Chapter 5: DISCUSSION

Phase I

Melanoma develops due to alterations in cellular machineries, such as induction of cell proliferation and/or impairment of the mechanisms controlling melanocyte senescence (both promoting primary clonal selection and expansion), and suppression of apoptosis (sustaining the cancer cell survival and tumor progression). Activation of the Ras pathway is an important intracellular event in many cancers and therefore it represents a promising target for treatment. The most reported is the common sequence of the RAS pathway - RAS-RAF-MEK-ERK for ERK activation and Ras-PI3K-AKT for AKT activation. Protein targeted therapies involving mitogen-activated protein kinase (MAPK) pathway have revolutionized management and improved outcomes for patients with advanced stage cancer.

5.1 Melanoma signaling and drug resistance

Development of resistance to anticancer therapy is a major challenge in the successful treatment of cancer. Evolution of resistance to monotherapy (treatment of cancer with a single anticancer agent) has been reported frequently in melanoma. Regardless of the encouraging results obtained by monotherapy with drugs such as dabrafenib or vemurafenib, the majority of the patients develop resistance to these inhibitors and the cancer relapses (181). In most cases, resistance is associated with the reactivation of the MAPK pathway (73, 182). In many instances, these drug-resistant tumors also often exhibit cross-resistance to other pathway inhibitors (183-185). Therefore, currently studies target multiple pathways, of which, PI3K-AKT and MEK-ERK are the most common. These pathways are known to cross-talk in drug resistance development (186, 187). Hence the present study aimed at developing a dual drug resistant melanoma model, which are resistant to a combination of inhibitors of two different signaling cascades/pathways and explored the alteration in its proteome/phosphoproteome. This strategy may be helpful in (i) understanding the mechanisms by which drug-resistant melanoma cells survive and (ii) planning newer treatment regimes in the future. The present study investigates the horizontal co-targeting of (i) ERK and (ii) PI3K/AKT/mTOR pathways in melanoma.
5.2 Development of drug-resistant cell line models

Human melanoma cell line, A375, and mouse melanoma cell line, B16F10, were used in this study. Since this study was designed to understand the alternate signaling mechanisms involved in drug resistance, using cell lines rather than patient-derived clinically relevant cell lines was a more practical option. Cell lines can be grown in media containing the drugs for continuous exposure to the treatment. A gradual increase in the concentration of the drugs also becomes feasible. The laboratory model cell lines can also attain a higher level of resistance which can be beneficial for studying the mechanisms of survival (134).

U0126 and LY294002 used in this study are already established inhibitors of these pathways and have been used to study melanoma as well. Earlier Smalley et al., (2007), have used U0126 against seven different melanoma cell lines to find the concentration of the drug to inhibit the growth of melanoma cells (188). Huang et al., (2015) have also used U0126 on A375 human melanoma for studying its anti-proliferative effects. LY294002 is also shown to inhibit the proliferation of melanoma cells (189). Smalley et al., (2006), had suggested that targeting multiple signaling pathways would be a better strategy than targeting a single pathway (188). Hence in this study, a combination of U0126 (ERK1/2 inhibitor) and LY294002 (PI3K-AKT kinase inhibitor) was used for the development of the dual drug resistant melanoma models for better understanding of the underlying mechanisms of drug resistance.

In the present study treatment of both B16F10 and A375 melanoma cells with either U0126 (ERK1/2 inhibitor) or LY294002 (PI3K-AKT kinase inhibitor) or in combination showed dose-dependent cytotoxic effects. In addition, the combination of the drugs gave a synergistic reduction in cell viability of both A375 and B16F10 cells. These results concur with the findings of Posch et al., (2013), who have also co-targeted MEK/ERK and PI3K/ mTOR pathways in NRAS mutant melanoma (190).

For developing the drug-resistant model, the B16F10 and A375 melanoma cells were treated with the drug combination starting at a concentration of 1/10th of the IC\textsubscript{50} value of the parental/control cells, with a gradual increase in the concentration of the drug combination. MacDermott et al., (2014), have given the guidelines for the generation of drug-resistant model and calculation of the resistance fold factor. They
have also used the guideline for developing a drug-resistant lung cancer model. According to this guideline, a resistance fold factor of ≥2 would be considered as ‘resistant’ to that drug (134).

