. Both compounds were found to possess PHD-2 activation potential. However, BBAP-6 was the more potent of the two. After evaluating PHD-2 activation potential of screened compounds we next determined the anticancer potential of screened compounds on *in-vitro* cell lines MCF-7 and MDA-MB-468. We first determined the IC50 values of BBAP-6 and BBAP-7 on each cell line using MTT assay and then progressed towards the determination of the apoptotic potential of screened compounds. We used DAPI and AO/EB staining to determine nuclear apoptotic changes induced by BBAP-6 and BBAP-7 as well as separate the cells according to the phase of apoptosis (early apoptotic or late apoptotic). To affirm the results AO/EB staining, we next performed JC-1 staining to determine the mitochondrial membrane potential. Loss of mitochondrial membrane potential is the characteristic feature of apoptosis. From the results of JC-1 staining it was confirmed that BBAP-6 and BBAP-7 induced apoptosis in MCF-7 and MDA-MB-468 cells. However, efficacy of BBAP-6 was more than BBAP-7.

From *in-vitro* studies it was confirmed that both BBAP-6 and BBAP-7 possess PHD-2 activation and apoptotic potential. We next determined the effect of BBAP-6 and BBAP-7 against DMBA-induced mammary gland carcinoma. Two different studies were conducted one involving BBAP-6 as primary compound under investigation and other involving BBAP-7. The animals were arbitrarily divided into 7 groups of 6 animal each in both the studies. The study extended its horizon by evaluating the effect of BBAP-6 and BBAP-7 in combination with Tirapazamine (TPZ) against DMBA induced mammary gland cancer. Since TPZ is selective hypoxia cytotoxin it will result in death of hypoxic cancer cells whereas, PHD-2 activator by inhibiting transcriptional activity of HIF-1α and NF-κB will curtail down growth of normoxic cancer cells. Henceforth, it was proposed that the combination may have a synergistic effect.

After the completion of the treatment protocol, the animals were recorded for ECG and HRV profile and subsequently, the blood was collected from the lateral tail vein of animals. The collected blood was centrifuged to obtain serum for metabolomic studies and biochemical estimations. Moving further, the animals were sacrificed by cervical dislocation under light chloroform anesthesia, and breast tissue was collected by making a median incision. The mammary gland was spread on frosted glass slides to prepare the mammary gland's whole mount for carmine staining. Some part of mammary gland tissue was preserved in formalin (10%) and the remaining tissue was stored at -20°C for western blotting and other biochemical assays. Both BBAP-6 and BBAP-7 exhibited inhibitory potential against mammary gland carcinoma and results are being discussed separately in the preceding section.

Cardiotoxicity associated with autonomic dysfunction is a well-established hallmark of chemotherapy in clinical cases and same has been reported in experimental models of carcinogenesis. DMBA treatment was apparent for the autonomic dysfunction when scrutinized through ECG and HRV analysis. The DMBA treatment produced significant increase in RR interval with decrease in HR. Treatment with BBAP-6 increased HR in dose dependent manner, however combination with TPZ most effectively reversed DMBA's induced decrease in HR. In line with the ECG, BBAP-6 dose dependently as well as in combination with TPZ favourably modulated time domain parameters like Average RR, Median RR and SDRR. However, no significant change in frequency domain HRV parameters was observed. All in all, BBAP-6 in combination with TPZ was recorded to be the most effective therapy in restoring the cardiac function and autonomic dysfunction.

Tissue morphology and pathology is markedly affected during carcinogenesis and therefore, we considered it worth to scrutinize the same using carmine and H&E staining. The DMBA treatment led to increased proliferation as perceived through increased LO, TEB, and AB after carmine staining. Treatment with BBAP-6 alone and in combination with TPZ reduced TEB, AB, and LO. The mammary gland tissue was further scrutinized histopathologically and DMBA treatment was conspicuous for hyperplasia with increased cellularity followed by dysplastic mammary glands with dilated ducts. Treatment with low dose BBAP-6 showed *in-situ* ductal carcinoma with degraded basal membrane, however, no inflammatory cell infiltration was seen. Treatment with high dose of BBAP-6 reduced aberrant cells in the lumen and marked basement membrane borders. However, disorganized ductal epithelial cells show early cellular injury. The combination therapy normalised mammary gland histology. However, few dysplastic and inspissated ducts were seen. The combination of BBAP-6 and

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TPZ efficiently cured DMBA-induced mammary gland cancer and reduced it to subacute inflammation.

Loss of apoptosis in cancer cells is the key phenomenon, that allows cancer cells to survive for longer duration and accumulate mutations for angiogenesis and metastasis. Therefore, authors preferred to evaluate the effect of BBAP-6 on various markers of apoptosis. Apoptosis is mediated through an extrinsic and intrinsic pathway. The DMBA treatment group showed increased BCL-2 expression and decreased BAX, caspase 3, caspase 8 and caspase 9 expression, suggesting inhibition of apoptosis. The results also affirm that DMBA impedes both extrinsic and intrinsic pathway of apoptosis. Treatment with BBAP-6 alone and in combination with TPZ favourably regulated extrinsic and intrinsic pathway apoptosis; wherein the combination of BBAP-6 and TPZ marked more favourable response.

The BBAP-6 has been screened as a PHD-2 activator and thus it was considered worth that the effect of BBAP-6 on PHD-2 expression may be scrutinized. The DMBA treatment reduced the expression of PHD-2 which could be attributed to the cellular proliferation and development of tumor mass. However, BBAP-6 upregulated the expression of PHD-2 in a dose-independent manner.

