Selective Elimination of Malignant Melanoma Using the Novel Anti-tumor Agents, OSW-1 AND PEITC

Kausar Begam Riaz Ahmed
SELECTIVE ELIMINATION OF MALIGNANT MELANOMA USING THE NOVEL ANTI-TUMOR AGENTS, OSW-1 AND PEITC

By

Kausar Begam Riaz Ahmed, B.Tech., M.S.

APPROVED:

______________________________
Peng Huang, M.D., Ph.D.
(Advisory Professor)

______________________________
Michael Davies, M.D., Ph.D.

______________________________
Varsha Gandhi, Ph.D.

______________________________
Elizabeth Grimm, Ph.D.

______________________________
Zahid Siddik, Ph.D.

APPROVED:

______________________________
Dean, The University of Texas
Graduate School of Biomedical Sciences at Houston
Selective Elimination of Malignant Melanoma Using the Novel Anti-tumor Agents, OSW-1 and PEITC

A DISSERTATION

Presented to the Faculty of

The University of Texas

Health Science Center at Houston

and

The University of Texas

MD Anderson Cancer Center

Graduate School of Biomedical Sciences

in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

By

Kausar Begam Riaz Ahmed, B. Tech., M.S.

Houston, Texas

August, 2014
Acknowledgements

I would like to thank my mentor, Dr. Peng Huang for his guidance and support during the course of my PhD study. I would also like to thank my PhD committee members Drs. Varsha Gandhi, Elizabeth Grimm, Michael Davies, Zahid Siddik, Nancy Poindexter, Sendurai Mani, David McConkey and Kevin Kim for their constructive feedback and scientific guidance throughout these years. I am grateful to all my colleagues in Dr. Huang’s lab for technical assistance and insightful discussions, which were instrumental in the completion of this work. I am immensely thankful to my parents and siblings who supported me in all possible ways during graduate school and always encouraged me to dream big and work hard.
Selective Elimination of Malignant Melanoma Using the Novel Anti-tumor Agents, OSW-1 and PEITC

Kausar Begam Riaz Ahmed, B. Tech., M.S.

(Advisory Professor: Peng Huang, M.D., Ph.D.)

Metastatic melanoma is amongst the most refractory of cancers. Drug resistance and lack of therapeutic selectivity are two main challenges to successful melanoma therapy. Herein, we investigated the mechanisms of anticancer activity and therapeutic selectivity of two novel agents, \(3\beta, 16\beta, 17\alpha\)-trihydroxycholest-5-en-22-one 16-\(O\)-[2-\(O\)-4-methoxybenzoyl-\(\beta\)-D-xylopyranosyl] \(\rightarrow\) 3]-2-\(O\)-acetyl-\(\alpha\)-L-arabinopyranoside (OSW-1) and \(\beta\)-Phenylethyl Isothiocyanate (PEITC) in melanoma.

OSW-1 inhibited melanoma cell viability at nanomolar concentrations with minimal toxicity to normal melanocytes. Mechanistic studies revealed that OSW-1 suppressed Disialoganglioside 3 Synthase (GD3S) gene expression in melanoma cells, leading to inhibition of gangliosides GD3 and GD2. GD3 is an abundantly expressed melanoma cell surface antigen with pivotal roles in cancer progression and invasion. OSW-1 promoted interaction between GD3 and mitochondrial voltage-dependent gating protein 1 (VDAC1). Subsequent VDAC1 activation and autophagic cell death was observed. Downregulation of VDAC1 ameliorated the cytotoxic effect of OSW-1 in melanoma, indicating that VDAC1 was critical to mediating OSW-1’s activity. Studies with OSW-1 resistant melanoma cells revealed that elevated ganglioside expression may form the mechanistic basis for the selectivity of OSW-1 towards melanoma.

Melanoma cells are characterized by mitochondrial dysfunction and intrinsic oxidative stress compared to normal melanocytes. Reactive oxygen species (ROS) activate
pro-oncogenic signaling pathways that promote cancer progression and metastasis. However, an excessive overload of ROS, beyond the cellular tolerance threshold may trigger oxidative damage and cell death. The natural product PEITC induced apoptosis in malignant melanoma. Biochemical studies revealed that PEITC depleted cellular glutathione leading to ROS accumulation, mitochondrial damage and cell death. Glutathione inhibition was crucial to mediating PEITC’s activity since the glutathione precursor, N-Acetyl Cysteine reversed ROS elevation and PEITC induced death. Normal melanocytes with a much lower basal ROS capacity were resistant to PEITC. Drug combination effect of PEITC with Carmustine and Temozolomide was synergistic, suggesting that redox modulating strategies may be effective in attenuating chemoresistance to these agents.