In this study, the mouse melanoma drug-resistant model, B16F10, took around 15 months of treatment - continuous with a gradual increase in the concentration of the drugs - to show a resistance fold factor of 2, and was considered as ‘Resistant/B16F10R’. This model was used for phase II proteomic / phosphoproteomic studies. A375 cells, after 15 months of drug combination treatment, showed a resistance fold factor of <2. Hence the drug treatment was continued. Mac Dermatt et al., (2014), (134), in their review on case studies have documented that it takes 2-18 months for attaining a resistance fold factor of ≥2 to single agent treatments of various cancer cell lines (134). However, in this study dual drug, co-targeting two major signaling pathways, were used for the development of the resistant model. Hence it is suggestive that horizontal co-targeting two pathways with inhibitors also develop resistance within the same duration.

The B16F10 and A375 cells showed fold resistance factor of 3.28 and 4.70, respectively, after 27 months of continuous drug treatment. These models were used for the phase III experiments. Clinically relevant resistant models obtained from patients undergoing or have undergone chemotherapy are also reported to have a resistant fold factor of 2-5. However, the durations for attaining the resistance are not very conclusive since most studies do not mention it (134).

The action of the U0126 (ERK1/2 inhibitor) or LY294002 (PI3K-AKT kinase inhibitor) in the B16F10R cells was confirmed by western blot probing for phospho-ERK and phospho-AKT proteins, which had decreased as expected, since the related kinases were inhibited. Furthermore, the cell viability and the cell proliferation abilities of the B16F10R cells were checked by live/dead assay and clonogenic assay, respectively. Although the B16F10C cells treated with the drug combination showed decreased proliferation, the cell viability and proliferation ability of the B16F10R cells treated with the drug combination was similar to that of the untreated B16F10C control cells. A similar effect has been shown in mutant BRAFV600E melanoma resistant to BRAF inhibitors by Villanueva et al., (2010), where they have co-targeted MEK and IGF-1R/PI3K (71). The growth rate of the drug combination treated
resistant cells was similar to that of the untreated control cells. Zhang et al., (2010), have also shown that drug-resistant cells grow/proliferate as well as the untreated parental cells in gastric carcinoma cell line (191). This confirms that the cells are resistant to the drugs/drug combinations treatment used.

**Phase II**

The diverse characteristics and a variety of adapting mechanisms in the resistant cells, are probably some of the reasons why a single biomarker for drug resistance has not been identified so far for melanoma (192). Proteomics gives the protein expression profiles of cells. Differences between the protein expression profiles of parental and drug-resistant cells could show distinct protein patterns of the two (193). While differences in the phosphoproteome profiling of the parental and drug-resistant cells will give an insight into the patterns of kinase related signaling cascades involved. Proteome/phosphoproteome profiling with the available tools of bioinformatics will probably give a better understanding of the alternate signaling cascades involved in the drug resistance mechanisms. Hence, in this study, changes in the proteome/phosphoproteome of a drug-resistant melanoma cell line, B16F10R, obtained in vitro after the continuous treatment with a combination of ERK and AKT kinase inhibitors, were studied using LC-MS/MS for proteomics and a single step phosphopeptide enrichment followed by LC-MS/MS for phosphoproteome analysis.

### 5.3 Proteomic profile of drug-resistant B16F10 melanoma cells

The functional and metabolic differences between B16F10C and B16F10R cells were investigated by proteomic analysis using LC-MS/MS. The proteome of the B16F10R showed 71 up-regulated and 150 down-regulated proteins. In an earlier study on the proteome of drug-resistant BRAF mutated melanoma, about 317 proteins were found down-regulated and 151 up-regulated. And the altered proteins were mostly associated with certain pathways reprogrammed in the resistant cells, i.e., amino acid metabolism and energy generation systems like glycolysis, citric acid cycle was down-regulated and cell cycle and DNA metabolism were upregulated (194). The proteome data of the present study also showed alteration in the proteins of glycolysis and citric acid cycle.
Among the ten most up-regulated proteins of the present study, matrix metalloproteinase 1 (MMP-1) and MMP-2 showed a 4.52 and 3.06 fold increase, respectively, in the B16F10R cells. Over-expression of MMP’s is known to be associated with the metastasis and invasion of cancers (195, 196).

In addition, although not one of top ten down-regulated protein the proteome profile also showed a down-regulation (down-regulated by 0.59 fold) of serine/threonine protein phosphatase 2A (Pppp2R1B) which is a dephosphorylating enzyme. This enzyme is known to dephosphorylate the kinases that are associated with signaling pathways in cells.