Hyperglycaemia and activated glutaminolysis have been established as pathophysiological features in cancer. In comparison to normal control; glucose, lactate and glutamine levels in serum were found to be significantly elevated in DMBA treatment and glutamine to glucose ratio (QGR) was considerably decreased. In contrast, serum levels of glucose, lactate, and glutamine were reduced after BBAP-6 administration, and the QGR was dose-dependently increased. Increased serum levels of pyruvate kinase M2 are reported in cancer, which turns phosphoenolpyruvate into pyruvate, ultimately leading to an increase in pyruvate concentration. DMBA treatment marked increased serum pyruvate which was restored by BBAP-6 alone and combination with TPZ. In addition to pyruvate; succinate and malonate are other TCA cycle metabolites were observed to be elevated in cancer. Tumor cells secrete succinate to facilitate polarization of tumor associated macrophages, promote cell migration and metastasis. Aberrantly, high serum levels of malonate and succinate were found toxic group which were restored by the BBAP-6. Moreover, tumor microenvironment convert toxic derivate of amino acid biotransformation, ammonia, into a non-toxic form urea and release in blood. Another nitrogenous product creatine was found to promote metastasis in colo-rectal cancer and breast cancer. Compared to normal control group, DMBA treated group showed

significantly elevated levels of creatinine, creatine and urea which were further reduced by BBAP-6 treatment and combination therapy. Malignant cells depend mainly on glucose and reductive glutamine metabolism for lipid and membrane synthesis. Apart from glucose and glutamine another important metabolite for fatty acid and membrane synthesis is glycerol which is primarily generated from glycolysis. In this study, DMBA treated group had considerably higher serum levels of glycerol. It is appropriate to point out that BBAP-6, both alone and in combination with TPZ, restored the metabolic profile of glycerol at high doses.

To assess comprehensive anticancer potential of BBAP-7, the efficacy of BBAP-7 was evaluated against DMBA induced mammary gland carcinoma. ECG analysis showed that despite significant apoptotic potential of BBAP-7 at high dose, the low dose and combination of high dose with TPZ favourably regulated time domain parameters like Average RR and Median RR. Similar observations were recorded with the frequency domain parameters like LF/HF ratio. Thus, one can derive that BBAP-7 in combination with TPZ can regulate the autonomic dysfunction more favourably.

Studies have reported that DMBA induced experimental carcinoma involves disruption of tissue redox balance, suggesting biochemical and pathophysiological disturbances. DMBA administration intensified lipid and protein peroxidation when scrutinized through TBARs and PC levels. The increase in TBARs and PC level suggest generation of ROS, which was also evident through downregulation of enzymatic oxidative defence of catalase and SOD. The SOD and catalase work in tandem to curtain the ROS, wherein SOD dismutase the $O^{\cdot}$ to molecular O_2 and $H_2O_2$and the H_2O_2 thus released is catalysed by the catalase to H_2O and O_2. Concomitant treatment with BBAP-7 and combination of BBAP-7 with TPZ restored the SOD and catalase towards normal. The above therapy also limited the lipid and protein peroxidation as evident through reduction in the TBARs and PC levels. The efficacy of BBAP-7 was further evaluated using carmine staining, H&E staining, and SEM. Malignant transformation and differentiation in mammary gland cancer are directly related to mammographic density because breast cancer in humans begins in terminal ductal lobular units (TDLUs), which resemble terminal end buds (TEBs) in rats. The carmine staining of the mammary gland tissue was very well evident for the increase in alveolar bud (AB), lobules (LB) and terminal end buds (TEB) after DBMA treatment. BBAP-7 and combination of BBAP-7 with TPZ embarked more significant effect of the cellular proliferation with decreased AB, LB and TEB count. The histopathological evaluation through H&E staining revealed typical features of DMBA induced mammary gland carcinoma. These features include ductal carcinoma in situ (DCIS), ductal cell

dysplasia, absence of basement membrane along with presence of infiltrating immune cells. Low dose of BBAP-7 restricted the dissemination of cancer cells solely inside the ductal structures, whereas at higher dose reduction in the atypical DEC was seen in lumen. Additionally, disorganised DEC were also seen, however, the basement membrane looked to be intact. The combination of BBAP-7 with TPZ demonstrated marked effect through restoration of typical morphology of the rat mammary gland with presence of subacute inflammation. The surface architecture was evaluated through the SEM. DMBA treatment was evident for the presence of small and large duct network, nodules along with degradation of collagen cover, which was restored towards normal after the BBAP-7 treatment. Cancer cells both in the presence and absence of oxygen undergo aerobic and anaerobic glycolysis to generate large amount of lactate, the phenomenon known as Warburg effect and Pasteur effect respectively. The anaerobic condition is cancer cells leads to activation of HIF-1 and consequently cell undergo repeated glycolysis. In other words, the major source of ATP for the hypoxic cells is glycolysis and consequently there is intracellular accumulation of the lactate. Similarly in the current study, DMBA treatment was very much evident for the increase lactate levels which was subsided towards normal after BBAP-7 and TPZ combination therapy. The decrease in the lactate levels is a direct marker for inhibition of repeated glycolysis and the same could be attributed to the proteasomal degradation of HIF-1 due to PHD -2 activation.

In a nut shell, screening results showed BBAP-6 and BBAP-7 as possible inducers of PHD-2. *In-vitro* and *in-vivo* studies revealed that both BBAP-6 and BBAP-7 possess anticancer potential, however, BBAP-6 had better efficacy than BBAP-7.

BBAP-6 effectively upregulated PHD-2 and restored apoptosis in mammary gland cancer caused by DMBA. Additionally, the study also records that BBAP-6 when combined with TPZ had greater anticancer efficacy. NMR metabolomics further revealed that BBAP-6 treatment significantly nudged down glycolysis and fatty acid synthesis in DMBA-induced mammary gland cancer. Henceforth, the present study reports BBAP-6 as a potential PHD-2 activator with anticancer activity.