Chapter 4: RESULTS

The study had three phases – Phase I, Phase II and Phase III. The results are described according to the phases

Phase I

In this phase the two cells lines B16F10 mouse melanoma and A375 human melanoma (i) were treated with varying concentration of the drugs of interest, U0126 (ERK1/2 inhibitor), LY294002 (PI3K-AKT kinase inhibitor), and their combination, (ii) IC\textsubscript{50} values for the drug combination were determined, (iii) After 15 months, resistance fold factor was calculated and (iv) the drug resistance was confirmed with western blot, live/dead assay and clonogenic assay.

4.1 Dose-response curves of U0126, LY294002, and their combination

The cytotoxic effects of varying concentrations of U0126 (ERK1/2 inhibitor), LY294002 (PI3K-AKT kinase inhibitor) and the drug combination, i.e., U0126 (10 µM) + LY294002 (10 µM), on the cell lines B16F10 mouse melanoma and A375 human melanoma was analyzed by MTT assay after 72 h of treatment. The dose-response curves of drug treatment showed a dose-dependent increase in cell death (Figure 4.1). The cell death percent was higher (Figure 4.1, panel C and F) in the drug combination than in the individual drugs. The IC\textsubscript{50} of each of the drugs and their combination was calculated.
Effects of U0126 and LY294002 on proliferation in B16F10 mouse melanoma cells (A-C) and A375 human melanoma cells (D-F) was done by MTT assay after 72 h of treatment. Data shown are mean ± SEM of three replicate wells. The B16F10 cells were treated with 5-60 µM of U0126 (A) and LY294002 (B). Drug combination (C) was done in the concentration range 5-50 µM, i.e., 5 µM U0126 + 5 µM LY294002 to 50 µM U0126 + 50 µM LY294002. The A375 cells were treated with 0.5-50 µM of U0126 (D) and LY294002 (E) and the drug combination (F) was done in the concentration range 0.5-40 µM. A graph was plotted with concentration of the inhibitor (X-axis) vs cell death % (Y-axis) and the IC$_{50}$ values were calculated using the formula $y = b + ax$. IC$_{50}$ value is the concentration of the drug which results in 50% cell death.

Figure 4.1 Dose-response curves of the drugs, U0126, and LY294002, and their combination
4.1.1 IC\textsubscript{50} values of U0126, LY294002, and their combination

As shown in Table 4.1, the IC\textsubscript{50} value of U0126, LY294002 and combination treatment of U0126 and LY294002 were 25.29 ± 1.4 µM, 16.43 ± 1.2 µM, and 10.19 ± 1.0 µM, respectively, for B16F10 cells and 1.79 ± 0.25 µM, 4.87 ± 0.68 µM and 1.54 ± 0.19 µM, respectively, for A375 cells. The IC\textsubscript{50} values of the drug combination were lower than the individual drug treatment in both cell lines. Human melanoma A375 cells were more sensitive to the drugs, both individually and in combination, than mouse melanoma B16F10 cells.

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Inhibitor</th>
<th>IC\textsubscript{50} values B16F10 (µM)</th>
<th>IC\textsubscript{50} values A375 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>U0126</td>
<td>25.29 ± 1.4</td>
<td>1.79 ± 0.25</td>
</tr>
<tr>
<td>2.</td>
<td>LY294002</td>
<td>16.43 ± 1.2</td>
<td>4.87 ± 0.68</td>
</tr>
<tr>
<td>3.</td>
<td>U0126+LY294002</td>
<td>10.19 ± 1.0</td>
<td>1.54 ± 0.19</td>
</tr>
</tbody>
</table>

B16F10 is mouse melanoma cell line and A375 is human melanoma cell line. Drug combination is U0126 (10 µM) + LY294002 (10 µM). IC\textsubscript{50} - Concentration of compound required for 50% inhibition of cell viability determined using MTT assay. The results represent the mean ± standard deviations of three repeats.

4.2 Establishment of drug-resistant melanoma cell lines

The cell viability of the drug resistance in the cell lines, B16F10R and A375R, were assessed by MTT assay after fifteen months of drug treatment and compared with the parental cells B16F10C and A375C, for calculation of the resistance fold factor.

4.2.1 IC\textsubscript{50} values of drug combination the parental and the resistant cell lines

The IC\textsubscript{50} value had increased to 21.08 ± 1.8 µM and 2.65 ± 0.82 µM (from 10.19 ± 1.0 µM, and 1.54 ± 0.19 µM, data shown in Table 4.1) for B16F10R and A375R, respectively (Figure 4.2), after 15 months of drug combination treatment with a gradual increase in the drug concentrations. The IC\textsubscript{50} of drug-resistant cell lines were higher than the parental cell lines.
Figure 4.2 Effects of the drug combination on the proliferation of B16F10 and A375 cells

The parental and resistant melanoma cells were treated with the drug combination at various concentrations (0.62–80 µM). This figure is the results of the MTT assay—parental (dark bars) and resistant (light bars). Panel A: on mouse melanoma B16F10; Panel B: on human melanoma A375. Data shown are means ± SEM of three replicate wells. Graphs were plotted with concentration of the inhibitor (X-axis) Vs cell viability % (Y-axis) and the IC\textsubscript{50} values were calculated using the formula \( y = b + ax \). IC\textsubscript{50} values are the concentration of the drug combination which results in 50% cell death.

4.2.2 Assessment of resistance fold factor of the drug-treated cells

The treatment with the drug combination was started at one-tenth of IC\textsubscript{50} values and gradually increased. B16F10, mouse melanoma cell line, attained a resistance fold factor of 2 after 15 months of drug combination treatment. Hence B16F10 was considered drug-resistant and designated as B16F10R (B16F10 resistant sub-line) and used for further experiments of phase II. The resistant B16F10R sub-line was maintained in the same dose of drug combination till the end of the study.