5.4 Phosphoproteomic profile of dual drug-resistant B16F10 melanoma model

5.4.1 Differentially phosphorylated proteins between resistance and control B16F10 cells

The phosphoproteome profile of B16F10R cells of the present study identified 263 phosphoproteins which were differentially phosphorylated, of which, 126 were hyperphosphorylated and 137 were hypophosphorylated. Pathway categorization of the altered phosphoproteins showed most of them belonged to either RNA metabolism or metabolic processes and a few were cell cycle regulators. Among the cell cycle proteins identified to be hyperphosphorylated in the dual drug resistant melanoma model B16F10R of this study the following proteins of the cell cycle were already reported to play a role in drug resistance

5.4.1.1 Structural maintenance of chromosome 3 protein (SMC3)

This study demonstrates the hyperactivation of SMC3, which might have triggered genomic instability or have stabilized the chromosomal architecture and separation (177, 178) resulting in drug resistance. The structural maintenance of chromosome 3 (SMC3) (formerly called Bamacan, Cspg6, HCAP, SmcD, or Mmip1) protein is a constituent of a number of nuclear multimeric protein complex, cohesin, that plays an essential role during the segregation of sister chromatids and is likely to be a component of the signaling network in which BRCA1 maintains genomic stability (177, 197). In addition to chromosomal segregation, SMC3 is also involved in DNA
recombination and repair (198). Recently SMC3, like p53 and BRCA1, has been identified as the target of the serine/threonine kinase Chk2, that plays a critical role in the DNA damage checkpoint pathway. SMC3 along with SMC1 has been shown to facilitate tumor genomic instability; progression and mutations in various subunits of cohesin are reported in sarcoma, melanoma, breast cancer, colon and glioblastoma tumors (199-201). The SMC3 knockdown can initiate genomic instability in cells (202), and in damaged axons (203). Down-regulation of SMC3 is part of the response of oncogenes to stress. The SMC3 up-regulation affects the expression of members of the ras-rho/GTPase and CREB oncogenic pathways that are key players in cell cycle regulation, microtubule dynamics, and in cell differentiation and survival.

5.4.1.2 SNW domain containing 1 (SNW1)

Phosphoproteomic analysis of this study showed hyperphosphorylation of SNW1. SNW1 is a highly conserved protein associated with splicing and transcription. It is recruited to the pre-mRNA when the U1 and U4 snRNPs (small nuclear ribonucleo proteins) dissociate from the spliceosome. Sato et al., (2015) demonstrated that SNW1 directly associates with EFTUD2 and snRNP200 and the disruption of SNW1 association with these proteins promote the apoptosis of breast cancer cells (179). However, the hyperphosphorylation of SNW1 possibly strengthen the interaction with EFTUD2 and snRNP200 and hence, failed to induce apoptosis and resistant cell become unresponsive to drugs.

5.4.1.3 Histone deacetylase 2 (HDAC2)

At present, histone deacetylases (HDAC) is one of the new targets that is being pursued in cancer therapeutics. This study indicates the hyperphosphorylation of histone deacetylase 2 (HDAC2) in B16F10R cells. And it is already reported that hyperphosphorylated HDAC indicates therapeutic failure due to drug resistance (176, 204-206). HDAC2 is an important regulator of compact chromosomes which means less gene expression, and the mechanism of HDAC inhibitors (HDACis) is the induction of chromosome de-condensation and sensitization to chemotherapy. Histone acetylation-deacetylation status of the cell is the most commonly studied regulatory processes of transcription. Histone acetyltransferases (HATs) and HDAC compete to add or remove acetyl groups on lysine residues of histones, respectively (207, 208). The acetylation of histones by HATs is associated with chromatin de-condensation.
and transcriptional activation; whereas the de-acetylation of histones by HDACs is associated with condensation of the chromosome and related transcriptional silencing. It is already reported that any change, either in the expression or activity, HDAC enzymes may lead to carcinogenesis or that specific HDAC enzyme is associated with particular malignancies. Inhibition of HDAC2 is reported to decrease cell proliferation which would be a potential target in conditions like cancer. Marchion et al., (2009), showed that the depletion of HDAC2 but not HDAC1 or HDAC6 sensitize the breast cancer cells and induce cell death. (209). Furthermore, HDAC2, but not HDAC1, confers resistance towards etoposide, the topoisomerase II inhibitor, in pancreatic ductal adenocarcinoma (PDAC) cells. (176). Suggesting that, HDAC2 may be considered as a therapeutic target for sensitization in nonresponsive tumor cells.

