Upregulating PHD2 activity to downregulate HIF-1 alpha and subsequently FASN in mammary gland carcinoma

Summary

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SUMMARY

The current study is ventured to delineate anti-cancer potential of Voacamine (VOA) in DMBA and MNU induced mammary gland carcinoma. The drug was selected on the basis of docking studies and obtained as a drug sample from National Cancer Institute (USA). A library of natural compounds similar in structure with Vincristine (VIN) was downloaded from ZINC database and then docked with PHD-2, VOA was one of them. In silico, toxicity (LD50) and ADME studies were performed with online software and afterward *In Vivo* studies were performed in albino wistar rats after the approval of protocol. Animals were divided into 7 groups in both the studies and treated with low and high doses of VOA alone and in combination with VIN and TMX. After the completion of the experiment, ECG and HRV of animals were recorded prior to the end of study. Afterward animals were sacrificed with cervical dislocation under ether anesthesia, blood and vital organs were collected and preserved in formalin (10%) in order to evaluate the histopathological changes. Mammary gland tissue was stretched on to the glass slide to perform carmine staining to evaluate the anti-angiogenic potential of the drug. Mammary gland tissue was also collected and stored in the -20 to carry out western blotting, gas chromatography and in vivo antioxidant biochemicals like TBARS, Protein carbonyl, SOD and Catalase. The collected blood was centrifuged and serum was carefully separated and used for serum metabolomic analysis. The result of both the studies have evidenced the mammary gland inhibitory potential of the VOA and discussed summarized in the preceding section separately.

In the DMBA study, results of western blotting shows that HIF-1α, SREBP-1c and FASN were significantly raised in TC groups that were observed to be decreased in subsequent treatment groups (treated with VOA and VIN). At the same time a raised level of PHD2 was found in treatment groups (with combination low and high dose therapy of VOA and VIN). It means treatment with VOA and VIN somehow enhancing protein expression of PHD2 and decreasing protein expression of HIF-1α, SREBP-1c and FASN. Metabolomic profile of TC control group shows a decreased level of glucose and increased level of lactate, glutamate and PUFAs that indicates excess glucose was converted into lactate which is true according to Warburg effect that cancerous cells prefer glycolytic pathway compared to oxidative pathway (Citric acid cycle) in hypoxic conditions. Excess of lactate formed disturbed the intra and extracellular pH in tumor cells. Disturbed pH in the intra and extracellular environment activated the SREBP-1c present in the endoplasmic reticulum. Western blotting has evidenced the increased expression of SREBP-1c which then enhanced the FASN expression and as result of this more and more synthesis of PUFAs resulted to construct the plasma membrane and to meet energy requirements. Treatment with VOA and VIN decreased in level of lactate, glutamate. From this it can be concluded that HIF-1α increased the intracellular lactate acidosis to activate SREBP-1c in order to enhance fatty acid synthesis that was reverted back when HIF-1α went degradation upon PHD2 to activation with VOA and VIN therapy. All in all, VOA and VIN activated PHD2 which down regulated HIF-1α, reduced lactate acidosis, inactivated SREBP-1c and FASN to check fatty acid synthesis needed for plasma membrane synthesis by the rapidly dividing breast cancer cells (Figure13). Further, decreased levels of TBARS, SOD, Catalase, and GSH evidenced the protective mechanism of VOA therapy. Combination therapy imparted much better protection compared to monotherapy of VOA and VIN. FAME analysis of mammary gland tissue also showed higher

levels of polyunsaturated fatty acid (PUFA) level in DMBA treated toxic treated groups which was also subsided back to normal. Carmine staining showed higher branching of mammary gland ducts, alveolar buds (ABs), lobules (Lo) and terminal end buds (TEB) that clearly demarcated the angiogenesis after DMBA administration. Very less branching of mammary gland ducts, ABs, TEB were observed in VOA and VIN treated groups which further support the anti-angiogenic potential of said therapy. Histopathology of mammary gland tissue further supported the anti-cancer potential of VOA alone and combination therapy with VIN. Excessive distortion of ductal epithelium (DE), myoepithelium (ME), and lactiferous ducts (LD) as observed in toxic control was not observed in treated groups which indicate VOA and VIN therapy in combination blocked further damage to the mammary gland tissue. Histopathology of vital organs like liver and kidney showed excessive damage to the Bowman's capsule, Glomerulus, proximal convoluted tubule (PCT) case of kidney and excessive damage to lobules (lo), enlargement of central vein (CV) and damage to sinusoids (dSn) indicates that combination therapy of VOA with VIN should be advocated cautiously in patients with renal and liver complications. Cardiotoxicity of drugs was assessed with ECG and HRV recording and large ECG changes like increase in P amplitude, R amplitude, T amplitude, Heart rate were noted after DMBA administration. Large increases in the same parameters were observed after the initiation of therapy which marked the cardio toxic potential of current therapy. Conclusively, combination therapy with (both low and high dose) VOA and VIN imparted much better protection to the mammary gland tissue but at the same time it caused noteworthy renal, liver and cardio toxicity. As far as monotherapy with VOA high dose was considered, it not only imparted protection to the mammary gland tissue but also caused negligible renal, liver and cardio toxicity.