In 15 months duration, the resistant fold factor of A375, human melanoma cell line, was <2.

However, drug combination treatment with a gradual increase was continued for A375.

4.3 Confirmation of resistance in B16F10R cells

The development of drug-resistance in B16F10R cells was confirmed with western blot, live/dead assay and clonogenic assay.
4.3.1 Western blot for phospho-ERK and phospho-AKT

Western blot showed complete inhibition of phospho-ERK and phospho-AKT in B16F10R cells (Figure 4.3, lane 1) as compared to the parental B16F10C cells (Figure 4.3, lane 2). The expression of total-ERK, total-AKT, and β-actin (loading control) did not show any differences between B16F10R and B16F10C cells.

![Western blot](image)

**Figure 4.3** Western blot for phospho-ERK and phospho-AKT in B16F10C and B16F10R

*Drug-resistant B16F10R cells were maintained in the continuous presence of the drug combination. B16F10C were untreated cells which served as control. When the cultures attained 80% confluency, cells were lysed using lysis buffer. Western blot was performed with the cell lysates. β-actin served as loading control. The arrows indicate the phospho-ERK and phospho-AKT bands.*

4.3.2 Live/Dead cell assay using acridine orange (AO) and propidium iodide (PI)

B16F10C cells treated with the drug combination, 10 µM each of U0126 and LY294002, (Figure 4.4; panel B), showed cell death as compared to untreated B16F10C cells (Figure 4.4; panel A). While treatment of B16F10R cells with the drug combination, did not show any significant cell death (Figure 4.4; panel C) and was comparable to untreated B16F10C cells (Figure 4.4; panel A).
Figure 4.4  Live/Dead assay of B16F10C and B16F10R cells

The live/dead assay was performed by acridine orange (AO) and propidium iodide (PI) staining. Panel A shows the result of untreated control B16F10C cells; Panel B shows the results control B16F10C cells treated with the drug combination of U0126 (10 µM) and LY294002 (10 µM); Panel C shows the results B16F10R cells treated with the drug combination. Images were taken phase-contrast microscopy using ZOE Fluorescent Cell Imager (Bio-Rad). Bright field images are from the normal light with no filter. Live cells appeared green in color (AO stain) and dead cells appear red in color.

4.3.3 Clonogenic cell survival assay B16F10R and B16F10C cells

Qualitative assessment of the clonogenic cell survival assay showed that the B16F10C were sensitive to the treatment of the drug combination, U0126 (10 µM) and LY294002 (10 µM) (Figure 4.5B), with cell death after incubation of the cells in the drug combination for 14 days. The growth of the B16F10R cells was unaffected by the treatment of the drug combination (Figure 4.5C). This result was similar to that of untreated B16F10C control cells (Figure 4.5A). The resistant subline was proliferating as well as the untreated controls.
Figure 4.5  Clonogenic assay with B16F10C and B16F10R cells

Abbreviation: ‘ULY’; combination treatment of U0126 (10 µM) and LY294002 (10 µM). Panel A shows the result of untreated control B16F10C cells; Panel B and C show the B16F10C and B16F10R cells with combination treatment U0126 (10 µM) and LY294002 (10 µM). The experiment was done in duplicates and was assessed qualitatively.

Phase II

Proteomic and phosphoproteomic analysis, of control B16F10C and resistant B16F10R cells, will help identify some of the proteins/phosphoproteins which are altered in the drug-resistant cells. Software programs have been used to identify the pathways in which proteins are associated and one hyperphosphorylated protein was selected for further experiments on radiosensitization.

4.4  Proteomic and phosphoproteomic analysis

For proteomic and phosphoproteomic analysis, four biological replicates (BR) each, of control B16F10C and resistant B16F10R cells were used.

4.4.1  Estimation of protein concentration in control and drug-resistant cells

The protein concentration of each BR was determined from a standard calibration curve (Figure 4.6) made with varying concentrations of BSA (bovine serum albumin). This standard curve was used for calculation of protein concentrations in the cell lysates of B16F10C and B16F10R.
The biological replicates (BR) of the B16F10 cells (Resistant BR1 – BR4) had lower protein content (average 1855.9 µg/5 µL) as compared to the B16F10C cells, (average 4017.9 µg/5 µL) (Control BR1 – BR4) as shown in Table 4.2.

### Table 4.2 Total protein concentration in B16F10C and B16F10R cell lysates

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Sample type</th>
<th>Estimated protein (µg) concentration in 5 µL of sample</th>
<th>Volume (µL) of sample needed for 20 µg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control BR1</td>
<td>4563.64</td>
<td>4.38</td>
</tr>
<tr>
<td>2</td>
<td>Control BR2</td>
<td>4594.74</td>
<td>4.35</td>
</tr>
<tr>
<td>3</td>
<td>Control BR3</td>
<td>4416.92</td>
<td>4.52</td>
</tr>
<tr>
<td>4</td>
<td>Control BR4</td>
<td>2496.65</td>
<td>8.01</td>
</tr>
<tr>
<td>5</td>
<td>Resistant BR1</td>
<td>1022.63</td>
<td>19.55</td>
</tr>
<tr>
<td>6</td>
<td>Resistant BR2</td>
<td>2303.94</td>
<td>8.68</td>
</tr>
<tr>
<td>7</td>
<td>Resistant BR3</td>
<td>2082.16</td>
<td>9.60</td>
</tr>
<tr>
<td>8</td>
<td>Resistant BR4</td>
<td>2015.22</td>
<td>9.92</td>
</tr>
</tbody>
</table>

Protein concentration was estimated in four biological replicates (BR) each of B16F10C and B16F10R by BCA method. The concentration was calculated from the standard calibration curve using the formula: \( y = ax + b \).