My study outlines novel therapeutic strategies to effectively eliminate malignant melanomas. The intrinsic differences in ganglioside expression and redox status between normal and cancer cells may be exploited to selectively kill melanoma cells using OSW-1 and PEITC, thus making these compounds worthy of further pre-clinical and clinical investigation.
# Table of Contents

Approval sheet .................................................................................................................. i
Title page .......................................................................................................................... ii
Acknowledgements ......................................................................................................... iii
Abstract ............................................................................................................................ iv
Table of contents ............................................................................................................. vi
List of illustrations .......................................................................................................... x
List of abbreviations ....................................................................................................... xiv

## Chapter 1: Introduction ................................................................................................. 1

1.1 Metabolic and biochemical alterations in melanoma................................................. 1

1.1.1 Dysregulated lipid metabolism: role of gangliosides............................................. 1

1.1.2 Mitochondrial dysfunction...................................................................................... 5

1.1.3 Redox homeostasis and oxidative stress............................................................... 6

1.2 Purpose and significance of this study.................................................................... 9

1.2.1 Rationale for this study......................................................................................... 9

1.2.2 Background information on OSW-1.................................................................. 11

1.2.3 Background information on PEITC ................................................................... 16

1.3 Research hypothesis and specific aims .................................................................. 20

## Chapter 2: Materials and Methods ............................................................................ 22

2.1 Chemicals and reagents.......................................................................................... 22

2.2 Cell culture............................................................................................................... 22

2.3 MTT assay for cell viability..................................................................................... 23
2.4 Colony formation assay ................................................................. 23
2.5 Mitochondrial membrane potential measurement by Rhodamine 123 staining ..... 24
2.6 Annexin-V/PI analysis for apoptosis .............................................. 24
2.7 Electron microscopy ...................................................................... 24
2.8 Immunoblotting .......................................................................... 25
2.9 Detection of ganglioside expression by flow cytometry...................... 25
2.10 Lipid raft / ganglioside enriched microdomain (GEM) isolation ............. 26
2.11 Real time RT-PCR ...................................................................... 26
2.12 Immunofluorescence staining and confocal microscopy..................... 27
2.13 Cytosolic calcium detection .......................................................... 28
2.14 Detection of cellular free radical expression by staining with the fluorescent dyes DCFDA, DAF-FM, Dihyroethidium and mitoSOX mitochondrial superoxide indicator 28
2.15 siRNA transfection ..................................................................... 28
2.16 Glutathione assay ................................................................------- 29
2.17 Reverse phase protein array analysis ............................................. 29
2.18 Statistical analysis ........................................................................ 30

Chapter 3: The anti-tumor agent, OSW-1, effectively eliminates melanoma cells through ganglioside GD3 and mitochondrial VDAC1 dependent mechanisms .... 31

3.1 Introduction .................................................................................... 31
3.2 Results .......................................................................................... 32
3.2.1 Cytotoxic effect of OSW-1 in melanoma .............................................................. 32
3.2.2 OSW-1 triggered cell death in melanoma is associated with autophagy .......... 37
3.2.3 Normal melanocytes are resistant to OSW-1 induced cytotoxicity .............. 48
3.2.4 OSW-1 inhibits ganglioside GD3/GD2 expression in melanoma cells .......... 52
3.2.5 OSW-1 promotes interaction of ganglioside GD3 with VDAC1 in the mitochondria ........................................................................................................................................ 61
3.2.6 OSW-1 eliminates melanoma cells through mitochondrial VDAC1 dependent mechanisms ........................................................................................................................................ 66
3.2.7 VDAC1 is a critical player in mediating the cytotoxic effect of OSW-1........... 78
3.2.8 Characterization of the mechanisms underlying the therapeutic selectivity of OSW-1 ........................................................................................................................................ 82
3.3 Discussion .............................................................................................................. 89

Chapter 4: PEITC effectively eliminates melanoma cells through redox mechanisms

4.1: Introduction .......................................................................................................... 94
4.2 Results ................................................................................................................... 95
4.2.1 Redox status of melanoma cells ....................................................................... 95
4.2.2 The cytotoxic effect of PEITC in melanoma .................................................... 98
4.2.3 PEITC exhibits minimal toxicity in normal melanocytes ............................... 106
4.2.4 The ROS mediated mechanisms of cell death by PEITC ................................. 111
4.2.5 Combination of Temozolomide / BCNU with PEITC attenuates drug resistance to these agents in melanoma ........................................................................................................ 119
Chapter 5: Summary and future directions ................................................. 132

5.1 Summary ................................................................................................. 132

5.2 Future directions ....................................................................................... 133

5.2.1 Mechanisms of selectivity of OSW-1 and PEITC against malignant melanoma cells .......................................................................................................................... 133