5.5 Inhibitors of HDAC (HDACi)

The class I selective HDACi, valproic acid (VPA), synergizes with etoposide to induce apoptosis of PDAC cells. Several reports have shown the association of hyperphosphorylated HDAC with the development of drug resistance (176, 204-206). The literature shows that suppressing HDAC2 activity can reverse the resistance towards the drug. Hence, VPA, a well known safe, anti-epileptic drug, and a weak inhibitor that can bind directly to HDAC2, was used to check whether the resistance of the dual drug resistant model developed in this study would be reversed. The resistant lines, B16F10R and A375R, developed in this study were sensitive to co-inhibition with HDAC2 inhibitor VPA. A dose-dependent reduction in cell growth over several concentrations of VPA was observed in both the dual drug resistant models of this study.

Phosphoproteomic data showed 1.73 fold increases in phosphorylation of pyruvate dehydrogenase E1 component subunit alpha (PDHA1). PDH converts pyruvate to acetyl CoA, which is the link between glycolysis and TCA. Hyperphosphorylation of this enzyme makes its inactive, therefore pyruvate accumulates which is known to inhibit HDAC2 enzyme (210).
5.6 Cross-resistance of dual drug resistant models to inhibitors of other pathway

Chemotherapeutic drug resistance is usually acquired by selection pressure of anticancer drugs and in many instances, the exposure to one drug induces cross-resistance to other chemotherapeutic drugs. In 'cross-resistance', the already resistant cells develop resistance to drugs of other signaling pathways as well. In our study, B16F10R and A375R, which were resistant to U0126 + LY294002 treatments showed cross-resistance against other pathway inhibitors such as SP600125 (JNK inhibitor), IWP-2 (WNT Inhibitor) and LDN193189 (BMP inhibitor). It was hypothesized that suppression of AKT and MAPK kinases or selective pressure could possibly force cells to use alternate pathways for their proliferation and survival, and the cells are expected to become addicted to the alternate pathway. The dual drug-resistant cells of this study showed less sensitivity to inhibitors for other pathways, compared to parental cells, indicating cross-resistance with other tested drugs as well. Possibly this is because PI3K and MAPK pathway are the most important for cell survival and proliferation and resistance developed by inhibition of these pathways cause significant changes in most of the other signaling pathways in drug-resistant cells. Development of cross-resistance has been reported earlier in mutant BRAF melanoma. Treatment with a specific BRAF inhibitor makes the mutant BRAF melanoma resistant to other BRAF inhibitors as well (211). Earlier Ribas et al., (2010), have also demonstrated in vitro that mutant BRAF melanoma cell lines resistant to specific BRAF inhibitors were cross-resistant to MEK inhibitors, in addition to other BRAF inhibitors (184). In the present study, the cross-resistance was seen in other signaling pathways, i.e., JNK, WNT, and BMP. And the highest cross-resistance was towards BMP inhibitor LDN193189. So this inhibitor was also used for sensitization studies along with VPA.

Phase III

Melanoma is known to be radioresistant cancer (76). After identifying the altered phosphoproteins in the dual drug resistant melanoma, the third objective of this study was to find a way to overcome the resistance of the dual drug-resistant cells. Since the dual drug resistant models showed cross-resistant with other signaling pathway inhibitors, radiation treatment was an alternative. Use of radiosensitizers prior to
radiation for increasing the susceptibility of cells to radiation may be of use for improving outcomes of radiotherapy especially in radioresistant cancers like melanoma (212).

VPA has already been used as a sensitizer/inducer followed by VPA + chemotherapy by Rocca et al., (2009) for the treatment of advanced stage melanoma (213). Hence in phase III of this study, the cells were sensitized with either VPA or LDN193189 and then exposed to radiation.

5.7 Radio-sensitization of drug-resistant melanoma cells using valproic acid and LDN193189

The hypothesis was that drug-resistant cells pretreated with HDACi may increase the cell-killing effect of radiation irrespective of the changes in cell signaling pathway or resistance status. In this study, we observed that the pretreatment of low doses (IC25) of VPA/LDN193189 enhanced the radiation damage in both parental and dual drug-resistant cells significantly than untreated cells. The cell death was due to apoptosis, since there was a significant change in the concentrations of the apoptotic markers cleaved caspases_3 and _9. Earlier studies have reported that pre-treatment with HDACi enhances the efficacy of radiation-induced apoptosis (126, 214).