### 4.4.2 Normalization of protein bands in SDS PAGE gels

SDS PAGE gel with protein samples (20 µg) of the four BR each of B16F10C and B16F10R showed unequal density banding patterns (Figure 4.7 A). Repetition of SDS-PAGE with 50 µg protein sample showed a similar pattern of non-normalization (Figure 4.7 B). Hence for normalization of protein concentrations in B16F10C and B16F10R cell lysates, the band intensity was determined using Image-J and adjusted.
manually. The normalization of protein bands in both, B16F10C and B16F10R, cell lysates are shown in Figure 4.7 C.

![Figure 4.7 Normalization of protein concentration in B16F10C and B16F10R cell lysates SDS PAGE gel run with (A) 20µg of protein; (B) 50 µg protein; (C) after manual adjustment of the protein concentration using Image-J.](image)

**Figure 4.7** Normalization of protein concentration in B16F10C and B16F10R cell lysates SDS PAGE gel run with (A) 20µg of protein; (B) 50 µg protein; (C) after manual adjustment of the protein concentration using Image-J.

4.4.3 Trypsin digestion of the proteins in the cell lysates

The SDS-PAGE gel with the samples of the cell lysates of B16F10C (PBR1 and 2) and drug-resistant B16F10R (RBR 1 and 2) digested with trypsin, showed complete digestion of protein in the trypsin-treated samples (Figure 4.8; lanes 2, 4, 6, 8). In comparison the pre-digestion samples separated into bands of proteins (Figure 4.8; lanes 1, 3, 5, 7).
4.4.4 Total proteome analysis of B16F10C and B16F10R cell lysates

Two technical replicates were used for the proteomic analysis of both, B16F10C and B16F10R cell lysates by LC-MS/MS.

4.4.4.1 Altered proteins in the total proteome

The data obtained for LC-MS/MS were searched against the mouse reference database using Proteome discoverer 2.1 (PD 2.1), and 9965 proteins belonging to 2990 groups were identified. The proteins within a group were ranked according to the number of peptide sequences, the number of protein sequence motifs (PSMs), their protein scores, and the sequence coverage. Of the 9965, 150 were down-regulated (fold change ≤ 0.66) while 71 were up-regulated (fold change ≥ 1.5). Up-regulated proteins: Metallothionein-1 (4.52 fold increase) and 2 (3.06 fold increase) have featured among the ten proteins with highest fold increase.

Down-regulated proteins: Proteins such as tropomyosin β chain isoform (0.25 fold decrease) and lysosomal acid lipase/cholesteryl ester hydrolase precursor (0.36 fold decrease) were among the 10 least altered proteins (Table 4.3). In addition to these serine/threonine protein phosphatase 2A (PP2R-1β), a dephosphorylating enzyme,
was found to be downregulated by 0.59 fold (since only the top 10 down-regulated are shown in table 4.3, this enzyme has not been shown).

### Table 4.3
Some of the altered proteins in B16F10R cells in comparison with the B16F10C cells

<table>
<thead>
<tr>
<th>Sl. no.</th>
<th>Gene Symbol</th>
<th>Description</th>
<th>Fold increase</th>
<th>Sl. no.</th>
<th>Gene Symbol</th>
<th>Description</th>
<th>Fold decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mt1</td>
<td>Metallothionein-1</td>
<td>4.52</td>
<td>1</td>
<td>Tpm2</td>
<td>Tropomyosin beta chain isoform Tpm2.2st</td>
<td>0.25</td>
</tr>
<tr>
<td>2</td>
<td>Garnl3</td>
<td>GTPase-activating Rap/Ran-GAP domain-like protein 3 isoform X1</td>
<td>3.76</td>
<td>2</td>
<td>Tmprss13</td>
<td>Transmembrane protease serine 13 isoform X1</td>
<td>0.29</td>
</tr>
<tr>
<td>3</td>
<td>Ak1</td>
<td>Adenylate kinase isoenzyme 1 isoform 1</td>
<td>3.41</td>
<td>3</td>
<td>Ptn</td>
<td>Prothymosin alpha</td>
<td>0.30</td>
</tr>
<tr>
<td>4</td>
<td>C4b</td>
<td>Complement C4-B isoform X1</td>
<td>3.32</td>
<td>4</td>
<td>Sfr1</td>
<td>Swi5-dependent recombination DNA repair protein 1 homolog Farnesyl pyrophosphate synthase isoform 1 precursor</td>
<td>0.31</td>
</tr>
<tr>
<td>5</td>
<td>Adh7</td>
<td>Alcohol dehydrogenase class 4 mu/sigma chain</td>
<td>3.28</td>
<td>5</td>
<td>Fdps</td>
<td>Lysosomal acid lipase/cholesteryl ester hydrolase precursor</td>
<td>0.34</td>
</tr>
<tr>
<td>6</td>
<td>Lcorl</td>
<td>Ligand-dependent nuclear receptor corepressor-like protein isoform X1</td>
<td>3.20</td>
<td>6</td>
<td>Lipa</td>
<td>Interleukin-1 receptor-associated kinase 4</td>
<td>0.36</td>
</tr>
<tr>
<td>7</td>
<td>Des</td>
<td>Desmin</td>
<td>3.12</td>
<td>7</td>
<td>Irik4</td>
<td></td>
<td>0.37</td>
</tr>
<tr>
<td>8</td>
<td>Mt2</td>
<td>Metallothionein-2</td>
<td>3.06</td>
<td>8</td>
<td>Espn</td>
<td>Espin isoform X1</td>
<td>0.37</td>
</tr>
<tr>
<td>9</td>
<td>Gripap1</td>
<td>GRIP1-associated protein 1 isoform X1</td>
<td>3.02</td>
<td>9</td>
<td>Tceal</td>
<td>Transcription elongation factor A protein 1 isoform 3</td>
<td>0.37</td>
</tr>
<tr>
<td>10</td>
<td>C7</td>
<td>Complement component C7 precursor</td>
<td>2.95</td>
<td>10</td>
<td>Ndufab1</td>
<td>Acyl carrier protein, mitochondrial isoform X1</td>
<td>0.38</td>
</tr>
</tbody>
</table>

The data obtained for LC-MS/MS was analyzed using Proteome discoverer 2.1, which compares the LC-MS/MS data against mouse database. This table shows 10 proteins with highest fold increase and 10 proteins with least fold increase, their gene symbol, and their fold change, i.e., concentration in B16F10R cells/concentration in B16F10C cells.