5.2.2 Characterization of the direct molecular target for OSW-1: SREBP and GD3S regulatory signaling pathways ................................................................................. 137

5.2.3 Oxidative stress based strategies to combat drug resistance in melanoma using PEITC .................................................................................................................... 139

References ......................................................................................................... 142

Vita ..................................................................................................................... 176
List of Illustrations

Chapter 1: Introduction ................................................................. 1
Figure 1.1: Convergence of growth factor receptor-mediated and integrin mediated
adhesion signaling pathways under GD3 expression in melanoma .................. 4
Figure 1.2: Altered redox dynamics between normal and cancer cells ............ 8
Figure 1.3: Structure of OSW-1 ......................................................... 14
Figure 1.4: Structure of PEITC ......................................................... 18

Chapter 2: Materials and Methods (no illustrations) ......................... 22

Chapter 3: The anti-tumor agent, OSW-1, effectively eliminates melanoma cells
through ganglioside GD3 and mitochondrial VDAC1 dependent mechanisms
Figure 3.1: Effect of OSW-1 on melanoma cell viability .......................... 33
Figure 3.2: OSW-1 inhibits colony formation in melanoma cells ............. 34
Figure 3.3: Effect of OSW-1 on mitochondrial membrane potential (ΔΨm) in melanoma
cells ...................................................................................................... 36
Figure 3.4: OSW-1 promotes autophagic vacuole formation in melanoma cells.... 39
Figure 3.5: Effect of OSW-1 on the autophagy marker, LC3B expression ....... 40
Figure 3.6: Autophagy induction is crucial to cell death induction by OSW-1 .... 41
Figure 3.7: Cytotoxic effect of OSW-1 ± Chloroquine drug combination in melanoma
cells ...................................................................................................... 44
Figure 3.8: Cytotoxic effect of OSW-1 ± Rapamycin drug combination in melanoma
cells ...................................................................................................... 46
Figure 3.9: Effect of OSW-1 on mitochondrial membrane potential in normal
melanocytes vs. melanoma cells ............................................................. 49
Figure 3.10: Selective killing of melanoma cells by OSW-1

Figure 3.11: Effect of OSW-1 on ganglioside expression in melanoma

Figure 3.12: Effect of OSW-1 on ganglioside GD2 expression in melanoma cells

Figure 3.13: OSW-1 represses gene expression of GD3S, FASN and HMGCR enzymes

Figure 3.14: Effect of OSW-1 on GD2 Synthase gene expression

Figure 3.15: Schematics of OSW-1 mediated modulation of ganglioside expression

Figure 3.16: Combination of OSW-1 with Triptolide partially reverses the OSW-1 induced mitochondrial membrane potential loss in melanoma

Figure 3.17: Effect of OSW-1 on GD3 and VDAC1 co-expression in lipid rafts

Figure 3.18: OSW-1 promotes co-localization of VDAC1 with ganglioside GD3

Figure 3.19: Correlation between OSW-1 induced mitochondrial membrane potential loss and ganglioside GD2 inhibition

Figure 3.20: OSW-1 induces VDAC1 upregulation in melanoma cells

Figure 3.21: Effect of cycloheximide on OSW-1 induced VDAC1 upregulation in melanoma cells

Figure 3.22: Effect of Triptolide on VDAC1 expression in melanoma cells

Figure 3.23: Induction of mitochondrial superoxide release by OSW-1 in melanoma

Figure 3.24: OSW-1 induces cytosolic calcium elevation in melanoma cells

Figure 3.25: Combination of OSW-1 with Cyclosporin A (CsA) causes elevated cell death effect in melanoma cells

Figure 3.26: Effect of OSW-1 and Cyclosporin A drug combination on VDAC1 expression and calcium homeostasis

Figure 3.27: OSW-1 treatment causes elevated expression of phosphorylated ERK1/2 in melanoma
Figure 3.28: MEF VDAC1-/- cells are resistant to the cytotoxic effects of OSW-1.

Figure 3.29: Down regulation of VDAC1 in A375SM cells confers resistance to OSW-1.

Figure 3.30: Combination of OSW-1 with the VDAC1 inhibitor, DIDS, indicates that VDAC1 does not regulate ganglioside expression.

Figure 3.31: The OSW-1 resistant WM35 cell model.

Figure 3.32: OSW-1 resistant WM35 DW cells have a decreased ganglioside GD3/GD2 expression profile compared to the parental WM35 melanoma cells.

Figure 3.33: OSW-1 resistant WM35 DW cells have an altered free radical expression profile compared to the parental WM35 melanoma cells.