In MNU study, MNU administration manifested an increase in expression of HIF-1α, SREBP-1c, FASN and reduced expression of PHD-2 which indicated development of severe hypoxia. Upon treatment with VOA and TMX the opposite relationship is obtained in the above proteins. Again, this indicated HIF-1α somehow increasing expression of SREBP-1c and FASN in order to increase fatty acid synthesis. The above finding was further supported by the serum metabolomic analysis of rats. High Glucose, lactate, Glutamate, Choline ,Betaine and myo-inositol were detected in toxic control which are very well known to enhance fatty acid required for plasma membrane of rapidly dividing cancer cells. From these findings it can be concluded that severe hypoxia activated HIF-1α which directed metabolism of glucose mainly through glycolysis due to lactate concentration was increased after MNU administration. Along with lactate, elevated levels of glutamate, choline, betaine and myo-inositol was also observed in the toxic group which clearly indicates that lactate somehow increases the rate of fatty acid synthesis. These changes were further supported that HIF-1α increased the lactate acidosis to enhance fatty acid synthesis in cancer cells under hypoxia. Interestingly, perturbed metabolites were restored back to normal upon initiation of therapy with VOA low dose, TMX and with combination therapy of both of these drugs which clearly indicates that VOA in low dose as well as in combination with TMX somehow reducing the level of HIF-1α, the ultimate culprit. Above we have already talked about the increased expression of PHD-2 in treatment groups treated with VOA low dose, TMX and combination of these two drugs. PHD-2 is a negative regulator of HIF-1α; it causes hydroxylation and the proteolytic degradation in normoxic cells but becomes inactive in hypoxia. From, it can be concluded that VOA and TMX monotherapy and combination therapy activated the PHD-2 in a hypoxic medium which enhanced the proteolytic degradation of HIF-1α and subsequently level of lactate and rate of glycolysis was subsided back to normal. Biochemical

analysis further supported the above finding. Higher level of TBARS, protein carbonyl was observed in MNU treated toxic groups which was restored back to normal. SOD, catalase and GSH are a group of enzymes which work together to keep the free radical level to minimum. Decreased level of SOD, catalase and GSH in toxic and increased level after treatment evidenced the antioxidant potential of therapy. Anti-angiogenic potential of therapy was evaluated by carmine staining. Results of carmine staining showed excessive branching of the mammary gland ductal tree after MNU administration which indicates initiation of angiogenesis in toxic control. Comparatively very less branching of mammary gland tissue was observed in treatment groups treated with VOA low dose, TMX and their combination evidenced by less number of AB, TEBs and lateral buds (Lb). Histopathological examination of mammary gland tissue further supports the above finding. Distorted arrangement of adipocytes, and lactiferous duct was observed in toxic control group animals after MNU treatment. Normal architecture of adipocytes and lactiferous duct in treatment groups shows that therapy with VOA low dose, TMX and combination of these two drugs worked well to protect the mammary gland tissue. ECG and HRV also recorded to assess the cardio toxic potential of therapy. Normal ECG and HRV were recorded in normal group rat but large ECG changes like increase in PR interval, QT interval, ST height, and JT interval were noted in toxic control which was found even more increased with VOA high doe, TMX and DMSO. From this it can be concluded that VOA high, TMX and DMSO somehow cause cardiac toxicity because increased ST height, JT interval and QT interval are associated with myocardial infarction. An ECG like normal rats were recorded in rats treated with low dose of VOA, and with combination therapy which indicates VOA is safe at this dose and can be used in heart patients also.

Cachexia in advanced cancers of mammary gland is another encountered problem and various studies have reported progressive loss of weight in cancer. MNU treated rat's documented constant decrease in body weight which is consistent with the previous studies (Figure 3). Further decrease in body weight was prevented by the low dose of VOA, TMX and combination therapy but excessive decrease in body weight like toxic control was observed with high dose of VOA which indicates its toxicity at high dose. Conclusively, we can say that VOA low dose is a comparatively safe and better drug to combat mammary gland carcinoma as well. Results of MNU study also proved that VOA has a potential to activate PHD-2 and which can further down regulate HIF-1α and its subsequent effect on fatty acid synthesis.