### 4.4.4.2 Pathway categorization of the altered proteins

The proteome data when analyzed by the Panther multiple pie chart tool, showed that the altered proteins belonged mostly to primary metabolic processes > carbohydrate metabolic process > glycolysis > Tricarboxylic acid cycle (Figure 4.9).
4.4.5  Phosphoproteomic analysis of B16F10C and B16F10R cell lysates

Two technical replicates were used for the phosphoproteome analysis of both, B16F10C and B16F10R cell lysates. LC-MS/MS and TiO$_2$-based enrichment strategy were followed for the enrichment of the phosphopeptides.

4.4.5.1  Altered phosphoproteins

The analysis of the phosphoproteome data, identified 263 phosphoproteins which were altered, of which, 126 were hyperphosphorylated (>1.5 fold) and 137 were hypophosphorylated (<0.66 fold) (Figure 4.10). The distribution of phosphorylated residues was highest on threonine residues (68%) followed by serine (29%) and tyrosine residues (3%).
Analysis of altered phosphoproteome in B16F10R cells

TiO₂-based enrichment strategy was followed to enrich the phosphopeptides. The boxes indicated altered, i.e., up-regulated and down-regulated proteins.

4.4.5.2 Pathway categorization of the altered phosphoproteins

Bioinformatic analysis of the differentially phosphorylated proteins by DAVID functional annotation chart software showed that some of the altered proteins belonged to five major pathways (Table 4.4). Some of the proteins, i.e., Rbm8a, Acin1, Casc3, Rnps1, Srrm, Pnn1, were associated with more than one pathway, while certain other proteins were found exclusively in one pathway, i.e., Cdk2, HDAC2, Mcm2, Smc3 associated with the cell cycle. One more protein, which is hyperphosphorylated by 1.73 fold is pyruvate dehydrogenase E1 component subunit alpha (PDHA1), which links glycolysis to TCA cycle. Hyperphosphorylation inactivates this enzyme and pyruvate accumulates since it is not converted to acetyl CoA.

Table 4.4 Pathway categorization of the phosphoproteins altered in B16F10R cells

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>KEGG Pathway</th>
<th>Protein count</th>
<th>Name of the protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Spliceosome</td>
<td>11</td>
<td>Ddx46, Rbm17, Rbm8a, SNW1, U2surp, Acin1, Srsf1, Srsf4, Snrnp70, Tra2a, Tra2b</td>
</tr>
<tr>
<td>2.</td>
<td>RNA transport</td>
<td>11</td>
<td>Rbm8a, Thoc5, Acin1, Casc3, Eif3b, Eif4g1, Eif4b, Eif5b, Pnn, Rnps1, Srrm1</td>
</tr>
<tr>
<td>3.</td>
<td>mRNA surveillance pathway</td>
<td>7</td>
<td>Fip111, Rbm8a, Acin1, Casc3, Pnn, Rnps1, Srrm1</td>
</tr>
<tr>
<td>4.</td>
<td>Protein processing in endoplasmic reticulum</td>
<td>5</td>
<td>Dnaja2, Nsf11c, Sec61b, Canx, Rrbp1</td>
</tr>
<tr>
<td>5.</td>
<td>Cell cycle</td>
<td>4</td>
<td>Cdk2, HDAC2, Mcm2, Smc3</td>
</tr>
</tbody>
</table>

DAVID Functional Annotation Chart software was used for the categorization of the altered phosphoproteins into various cellular pathways. KEGG: Kyoto Encyclopedia of Genes and Genomes is bioinformatics software for associating genomes to metabolism.
In addition, the data revealed that the differentially phosphorylated proteins in B16F10R cells had diverse molecular functions, RNA metabolism (regulation of spliceosome activity, RNA transportation, mRNA surveillance pathway), protein processing in the endoplasmic reticulum and cell cycle regulators.

### 4.4.5.3 Functional categorization of the altered proteins

Panther multiple pie chart tool (175) compared the phosphoproteomic data against the Mus musculus database as background and suggested the involvement of four major pathways in the development of drug-resistance (i) RNA metabolism/spliceosome, (ii) developmental process, (iii) metabolic process and (iv) cell cycle proteins (Figure 4.11).

![Pie chart showing biological function analysis of the phosphoproteins altered in B16F10R](image)

**Figure 4.11 Biological function analysis of the phosphoproteins altered in B16F10R**

Annotation enrichment analysis of altered phosphoproteins in B16F10R was done using the Panther Multiple Pie Chart tool.

Comparing the phosphoproteomic datasets of the present study with the Human Protein Reference Database (HPRD) database identified 71 novel phosphosites on 43 proteins in the B16F10R as compared to B16F10C.

### 4.4.5.4 Protein-protein interaction of the altered phosphoproteins

Identification of the protein-protein interaction of the altered phosphoproteins in B16F10R cells using the STRING database (169), showed a well-annotated protein-protein interaction network of: (i) RNA splicing-regulatory proteins - Hnrnpa2b1,
Snrnp70, Rbm17, Rbm17, Tra2b, U2surp, Srsf4, SNW1, Acin1, and (ii) Cell cycle proteins - HDAC2, Mcm2, Cdk2, Smc3 (Figure 4.12).