Figure 3.34: Differential protein expression between WM35 vs. WM35 R vs. WM35 DW cells.

Figure 3.35: Differential expression of lipogenesis and mitochondria signaling proteins in WM35 vs. WM35 R vs. WM35 DW cells.

Chapter 4: PEITC effectively eliminates melanoma cells through redox mechanisms.

Figure 4.1: Basal free radical expression profile in melanoma cells.

Figure 4.2: Effect of PEITC on melanoma cell viability.

Figure 4.3: PEITC induced cytotoxicity is through apoptosis in melanoma cells.

Figure 4.4: PEITC induces mitochondrial membrane potential loss in melanoma cells.

Figure 4.5: Correlation between cellular basal ROS levels and cell death induced by PEITC.

Figure 4.6: Effect of PEITC on JNK and p38 expression in melanoma.

Figure 4.7: Preferential killing of melanoma cells by PEITC.

Figure 4.8: Induction of ROS accumulation in melanoma cells and normal melanocytes by PEITC.
Figure 4.9: Comparison of ROS expression between melanoma cells and normal melanocytes................................................................. 110
Figure 4.10: Effective killing of melanoma cells through ROS induced damage........ 112
Figure 4.11: Correlation between cell death and PEITC induced ROS stress.......... 114
Figure 4.12: PEITC causes depletion of cellular glutathione............................... 116
Figure 4.13: N-Acetyl Cysteine (NAC) reverses the inhibitory effect of PEITC......... 117
Figure 4.14: Effect of PEITC ± TMZ/BCNU treatment on melanoma cell viability.... 121
Figure 4.15: Combination of Temozolomide / BCNU with PEITC inhibits colony formation in melanoma cells................................................................. 122
Figure 4.16: Combination index analysis of PEITC ± TMZ/BCNU treatment........... 123
Figure 4.17: Redox mechanisms mediate the combinatorial cell death effect of TMZ/ BCNU ± PEITC treatment in melanoma......................................................... 125

Chapter 5: Summary and future directions (no illustrations).............................. 132
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADM</td>
<td>Adriamycin</td>
</tr>
<tr>
<td>APP</td>
<td>Amyloid Precursor Protein</td>
</tr>
<tr>
<td>BSO</td>
<td>L-Buthionine (S, R)-Sulphoximine</td>
</tr>
<tr>
<td>CDDP</td>
<td><em>cis</em>-Diamminedichloroplatinum(II) or cisplatin</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin Immunoprecipitation</td>
</tr>
<tr>
<td>CM-H$_2$DCFDA</td>
<td>5, 6-Chloromethyl-2′,7′-Dichlorodihydrofluorescein Diacetate</td>
</tr>
<tr>
<td>CsA</td>
<td>Cyclosporin A</td>
</tr>
<tr>
<td>DAF-FM</td>
<td>4-Amino-5-Methylamino-2′,7′-Difluorofluorescein</td>
</tr>
<tr>
<td>DCF</td>
<td>Dichlorohydrofluorescein</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal Adhesion Kinase</td>
</tr>
<tr>
<td>FASN</td>
<td>Fatty Acid Synthase</td>
</tr>
<tr>
<td>FCCP</td>
<td>p-Trifluoromethoxyphenyl Hydrazone</td>
</tr>
<tr>
<td>GCS</td>
<td>γ-Glutamyl Cysteinyl Synthetase</td>
</tr>
<tr>
<td>GD</td>
<td>Disialoganglioside</td>
</tr>
<tr>
<td>GD3S</td>
<td>Disialoganglioside 3 Synthase</td>
</tr>
<tr>
<td>GM</td>
<td>Monosialoganglioside</td>
</tr>
<tr>
<td>GNE</td>
<td>UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase</td>
</tr>
<tr>
<td>GPX</td>
<td>Glutathione Peroxidase</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-Transferase</td>
</tr>
<tr>
<td>GS-X</td>
<td>Glutathione Efflux Pump</td>
</tr>
</tbody>
</table>
GR  Glutathione Reductase
HEt  Hydroethidium
HMGCR 3-Hydroxy-3-Methylglutaryl-CoA Reductase
iNOS Inducible Nitric Oxide Synthase
i.p. Intraperitoneal
JNK c-Jun N-terminal Kinase
MEF Mouse Embryonic Fibroblasts
MMP Mitochondrial Membrane Potential
MTP Mitochondrial Transition Pore
MTX Methotrexate
NAC N-Acetyl Cysteine
NCX1 Sodium-Calcium Exchanger 1
NF-κB Nuclear Factor κB
OSBP Oxysterol Binding Protein
ORP4L OSBP-related Protein 4L
OSW-1 3β, 16β, 17α-trihydroxycholest-5-en-22-one 16-O-[2-O-4-methoxybenzoyl-β-D-xylopyranosyl]-[1→3]-2-O-acetyl-α-L-arabinopyranoside
p450scC Cholesterol side chain cleavage cytochrome P-450
PBS Phosphate Buffered Saline
PGC1α Peroxisome Proliferator-activated Receptor Gamma Coactivator 1α
PEITC β-Phenylethyl Isothiocyanate
PI Propidium Iodide
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>RPPA</td>
<td>Reverse Phase Protein Array</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcriptase-Polymerase Chain Reaction</td>
</tr>
<tr>
<td>SAR</td>
<td>Structure Activity Relationship</td>
</tr>
<tr>
<td>SREBP</td>
<td>Steroid Regulatory Element Binding Protein</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VDAC1</td>
<td>Voltage Dependent Anion Channel 1</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>TAX</td>
<td>Taxol</td>
</tr>
</tbody>
</table>
Chapter 1: Introduction

