Chapter 6: SUMMARY AND CONCLUSIONS

In an attempt to identify the alternate signaling pathways/key phosphoproteins utilized by the dual drug-resistant melanoma models, the study was divided into three phases.

6.1 Phase I

Currently, most of the research is focused on targeting multiple pathways and among all PI3K-AKT and MEK-ERK are the most common. Treatment of melanoma cells, B16F10 and A375, with U0126 (ERK1/2 inhibitor) or LY294002 (PI3K-AKT kinase inhibitor) alone showed appreciable cytotoxic activity.

Hence, the present study established dual drug-resistant melanoma sublines (A375R and B16F10R) from the parental melanoma cell line (A375C and B16F10C) by co-targeting key signaling pathways Raf/MEK/ERK and PI3K/PTEN/AKT/mTOR pathways - which are best known for their crosstalk - to understand the alternate pathways utilized by the dual drug-resistant cells for their proliferation and survival.

a) Two melanoma cell lines, B16F10 and A375 were used for the development of dual drug resistance.

b) The combined treatment U0126 (ERK inhibitor) and LY294002 (PI3K/AKT inhibitor) showed a dose dependent increase in cytotoxicity. The 50% inhibition concentrations (IC50) values were determined.

c) 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 1/10th the IC50 value with a gradual increase until they become resistant to the drug combination.

d) The B16F10 cells attained a resistance fold factor of >2 in 15 months. And was called as B16F10R subline.

e) Development of drug-resistant cell lines was confirmed by different techniques. Drug-resistant cells were able to proliferate even in absence of phosphorylated ERK and AKT, which was confirmed by colony formation assay and live/dead cells assay.
f) The human melanoma A375 cells, however, did not attain a resistance fold factor of $\geq 2$ in 15 months. Hence the treatment with continued.

g) This dual drug resistant B16F10R model was used for the proteomic/phosphoproteomics analysis in phase II.

### 6.2 Phase II

The proteome analysis of B16F10C (control) and B16F10R (drug-resistant) cells were done using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The phosphoproteome analysis was performed using titanium dioxide (TiO$_2$) phosphopeptide enrichment followed by Q-Exactive hybrid quadrupole-Orbitrap mass spectrometry. Data were analyzed using several data analysis tools. The present study has identified several altered proteins and phosphoproteins

a) The proteome of the B16F10R showed 71 upregulated and 150 downregulated proteins belonging to primary metabolic processes, carbohydrate metabolic process, glycolysis and tricarboxylic acid cycle.

b) Matrix metalloproteinase (MMP) isoforms were among the top 10 up-regulated proteins and serine/threonine protein phosphatase 2A (PP2A), a dephosphorylating enzyme is downregulated.

c) The phosphoproteome analysis had identified 363 differentially phosphorylated phosphoproteins - 126 hyperphosphorylated and 137 hypophosphorylated.

d) The molecular functions of the differentially phosphorylated proteins of B16F10R identified by software belonged to RNA metabolism (regulation of spliceosome activity, RNA transportation, mRNA surveillance pathway), protein processing in the endoplasmic reticulum and as cell cycle regulators.

e) In the pathway characterization of the altered phosphoproteins using software, some were associated with more than one pathway. The cell cycle regulatory proteins Cdk2, HDAC2, Mcm2, Smc3 were seen associated only with the cell cycle.
f) The protein-protein interactions showed two major groups – RNA spliceosome and cell cycle regulators.

g) Literature search on HDAC2, Mcm1, and Smc3, the cell cycle regulators, showed that these proteins were reported to play a role in drug resistance in melanoma.

h) Histone deacetylase (HDAC2) being one of the currently pursued targets for melanoma therapy and its already established inhibitor valproic acid (VPA) was used in an attempt to reverse the resistance to dual drugs in A375R and B16F10R.

i) VPA showed a dose-dependent cytotoxic effect on the resistant cells. The cell death was mediated by apoptosis which was confirmed using western blotting for the apoptotic markers (cleaved caspase_3 and cleaved caspase_9) and cell morphology analysis.

j) Dual drug-resistant cells, when tested against other pathway inhibitors, showed cross-resistance to them.

k) 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 were studied in phase III

6.3 Phase III

VPA has been used as a sensitizer/inducer followed by chemotherapy + VPA for treatment of melanoma in an advanced stage. Hence in this phase, an attempt was made to sensitize the cells with VPA/ LDN193189 and then exposed to radiation.

a) The optimal radiation dose for the sensitization experiments was found to be 2 Gy (25 – 30% cell death). The cells were pretreated with VPA (a well-known target for HDAC2) or LDN193189 at IC_{25} concentration as sensitizers for radiation treatment. Following which the cells were exposed to radiation.
b) The VPA pretreated control cells of both A375C and B16F10 as well as the B16F10R cells when exposed to 2 Gy radiation showed synergistic cell death (cell death% > Cell death% with VPA + Cell death% with 2 Gy). While the A375R resistant cells showed only an additive effect (cell death% = Cell death% with VPA + Cell death% with 2 Gy).

c) The cell counts decreased as compared to the untreated controls. Least viable cell counts were found in the VPA + 2 Gy treated cells in both the control and resistant cells. Live/dead assay also showed maximum cell death in the VPA + 2 Gy treatments.

d) The melanin content of the resistant cells was found to be higher in the resistant cells which decreased on each of the treatment, i.e., 2 Gy, LDN193189 + 2 Gy, and VPA + 2 Gy. Least melanin was found in the VPA + 2 Gy treated cells.

e) The clonogenic assay with the B16F10C and B16F10R cells showed a maximum decrease in the cell viability in the VPA + 2 Gy treated cells.

f) All these data suggest that VPA can be a sensitizer for radiation treatment in the dual drug resistant model of this study.

g) In silico docking of VPA on the PDB structure of HDAC shows that it binds to the Arg39 residue of HDAC2. Arg39 is shown to be involved in interactions with other inhibitors in docking studies reported earlier.

The data described above provides an insight into the biology of the chemoresistance in melanoma and provide an approach to tackle the drug resistance issue. And, it reveals the need for further studies by using in vivo models for confirmation and treatment to provide conclusive valuable information for future use in the clinic.

Our study highlights the possible role of HDAC2 in the development of drug resistance in melanoma. Therefore, designing leads for targeting HDAC2 along with other key signaling pathways may be helpful in overcoming drug resistance.
Figure 6.1 Proposed mechanism of cell death on VPA treated dual drug-resistant model B16F10 R

Key to the figure: Suppression of the reaction; Activation; Inhibition. The dotted arrows indicate inhibition of the reaction. Solid arrows indicate active reaction.