Figure 4.12  Protein-protein interactions of the proteins identified in B16F10R cells
The protein-protein interaction of the phosphoproteins identified by DAVID Functional Annotation Chart software were analysed with the STRING software. Colour coding: Red circles indicate the spliceosome and mRNA surveillance pathway; Green circles indicate cell cycle proteins; Light green circles indicate RNA transport proteins; Blue circles indicate protein processing in endoplasmic reticulum. Thick lines indicate strong interaction and the dotted lines indicate predicted interactions.

4.4.5.5 Placement of the altered phosphoproteins in the cell signaling network

Figure 4.13 shows the placement of the altered phosphoproteins in B16F10R in the cell cycle pathways. Cdk2, HDAC and MCM are in the ‘S’ phase, while the SMC3 is from the ‘M’ phase of the cell cycle. The LC-MS/MS spectra of the hyperphosphorylated protein, i.e., HDAC2 (Figure 4.14)
Figure 4.13  Placement of the altered phosphoproteins of the cell cycle pathway
Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of the database produced by DAVID 6.7 was used to generate this picture. The red star indicates dysregulated proteins (from left to right) Cdk2, HDAC, MCM and Smc3 in drug-resistant cells.

Histone deacetylase 2 (HDAC2)

Figure 4.14  LC-MS/MS spectra of hyperphosphorylated HDAC2 in B16F10R cells
The LC-MS/MS ion spectra of phosphopeptides identified in drug-resistant melanoma model. TMT labeling was done. LC-MS/MS was done on an Orbitrap Fusion tribrid mass spectrometer (Thermo).
4.4.5.6 Literature-based validation identified altered phosphoproteins

Literature-based validation of some of the identified hyperphosphorylated proteins showed that histone deacetylases 2 (HDAC2), structural maintenance of chromosome 3 (SMC3) and SNW domain containing 1 (SNW1) proteins have been well reported for their role in drug-resistance. Some of the references are as follows:

HDAC2: is involved in etoposide mediated resistance in pancreatic ductal adenocarcinoma (PDAC) cells. (176)

SMC3: triggers genomic instability or have stabilized the chromosomal architecture and separation resulting in drug resistance. (177, 178).

SNW1: hyperphosphorylation of SNW1 suppresses apoptosis and the resistant cells become unresponsive to drugs (179).

4.5 Role of HDAC2 in the drug resistance of B16F10R

HDAC2 was one of the hyperphosphorylated proteins in the B16F10R melanoma model developed in this study. It plays a role in the epigenetic mechanism, i.e., DNA modeling – demethylation, in drug resistance of melanoma (180). Valproic acid (VPA), a well-established inhibitor of HDAC2, was used for validation of the role of HDAC2 in the B16F10R cells. The rationale for the use of VPA: If HDAC2 is crucial for the sustenance of the drug resistance status, inhibition of this enzyme should decrease the cell survival, i.e., cell death should increase and if the cell death is by apoptosis there will be an increase in the markers of apoptosis, cleaved caspase_3, and cleaved caspase_9.

4.5.1 Dose-dependent curves of valproic acid (VPA) treated B16F10 cell lines

Dose-dependent curves of VPA treated B16F10C and B16F10R cell lines showed a concentration-dependent reduction in cell viability (Figure 4.15).
Both parental and resistant B16F10 cell lines were treated with varying concentrations of VPA (0.1-15 mM) for 72 h and MTT assay was performed. Data shown are means ± SEM of three replicate wells. A graph was plotted with concentration of the VPA (X-axis) vs cell viability % (Y-axis) and the IC$_{50}$ values were calculated using the formula $y = b + ax$. An IC$_{50}$ value is the concentration of the drug which results in 50% cell death. Control (dark bars) and resistant (light bars).

4.5.2 Western blot of the VPA treated B16F10R cells

Western blot showed that (i) The level of cleaved caspase$_3$ and caspase$_9$ were (markers of apoptosis) higher in the B16F10R cells compared to the B16F10C cells (Figure 4.16, B, C). (ii) Treatment of B16F10C and B16F10R cell lines with VPA (2 mM) increased the concentration of cleaved caspase$_3$ and cleaved caspase$_9$ (Figure 4.16, A, B, C), suggests that inhibition of HDAC2 with VPA induces apoptosis of the cells. (iii) The increase was highest in the VPA treated B16F10R cells (iv) In addition, there was a decrease in the pyruvate dehydrogenase enzyme complex (PDH), which 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.
Control (B16F10C) and drug-resistant (B16F10R) cells were treated with VPA (2 mM) for 24 h and cells were lysed using lysis buffer. Apoptotic proteins-cleaved Caspase-3 (Cl_Caspase3), cleaved Caspase-9 (Cl_Caspase9) and pyruvate dehydrogenase complex (PDH) were detected by western blots in both untreated (B16F10C and B16F10R) and VPA treated cells (B16F10C+VPA and B16F10R+VPA). (A) Western blot of the different proteins. β-actin served as loading control. The comparison of the quantified proteins Cl_Caspase3, Cl_Caspase9 and PDH are shown B, C, and D, respectively. *indicates the statistically significant difference (p<0.05).

4.5.3 Cell morphology changes of cells on treatment with VPA

B16F10R cells showed marked changes in morphology, upon treatment with VPA (Figure 4.17 F). The VPA treated cells showed varied morphologies like irregular star-like or spindle-shaped (4.17 E and F) compared to untreated B16F10R cells (Figure 4.17 B and C). The number of cells in the VPA treated B16F10R were considerably less than in untreated B16F10R (4.17 D and A).
Figure 4.17 Morphological changes in drug-resistant melanoma (B16F10R) cells upon VPA treatment

B16F10R cells were treated with VPA (2mM) for 24 h. Image of untreated B16F10R in objectives magnification of, A) 4X; B) 20X; and C) 100; after 24 h after VPA treatment: D) 4X; E) 20X; and F) 100X. Cells were stained using Pap stain method. Cell morphology was captured under a light microscope (Olympus CX41).