1.1 Metabolic and biochemical alterations in melanoma

1.1.1 Dysregulated lipid metabolism: role of gangliosides.

Altered metabolism is a hallmark of cancer cells (1, 2). In addition to exhibiting the warburg effect phenotype, aggressive cancers are also characterized by increased de novo lipogenesis which provides membrane building blocks for rapid proliferation, post-translational protein modifications and cell signaling (3). The expression of critical lipogenic enzymes such as fatty acid synthase (FASN), HMG Co-A reductase (HMGCR), acetyl-coA carboxylase (ACC) is upregulated in human cancers, including malignant melanoma (4, 5).

Increased expression of the key fatty acid synthesis enzyme, FASN is correlated with poor prognosis in breast cancer and melanoma (6, 7). Elevated fatty acid synthesis has been associated with a high proliferative index in prostate cancers (8). Aberrant fatty acid biosynthesis induced by FASN overexpression has been known to induce a cancer-like phenotype in non-cancer epithelial cells (9). Further, oncogenic signaling pathways also activated FASN leading to increased fatty acid biosynthesis. Yamauchi et al. (2011) demonstrated that the PI3K-Akt-mTORC1 oncogenic signaling in melanoma cells activates the steroid regulatory element binding protein (SREBP), which is the master transcription factor for lipid metabolism (10). SREBP, in turn, activates the downstream HMGCR enzyme leading to increased cholesterol biosynthesis. Inhibition of the PI3K-Akt-mTORC1-SREBP signaling axis resulted in reduced cholesterol levels in the cell membrane lipid rafts. Importantly, inhibition of this SREBP mediated cholesterol biosynthesis using pharmacological inhibitors suppressed Akt activation in lipid rafts and attenuated tumor
growth (10). Ganglioside GD3 positively regulated the lipogenic phenotype in melanoma cells by mediating Akt activation and thus the downstream cholesterol biosynthesis.

Gangliosides are sialic acid containing glycosphingolipids that are ubiquitously expressed in human cancers and regulate signals transduced at the membrane microdomains (11). In general, while disialic acid containing gangliosides (GD3, GD2, GD1) promote tumor cell proliferation, invasion and motility and are considered to be pro-oncogenic (11, 12), monosialic acid containing gangliosides (GM3, GM2, GM1) are tumor suppressive and inhibit cell growth and metastasis (11, 13, 14). In melanoma, elevated levels of gangliosides GD3, GD2, GM3 and GM2 were reported (15). Normal melanocytes exhibited high expression of ganglioside GM3 and minimal levels of GD3 (16). This differential ganglioside expression was due to a very low expression of the GD3 synthesis regulating enzyme GD3 synthase in normal melanocytes (16). Using GD3-negative mutant of SK-MEL-28 cells, Furukawa et al. (2011), demonstrated that GD3 expression contributed to increased phosphorylation of adaptor molecules, p130Cas, paxillin, and focal adhesion kinase (FAK). Further, higher localization of the Src kinase, Yes, to the lipid rafts in the cell membrane of GD3-positive SK-MEL-28 cells was observed. Also, enhanced integrin function and a higher localization of integrin to the ganglioside enriched microdomains (GEM)/lipid rafts in the GD3-positive SK-MEL-28 cells, where it clusters with GD3 on the rafts, was observed. (17-19). Thus ganglioside GD3 acts as a point of convergence for growth factor receptor-mediated signaling and integrin mediated adhesion signaling, leading to activation of enhanced malignant signals and cancer phenotypes (figure 1.1) (18, 20). More significantly, injection of anti-GD3 monoclonal antibody suppressed tumor progression in melanoma tissues and patients making it an important target for melanoma therapy (21, 22).
The ganglioside GD2 is overexpressed in breast and lung cancers. Battula et al. reported that GD2 is a stem cell marker in human breast cancer and may contribute to c-Met activation and chemoresistance in these cells (23, 24). Anti-GD2 antibody treatment of small cell lung cancer (SCLC) cells led to dephosphorylation of FAK, activation of p38 MAPK and finally cell death by anoikis (25).

