ABSTRACT

Currently, most of the research is focused on targeting multiple signalling pathways and among them RAS signaling pathway, PI3K-AKT and MEK-ERK are the most common targets. However, drug resistance to these agents continues to present challenges. The present study aimed to develop dual drug resistant melanoma models, study the altered proteome and phosphoproteome profiles by LC-MS/MS analysis, identify the key molecules for sustaining resistance and characterize the drug resistance mechanism.

In Phase I, two melanoma cell lines, B16F10 and A375 were used for the development of dual drug resistant models. Cells were treated with a combination of U0126 (ERK inhibitor) and LY294002 (PI3K/AKT inhibitor), and from dose response curve the 50% inhibition concentrations (IC$_{50}$) values were determined. To establish the drug-resistant models, the parental melanoma cells (referred as B16F10C and A375C) were treated with a combination of U0126 and LY294002 continuously, starting from the 1/10$^{th}$ the IC$_{50}$ values with a gradual increase until they become resistant to the drug combination. The B16F10 cells attained a resistance fold factor of $>2$ in 15 months and were named as B16F10R subline. To confirm the development of drug-resistant cell line, ERK and AKT phosphorylation estimation, live/dead cells assay and clonogenic assay were performed. Even after treatment with the combination of U0126+ LY294002, B16F10R cells was able to survive, proliferate, and form colonies. Since A375 cells, however, did not attain a resistance fold factor of $\geq 2$ in 15 months, the treatment with continued and A375R subline was used in Phase III when it attained a resistance fold factor of $\geq 2$.

In Phase II, dual drug resistant B16F10R model and parental B16F10C cells were used for the proteomic and phosphoproteomics analysis. Phosphopeptide enrichment was done with titanium dioxide prior to LC–MS/MS analysis. The proteome analysis of B16F10R/B16F10C showed 71 upregulated and 150 downregulated proteins belonging to primary metabolic processes, carbohydrate metabolic process, glycolysis and tricarboxylic acid cycle. Matrix metalloproteinase (MMP) isoforms were among the top 10 up-regulated proteins and serine/threonine protein phosphatase 2A (PP2A), a dephosphorylating enzyme was down-regulated. Phosphoproteome analysis had identified 363 differentially phosphorylated phosphoproteins—
hyperphosphorylated and 137 hypophosphorylated. Data analysis using online tools suggested the altered phosphoproteins belonged to RNA metabolism (regulation of spliceosome activity, RNA transportation, mRNA surveillance pathway), protein processing in the endoplasmic reticulum and some were cell cycle regulators. Among these proteins, histone deacetylases 2 (HDAC2), SNW domain containing 1 (SNW1) and Structural maintenance of chromosome 3 protein (SMC) were known to be associated with drug resistance. Further inhibition of HDAC2 (using valproic acid, VPA) in the B16F10R resistant sublines showed induction of apoptosis and irregular cell morphology. Since the alteration was in the nuclear proteins, drug-resistant cells showed cross-resistance with inhibition of other pathways and cell surface targets.

In Phase III, after twenty-seven months of drug combination treatment, the A375R subline showed a resistance fold factor of 4.7 while the B16F10R had increased to 3.28. Both these resistant sublines used for further experiments. VPA which is known inhibitor of HDAC was used for radiation sensitization. Treatment of low dose of VPA with a low dose of radiation has shown the potential for enhancing the efficiency of radiotherapy in both parental and drug-resistant cells. This study demonstrated that HDAC inhibitor at a safe dose was a radiosensitizer for drug-resistant melanoma model developed in this study. *In silico* docking of VPA and pyruvate has shown stable interactions of the two molecules with HDAC2 forming two hydrogen bonds with Arg39 which maybe one of the active site residues, since it is involved in binding other inhibitor of HDAC2 also.

In conclusion, our study has shown that cell cycle regulators are hyperphosphorylated in the in the dual drug-resistant in melanoma model. Hyperphosphorylated HDAC2 has been shown to contribute to the drug resistance. Therefore, designing leads for targeting them along with key signaling pathways may be helpful in overcoming the drug resistance. Pretreatment with low dose of HDAC inhibitor has a potential for sensitizing drug resistant cancer cells to low doses of radiation.

**Key words:** Cytotoxic, drug-resistance, HDAC2, melanoma, proteomic, radiosensitizer