4.6 Resistance fold factor of B16F10R and A375R cells after 27 months of drug combination treatment

After 27 months of drug combination treatment, the IC<sub>50</sub> values of

a) B16F10 mouse melanoma cells: B16F10C cells 9.14 ± 1.62 µM and B16F10R cells 29.99 ± 3.21 µM (Figure 4.18 A). The resistance fold factor was 3.28.

b) A375 human melanoma cells: A375C cells 1.76 ± 0.20 µM and A375R cells 8.28 ± 2.1 µM (Figure 4.18 B). The resistance fold factor was 4.7.
The drug combination treatment was continued for B16F10 and A375 cells for 27 months. After 27 months, the parental and resistant melanoma cells were treated with the drug combination at various concentrations (0.62-80 µM). This figure is the results of MTT assay- parental (dark bars) and resistant (light bars). Panel A - on mouse melanoma B16F10; Panel B: on human melanoma A375. Data shown are means ± SEM of three replicate wells. Graphs were plotted with concentration of the inhibitor (X-axis) vs cell viability % (Y-axis) and the IC\textsubscript{50} values were calculated using the formula: \( y = b + ax \). IC\textsubscript{50} values are the concentration of the drug combination which results in 50% cell death. Control (dark bars) and resistant (light bars). C.

The resistant fold factors of both the drug-resistant cell lines, A375R and B16F10R, were increased to 4.7 and 3.28 from 1.72 and 2.07 (data from table 4.1), respectively, over a period of 12 months. The A375R showed higher resistance than B16F10R for U0126 + LY294002 drugs combination at the end of 27 months. Hence A375R was included in the experiment on cross-resistance with other inhibitor of other pathways and phase III.

4.7 Assessment of cross-resistance of drug-resistant melanoma cells to inhibitors of other pathways

To assess the cytotoxic effect of inhibitors of other pathways, SP600125 (JNK inhibitor), LDN193189 (BMP inhibitor) and IWP 2 (WNT inhibitor), on the drug-resistant models, they were tested with parental cell lines B16F10C and A375C, and drug-resistant cell lines B16F10R and A375R.

4.7.1 Cytotoxic effects of the inhibitors of other pathways on the drug-resistant melanoma cells

The growth inhibition curves in presence of drugs showed a dose-dependent inhibition of cell viability as measured by MTT assay after 72 h of treatment with the
drugs SP600125 (Figure 4.19, A and B) and LDN193189 (Figure 4.19, C and D). The least effective among the three was IWP2 (Figure 4.19, E and F).

Figure 4.19  Cytotoxic effect of inhibitors of other pathways on B16F10 and A375 cell lines

MTT assay was performed with the control and drug-resistant cell lines of A375 and B16F10 treated with SP600125 - JNK inhibitor (A and B), LDN193189 - BMP inhibitor (C and D), and IWP 2 - WNT inhibitor (E and F), respectively. The drug concentration ranges were 0.3-40 µM for A375 and 0.16 - 20 µM for B16F10. Data shown are means ± SEM of three replicate wells. Graph was plotted with concentration of the inhibitor (X-axis) vs cell viability % (Y-axis) and the IC$_{50}$ values were calculated using the formula $y = b + ax$. IC$_{50}$ value is the concentration of the drug which results in 50% cell death. Control (dark bars) and resistant (light bars).
4.7.2 Resistance fold factors of B16F10R and A375R cells with the inhibitors tested in this study

The A375R cell line had higher fold resistance than B16F10R cell line to LDN193189 and lower resistance fold factor to SP600125 and IWP2 (Table 4.5). The resistance fold factors were IWP-2<SP600125<LDN193189 <U0126+LY294002 (after 27 months of treatment) in both A375R and B16F10R cell lines.

Table 4.5 IC₅₀ values and fold resistance of all the inhibitors used on A375 and B16F10 models

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Inhibitor</th>
<th>Human melanoma cells (A375)</th>
<th>Mouse melanoma cells (B16F10)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC₅₀ (μM)</td>
<td>Control</td>
<td>Resistant</td>
</tr>
<tr>
<td>1.</td>
<td>U0126 + LY294002*</td>
<td>1.8 ± 0.2</td>
<td>8.3 ± 2.1</td>
</tr>
<tr>
<td>2.</td>
<td>SP600125</td>
<td>5.6 ± 1.3</td>
<td>8.0 ± 1.0</td>
</tr>
<tr>
<td>3.</td>
<td>LDN193189</td>
<td>1.5 ± 0.4</td>
<td>3.1 ± 0.8</td>
</tr>
<tr>
<td>4.</td>
<td>IWP-2</td>
<td>12.8 ± 1.1</td>
<td>12.1 ± 1.9</td>
</tr>
</tbody>
</table>

IC₅₀: 50% growth inhibition. The fold resistance was calculated from IC₅₀ values using the following formula: Fold resistance = IC₅₀ drug-resistant cells/IC₅₀ parental cells. The IC₅₀ values were calculated from the dose-response curves in figure 4.18 (for U0126 + LY294002) and figure 4.19 (for SP600125, LDN193189, and IWP2). *The fold resistance of U0126 + LY294002 combination was assessed after 27 months of drug treatment.