Majority of gangliosides are constituted in the lipid rafts, where they interact with the receptors and other downstream proteins. It was observed that overexpression of the lipid raft-resident protein, caveolin-1, led to the dispersion of GD3 from the lipid raft domains (26). Further, the fine structure in the ceramide group of GD3 expressed in lipid raft was reported to be different from GD3 located in non-raft cell fractions (26). Further, a translocation of GD3 to the mitochondria during cell death signaling, where it regulates microtubule polymerization and apoptotic induction, has also been reported (27). Further studies need to be undertaken to shed light on the complex roles of GD3 in melanoma biology.
Figure 1.1: Convergence of growth factor receptor-mediated and integrin mediated adhesion signaling pathways under GD3 expression in melanoma.

Reprinted by permission of Oxford University Press on behalf of the Japanese Biochemical Society.

1.1.2 Mitochondrial dysfunction

Mitochondria are dynamic cellular organelles which act as functional sites for oxidative phosphorylation, fatty acid oxidation, tricarboxylic acid cycle and execution of apoptosis (28, 29). Cancer cells are characterized by Warburg effect, wherein the tumor cells rely on glycolysis rather than oxidative phosphorylation for their energy and metabolic needs due to defects in their mitochondrial respiratory machinery, decreased oxidation of NADH-linked substrates or mitochondrial mutations. Increase in reactive oxygen species (ROS), due to various causes such as defects in electron transport, reduced ROS scavenging, environmental or oncogene induced stress etc., is a characteristic feature of cancer cell mitochondria (28). Enhanced ROS contributes to stabilization of hypoxia inducible factor-α, loss of p16, further oxidative damage and metastasis of cancer cells. In addition, mutations in mitochondrial DNA have also been observed. For instance, Tan et al. (2002) identified somatic mutations in the D-loop region, 16sRNA, ND2 and ATPase 6 genes of mitochondrial DNA in breast cancer patients (29, 30). In melanoma, a comprehensive analysis by Poetsch et al. (2004) discovered mitochondrial instability in the D-loop region in 13% of nodular primary cancers and 20% metastatic melanomas (31). Interestingly, the pro-oncogenic mutant BRAF, BRAF V600E localized to mitochondria in thyroid cancer cells leading to inhibition of oxidative phosphorylation, increased glycolytic activity and a high glucose uptake (32).

Mitochondrial autophagy or mitophagy is also activated in cancer cells as a cellular response mechanism to nutrient depletion (28). Various studies indicate that mitophagy is preceded by mitochondrial fragmentation and membrane permeabilization. The mitochondrial transition pore protein, voltage dependent anion channel 1 (VDAC1) has been
reported to induce apoptotic and autphagic cell death in cells (33-35). Recent studies by De Pinto et al. (2007) and Tomasello et al (2009) demonstrated that overexpression of human (h)VDAC1 caused depolarization of the inner membrane and triggered mitochondrial permeability transition (MPT), which may be followed by autophagy (33, 36). In cancer cells, the hypoxia regulated BNIP3/NIX proteins have been recognized as key players in mediating mitochondrial autophagy (28, 37).

1.1.3 Redox homeostasis and oxidative stress

A unique feature of melanoma biology is the free radical generation by aberrant melanosomes (38). Reactive oxygen species play important roles in melanoma development, immune response, metabolism, and metastasis (38). Loss of PTEN and decreased PTEN activity is observed in about 20% of melanomas (39). Activation of Akt signaling can promote ROS generation by inducing the expression of the ROS generating enzyme NOX4 in melanoma cells and mice (40). Also, Akt may inhibit the activity of various pro-apoptotic proteins such as BAD and forkhead transcription factor (FOXO) thus stabilizing the cells with mitochondrial damage and potentiating ROS production. Further, MAPK which are activated in melanoma due to BRAF V600E activating mutation also modulate ROS generation in cells (41, 42). Further loss of function of the tumor suppressors, p53 and p16 may also contribute to ROS stress phenotype (43). Chronic oxidative stress in combination with ultraviolet (UV) exposure may lead to malignant transformation of cells (38, 44, 45). ROS can activate the transcription factors, NF-κB and AP-1 thus promoting melanoma progression (46, 47). ROS generated by hypoxic mitochondria and NADPH oxidases may stabilize hypoxia inducible factor 1-α (HIF1-α) and potentially block PTEN mediated inhibition of Akt signaling leading to activation of downstream anti-apoptotic proteins.
including Bcl-1 and Bcl-xL (48). Thus ROS plays pivotal roles in melanoma transformation and metastasis.