In addition, in this study, VPA treated resistant cells, have shown a decrease of pyruvate dehydrogenase (PDH) complex. PDH converts pyruvate (a product of glycolysis) to acetyl CoA, starting material of TCA cycle, which is the final common metabolic pathway and source of energy to the cells. Hence PDH plays a central role in the energy status of the cells. Thus a decrease in energy production may also contribute to cell death. This finding is in line with an earlier report by Naia et al., (2017). They showed that inhibition of HDAC2 indirectly involves reduction of PDH (215). As a result pyruvate levels increase in the cell. Pyruvate is also known to inhibit HDAC and induce apoptosis in tumor cells (216).

Although melanin was marginally high in the resistant cells of A375, the melanin content decreased after exposure to radiation in of LDN193189 treated, VPA treated cells more than those with 2 Gy radiation alone. The highest melanin decrease was found in the VPA + radiation treated cells. That melanosomes play a role in drug resistance of melanoma cells has been reported earlier by Chen et al., (2009) (217).
They also hypothesized that manipulation of melanosomes, i.e., either alter density or function, may help in better outcomes of anti-melanoma drugs.

Furthermore, the clonogenic survival assay of B16F10 cells, both parental and resistant, also showed that exposure to radiation of cells pretreated with VPA reduced the colony formation compared to untreated, just radiation exposed, or LDN193189 + 2 Gy radiation treated cells. Heinemann et al., (2015), showed that combined treatment of BET and HDAC inhibitors has similar synergistic effects on both patient-derived vemurafenib-resistant melanoma cell line and sensitive cell line (218). Several mechanisms have been proposed for the role of HDACi, such as formation of DNA-Pt adducts, down-regulation of the efflux transporter ABCC2 and the DNA repair gene ERCC1 (129), suppression of the homologous recombination repair and synchronization of the cells to G1 phase, all of which increase the sensitivity of the drug-resistant cells to radiation (219).

The results obtained in this study have been summarized in Figure 5.1.

---

**Figure 5.1  Proposed mechanism of the resistance to dual drugs**

*The development of dual drug-resistant cells takes about 15 months of time. Among different proteins identified to be altered in drug-resistant cells, RNA splicing-regulatory proteins and cell cycle proteins shown to play a role in maintaining its drug-resistant state. Pretreatment of drug-resistant cells with HDAC2 inhibitor enhances radiation-induced apoptosis.*
5.8 Molecular docking of valproic acid on HDAC2

*In silico* docking of VPA on HDAC2 revealed that VPA has two interactions with Arginine 39 residue. Uba and Yeleski, (2017), in their studies on active site of HDAC isoforms and their inhibitor binding properties have shown that Arg39 of HDAC2 is one of the residues that binds to a compound KA_025, which is an HDAC2 inhibitor (220). Arg39 appears to be an important inhibitor binding residue. Hence it is proposed that VPA inhibits HDAC2 enzyme due to the two interactions with Arg39 as shown by the *in silico* docking. Pyruvate also has similar interactions with Arg39 of HDAC2, which explains the cell death in the VPA treated dual drug resistant cell model developed in this study.

5.9 Mechanism of resistance in the dual drug resistant model and role of VPA as a sensitizer

With the results obtained in this study validation of the findings with the available literature, it is proposed that the drug resistance and the sensitization to the same with VPA may have the following mechanism:

- HDAC2 is activated by phosphorylation, catalyzed by enzyme casein kinase 2(CK2). Although the proteome data shows there is no overexpression of the HDAC2 protein in the dual drug resistant model developed in this study, the phosphoproteome data shows there is a 4.7 fold increase in its phosphorylation. Hence enzyme HDAC2 is active in the dual drug resistant model.

- Proteome data also shows that serine/threonine protein phosphatase 2A (PP2A), which dephosphorylates HDAC2, is down-regulated. This suggests that HDAC2 remains in the phosphorylated active state.

- It is reported that HDAC2 suppresses apoptosis /cell death in HeLa cells (Lei *et al.*, 2000), which explains the resistance developed to the dual drug treatment (221).

On VPA treatment:

- VPA is a small molecule inhibitor molecule, which interacts with A39 of HDAC2 by two interactions.
VPA has two roles (i) inhibits that activity of HDAC2 (222), (ii) activates p38 which are pro-apoptotic protein (223). Both of which cause cell death.

Cell death is seen in the dual drug resistant model after treatment of VPA, probably due to apoptosis mediated by (i) inhibition of HDAC2 and (ii) activation of p38 which activates cleavage of caspases_3 and _9, which have increased in the VPA treated dual drug-resistant cells.

The mechanism of action of the cell death in VPA treated cells of the dual drug resistant model developed in this study maybe p38 mediated mitochondrial apoptosis.