Phase III

4.8 Assessment of radiation sensitization

Melanoma is known to be radioresistant. HDAC2 is hyperphosphorylated in the drug-resistant model. VPA is an inhibitor of HDAC2. The rationale of this phase: pre-treatment of the drug-resistant cells with a low dose of VPA (IC₂⁵ value) would sensitize the drug resistance melanoma cells and make them more susceptible to radiation-induced cell death. In addition, LDN193189, which had the least IC₅₀ values among the inhibitors of the pathways tested for cross-resistance, was tested for radiation sensitization.
4.8.1 Radiation dose optimization with A375C and B16F10C cell lines

B16F10C and A375C cells along with sham-irradiated cells were exposed to different doses, 2, 4, 6, and 8 Gy, of electron beam irradiation. A dose of 2.0 Gy radiations (Figure 4.20) which showed 25 – 30% cell death in both A375C and B16F10C cells was considered optimum for radiation sensitization experiments.

Figure 4.20 Radiation dose optimization with A375C and B16F10C cell lines

Cells were grown in T-25 flask and irradiated with 2, 4, 6, and 8 Gy electron beam radiation (CAART Centre, Mangalore University, India) at a dose rate of 8.8 Gy/min at room temperature. After irradiation, cells were further incubated with drugs for 24 h. Sham served as untreated control. The dark bars represent mouse melanoma B16F10 cells and the light bars human melanoma A375 cells.

4.8.2 IC<sub>50</sub> values of valproic acid (VPA) with A375 and B16F10 cell lines

Dose-dependent curves of VPA on A375 and B16F10 cell lines showed a concentration-dependent reduction in cell viability (Figure 4.21).

The IC<sub>50</sub> values of VPA were calculated from the dose dependent curves (Figure 4.21): 2.59 ± 0.14 mM, 3.56 ± 0.44 mM; 1.44 ± 0.08 mM and 1.91 ± 0.12 mM for A375C, A375R, B16F10C, and B16F10R, respectively.
The A375 (panel A) and B16F10 (panel B), both parental and resistant, were treated with varying concentrations of VPA (0.1-15 mM) for 72 h and MTT assay was performed. Data shown are means ± SEM of three replicate wells. A graph was plotted with concentration of the VPA (X-axis) vs cell viability % (Y-axis) and the IC<sub>50</sub> values were calculated using the formula y = b + ax. An IC<sub>50</sub> value is the concentration of the drug which results in 50% cell death. Control (dark bars) and resistant (light bars).

4.9 Sensitization of drug-resistant melanoma cells with VPA and LDN193189 for radiation treatment

The parental and drug-resistant cells of A375 and B16F10 cells were pre-treated with an IC<sub>25</sub> concentration of VPA and LDN193189 for 24 h and irradiated with 2.0 Gy radiation. The following experiments were done 24 h after exposure to radiation: (i) cytotoxicity by MTT assay, (ii) cell viability by trypan blue, (iii) Live/Dead assay for apoptosis, (iv) Melanin production and (v) Clonogenic assay for cell proliferation.

4.9.1 Cytotoxicity of the drug-resistant melanoma cells sensitized with VPA and LDN193189 prior to radiation

The cell death% in the A375 and B16F10, parental and drug-resistant, cells with either 2.0 Gy of radiation or VPA or LDN193189 was in the range of 3-25% (Figure 4.22; Table 4.6). A375 cells became more sensitive to radiation when pretreated with VPA than LDN193189. The combined treatment of VPA + 2 Gy on A375C cells showed synergistic effects, i.e., cell death% > cell death% with 2 Gy + cell death% with VPA, while the resistant cells showed an additive effect (cell death% on VPA + 2Gy treatment = cell death% with 2 Gy + cell death% with VPA). However, the
B16F10 cells pre-treated with VPA followed by irradiation (2 Gy) showed a synergistic effect in both control and resistant cells. While LDN193189 pre-treated B16F10C cells showed the synergistic effect and the resistant B16F10R cells showed only additive effect (Table 4.6).

Figure 4.22  Radiation sensitization effects of VPA and LDN193189 on A375 and B16F10 cell lines

A375 and B16F10 cells, both parental and resistant, were pre-treated with IC_{25} dose of VPA or LDN193189. After 24 h of incubation, the flask containing cells were irradiated with an electron beam (CAART Centre, Mangalore University, India) at a dose rate of 8.8 Gy/min for 14 sec (equivalent to 2 Gy) at room temperature. After irradiation, cells were further incubated with drugs for 24 h and MTT assay was performed. Respective sham irradiation and control groups were used for comparison and data shown are mean ± SEM of three replicate wells. The results of MTT assay: Panel A - human melanoma A375 and panel B - mouse melanoma B16F10; control (dark bars) and resistant (light bars)

### Table 4.6  Effect of drug, radiation and combination treatment on the B16F10 and A375 cells

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Cells</th>
<th>Treatment →</th>
<th>2 Gy</th>
<th>LDN</th>
<th>VPA</th>
<th>2Gy+LDN</th>
<th>2Gy+VPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>A375</td>
<td>Control</td>
<td>20</td>
<td>23</td>
<td>22</td>
<td>33</td>
<td>54 **</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Resistant</td>
<td>19</td>
<td>25</td>
<td>24</td>
<td>29</td>
<td>43 *</td>
</tr>
<tr>
<td>2.</td>
<td>B16F10</td>
<td>Control</td>
<td>8</td>
<td>23</td>
<td>24</td>
<td>35**</td>
<td>57 **</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Resistant</td>
<td>3</td>
<td>21</td>
<td>20</td>
<td>25*</td>
<td>39 **</td>
</tr>
</tbody>
</table>

Drugs used are LDN193189 (LDN) and valproic acid (VPA). Radiation dose was 2 Gy. Effect of the various treatments was studied in terms of cell death%. Data is the cell death % of the experiment shown in Figure 4.22; *additive effect; ** synergistic effect (more than the additive effect of 2Gy and VPA).
4.9.2  Cell viability of the drug-resistant melanoma cells sensitized with VPA and LDN193189 prior to radiation

Trypan blue cell counts have shown (Figure 4.23), that there was a decrease in the number of cell viability % in the all treated cells, as compared to the untreated cells, both in the control and resistant cells of A75 and B16F10. The decrease in viable cells was more in A375R cell than in B16F10 R cells.