Reactive oxygen species function as a doubled-edged sword. Intrinsically high ROS levels in melanoma cells promote tumor development. However, if the ROS stress in melanoma is at a level that is beyond the cell’s toxicity tolerance threshold, then it may trigger cell death. Normal cells which have lower basal ROS stress levels and a reserve antioxidant capacity will be resistant to this ROS accretion since their antioxidants will be mobilized to neutralize the ROS increase and prevent it from reaching the cell-death threshold. The pro-oncogenic signaling in melanoma cells triggers increased ROS production and elevates the antioxidant capacity of the cells so as to neutralize the high level of free radicals being generated. Eventually, this results in a shift of redox dynamics and a high dependence of cancer on antioxidants to sustain the ROS levels within the cell’s toxicity threshold (49). Hence cancer cells are more vulnerable to redox modulatory or pro-oxidant based therapeutic strategies that by either scavenging the antioxidants or instigating excessive ROS production (for instance through inhibition of oxidative phosphorylation) may drive the ROS levels above the toxicity tolerance threshold of the cell leading to massive cell death (49). This therapeutic strategy is illustrated in figure 2.

This therapeutic strategy is relatively novel and may find great utility in inducing cell death as well as combating drug resistance to other conventional melanoma chemotherapeutic agents. Currently, Elesclomol, a pro-oxidant compound that utilizes a similar mechanism of action is in clinical trials for melanoma (50-52).
Figure 1.2: Altered redox dynamics between normal and cancer cells.


1.2 Purpose and significance of this study

The purpose of this PhD dissertation was to characterize novel therapeutic strategies based on altered lipid metabolism and reactive oxygen species mediated stress in melanoma cells to selectively eliminate malignant melanomas using the novel anticancer agents, OSW-1 and PEITC.

The rationale for this research design, study hypothesis and specific aims have been described in the sections below.

1.2.1 Rationale for this study

Selective elimination of malignant melanomas by OSW-1:

The ganglioside GD3 is highly expressed in a majority of melanoma cell lines and tissues (53, 54) and mediates cell proliferation and metastasis (17, 19). Hamamura et al. (2005) demonstrated that GD3 enhanced cell growth and invasion through FAK, p130cas and paxicillin signaling in melanoma cells (17). Enhanced integrin cluster formation in lipid rafts on the melanoma cell membrane was observed under GD3 expression suggesting that it contributed to adhesion signaling in malignant melanoma (19). Thus GD3 acts as a convergence point for growth factor and integrin-mediated adhesion signaling in melanoma cells (18, 20). Further, GD3 expression induced cholesterol biosynthesis, lipogenic phenotype and activation of Akt signaling in melanoma (10). Significantly, only trace amounts of GD3 were detected in normal melanocytes as compared to melanoma cells (16), thus highlighting its potential as a surface antigen that can be targeted to selectively eliminate melanoma cells with limited toxicity to the surrounding normal melanocytes. Currently, GD3 is being investigated in melanoma immunotherapy (21, 22, 55).
Recent studies identified that Triptolide, which bears structural similarities with OSW-1, inhibited ganglioside GD3 and GD2 expression in melanoma cells (56). Yokoyama et al. (2005), discovered that OSW-1 encapsulation in GM3 liposomes led to melanoma cell killing (57). These results suggested that OSW-1 might potentially inhibit ganglioside expression in melanoma cells. Previous studies from our lab have demonstrated that OSW-1 induces cytotoxicity through mitochondrial dependent mechanisms in leukemia cells (58, 59). Ganglioside GD3 translocates to mitochondria during cell death and through its interactions with the raft-like mitochondrial microdomains may have a regulatory function in inducing mitochondrial membrane permeabilization and apoptosis (27, 60).

The rationale for my research study was that OSW-1, because of its similarity to Triptolide and higher affinity towards GM3 may have an inhibitory effect on ganglioside (GM3, GD3, GD2) expression. Inhibition of ganglioside GD3, which plays important roles in melanoma proliferation and invasion, would promote melanoma cell death. Further, because of the melanoma cell specific expression of GD3, cell death due to GD3 inhibition by OSW-1 may be selective towards cancer cells. It was essential to investigate the effect of GD3 in mitochondrial signaling since OSW-1 was previously to act through mitochondria dependent mechanisms.