![Graph showing cell viability](image)

Figure 4.23  Cell viability of drug-resistant melanoma cells sensitized with VPA and LDN193189 for radiation treatment

Cells were pre-treated with IC_{25} dose of VPA or LDN193189. After 24 h of incubation, the flask containing cells were irradiated with an electron beam (CAART Centre, Mangalore University, India) at a dose rate of 8.8 Gy/min for 14 sec (equivalent to 2 Gy) at room temperature and cells were further incubated with drugs for 24 h. Following incubation, cells were trypsinized and counted by trypan blue method. Respective sham irradiation and control groups were used for comparison and data shown are mean of duplicate counts. Panel A, shows the results on human melanoma A375 and panel B, shows the results on mouse melanoma B16F10; control (dark bars) and resistant (light bars).

4.9.3  Live/Dead assay of the drug-resistant melanoma cells sensitized with VPA and LDN193189 prior to radiation

The live/dead assay showed that pre-treatment of B16F10C and B16F10R with VPA had more cell killing effect in both parental and resistant cells compared to untreated, 2 Gy treated and LDN193189 pre-treated (Figure 4.24).
A. B16F10C

Figure 4.2 Live/Dead assay of (A) B16F10C and (B) B16F10R cells with acridine orange (AO) and propidium iodide (PI) staining. Images were taken phase-contrast microscopy using ZOE Fluorescent Cell Imager (Bio-Rad). Bright field images are from the normal light with no filter. Live cells were stained with green color (AO stain) and dead cells give red color (PI stain).
4.9.4 Melanin production in the drug-resistant melanoma cells sensitized with VPA and LDN193189 prior to radiation

Melanin the pigment in the melanocytes has a protective effect against radiation. The rationale for this experiment was to check if the pretreatment with drugs VPA and LDN193189 will decrease the melanin content and thus make them susceptible to radiation.

The absorbance at 470 nm was maximum for all untreated cells in VPA+2Gy treated cells (Figure 4.25 A and B). The absorbance value decrease in treatment with 2Gy, LDN+2G, and VPA+2Gy as compared to the untreated controls all the four cell lines. Pre-treatment of VPA showed the least absorbance indicating least concentration of melanin.

![Graph showing melanin production](image)

**Figure 4.25** Melanin content in the resistant cells sensitized with VPA/LDN193189 before radiation

*Cells were pretreated with IC25 dose of VPA or LDN193189 and were irradiated. After 24 h of incubation, the flask containing cells were irradiated with an electron beam (CAART Centre, Mangalore University, India) at a dose rate of 8.8 Gy/min for 14 sec (equivalent to 2 Gy) at room temperature and cells were further incubated with drugs for 24 h. Following incubation, cells were lysed and the color due to melanin release was measured at 470 nm. Respective sham irradiation and control (untreated) groups were used for comparison and data shown are mean ± SD of three replicate wells. Panel A, shows the results on human melanoma A375 and panel B, shows the results on mouse melanoma B16F10.*
4.9.5 Clonogenic assay with the drug-resistant melanoma cells sensitized with VPA and LDN193189 prior to radiation

The clonogenic survival assay also confirmed that the pre-treatment of a low dose of VPA (2 mM) followed by exposure to low dose of radiation (2 Gy) increased cell death significantly in B16F10C and B16F10R cells (Figure 4.26A and B). The cell death was synergistic in the VPA pretreated cells exposed to radiation (Figure 4.26C).

![Figure 4.26 Clonogenic survival assay for parental melanoma cells (B16F10C) and drug-resistant melanoma cells (B16F10R)](image)

*Cells were pretreated with an IC25 dose of VPA (IC25) and incubated for 24 h. After irradiation with 2 Gy, cells were further incubated with drugs for 24 h. The colonies in control B16F10C (Panel A) and drug-resistant B16F10R (Panel B) were counted and respective % viability was calculated with the sham considered as 100% and plotted in (C).*
4.9  **In silico docking of valproic acid on HDAC2**

Among the listed interactions generated from PDBsum, two H-bonds involving His145 and Gly154 (Figure 4.27A), with an energy of -9.5 kcal/moL were generated between HDAC2 and its inhibitor IWX (used as a standard to check for reliability of the docking). The negative energy shows the stability of the interaction. There was no interaction between valproic acid and His145 and Gly146 of HDAC2 as seen in the case of the inhibitor IWX; however, valproic acid is deeper in the active site and makes an H-bond with Arg39 (Figure 4.27 B and C).

![Figure 4.27 Docking of IWX, VPA, and pyruvate with HDAC2](image)

*Molecular interaction of HDAC2 with (A) IWX inhibitor, (B, C) VPA, (D, E) pyruvate. The black dashed lines indicate the interactions of the ligand with the labeled amino acid residues.*
The interaction of HDAC2-valproic acid complex is stabilized by energies of -4.61 kcal/mol. This docking study shows that although the binding energy of VPA to HDAC2 is comparatively more than the interaction of IWX to HDAC2. VPA can make interactions with Arg39.

Since the earlier result showed decreased PDH enzyme (Figure 4.27, D and E), which means there is an accumulation of its substrate pyruvate and pyruvate is also reported to be an HDAC inhibitor, *in silico* docking of pyruvate on HDAC2 was done. The result shows that pyruvate can bind to Arginine39 with two interactions similar to VPA.