Effective elimination of melanoma by PEITC:

Increased ROS generation and altered redox status is observed in cancer cells compared to normal cells. Recent studies have suggested that this differential redox biology may be exploited to selectively target malignant cells, overcome drug resistance and improve the therapeutic activity of current clinically available anticancer agents (61).
Melanoma cells are under a higher intrinsic oxidative stress compared to normal melanocytes due to oncogene activation and aberrant melanosome function (62). This elevated ROS promotes melanoma oncogenic transformation, invasion, metastasis and metabolism. However, if the ROS stress in cells is pushed beyond the cells’ tolerance limit either by using pro-oxidant compounds or by abrogating the cellular antioxidant capacity, it would lead to massive toxicity and cell death.

The anticancer agent PEITC causes ROS accumulation in cancer cells either through depletion of the antioxidant glutathione or by inhibiting oxidative phosphorylation (63, 64). In my research, I postulated that melanoma cells would be at a higher intrinsic oxidative stress than normal melanocytes, making them vulnerable to ROS stress based therapeutic strategies. PEITC, because of its ability to potentiate further ROS increase, will effectively eliminate melanoma cells. Normal melanocytes, which maintain basal redox homeostasis, will be able to withstand the exogenous ROS stress induced by PEITC to a certain extent, and thus would be less sensitive to PEITC or other redox modulating agents. Further, these principles of redox modulation by PEITC may be utilized in attenuating drug resistance to currently used anticancer agents such as Temozolomide and Carmustine.

1.2.2 Background information on OSW-1

The natural product, 3β, 16β, 17α-trihydroxycholest-5-en-22-one 16-O-[2-O-4-methoxybenzoyl-β-D-xylopyranosyl]-[1→3]-2-O-acetyl-α-L-arabinopyranoside (OSW-1), an acylated disaccharide cholestane saponin molecule was originally isolated as a main constituent of the bulbs of Ornithogalum saundersiae (Liliaceae) (65-67). It is highly cytotoxic against several human cancer cell lines with half maximal inhibitory concentrations in the nanomolar range (58, 59, 68). It exhibited significantly higher inhibitory effect than
many clinically used anticancer agents including etoposide, adriamycin (ADM), taxol (TAX), cisplatin (CDDP), mitomycin c and methotrexate (MTX) in leukemia, pulmonary adenocarcinoma, mouse mastrocarcinoma, pancreatic and brain cancers (59, 67).

Importantly, adriamycin-resistant and camptothecin-resistant p388 leukemia as well as fludarabine refractory primary leukemia cells from chronic lymphocytic leukemia (CLL) patients were sensitive to OSW-1 suggesting that it had a unique mechanism of action that was different from these clinically used agents (59, 67). In vivo evaluation showed that it was extremely potent against mouse p388 tumors with a single 0.01 mg/kg dose increasing life span by 59% (67). Importantly, OSW-1 was therapeutically selective towards malignant cells with half-maximal inhibitory concentrations (IC$_{50}$) 40- to 150-fold higher in normal cells than cancer cells (59).

An in vitro screen against the National Cancer Institute (NCI)-60 cell line panel indicated that OSW-1 exhibited significant toxicity with an IC50 of 0.78 nM (67). Melanoma cells were specifically sensitive to OSW-1. Further Pearson’s correlation analysis using NCI’s COMPARE algorithm generated a Pearson’s correlation co-efficient between 0.6 and 0.83 for OSW-1 vs. cephalostatin 1 suggesting that they had related mechanisms of action and shared cellular targets (67, 69). Structure activity relationship (SAR) analysis by Guo et al., identified that OSW-1 was analogous to one-half of the cephalostatin molecule with C22-oxocarbenium ions playing a common role in the bioactivity of both OSW-1 and cephalostatin (68). Structural studies also revealed that the acetyl (Ac) and the 4-methoxybenzoyl (MBz) groups on the disaccharide moiety of OSW-1 were essential as their removal diminished its cytotoxicity by 1000 fold (67, 70). In addition, the steroidal aglycone was critical to OSW-1’s potent cytotoxicity since SAR analysis indicated that substituting the
aglycone group on the steroidal backbone of OSW-1 with a totally disparate synthetic aglycone, while still retaining the disaccharide moiety, resulted in the compound not exhibiting any cytotoxic effects even at a concentration of 10 µM (66, 68, 71, 72). Thus both the disaccharide and aglycone groups are important to the antineoplastic activity of OSW-1. The first chemical synthesis of OSW-1 was reported by Deng et al. (1999), with an overall yield of 6% and 14 steps (73). Later, Yu et al. (2002), published an improvised synthesis procedure which reduced the total chemical synthesis steps to 10 and improved the overall yield to 28% (74). These findings inspired several studies on the synthesis and in vitro cytotoxic activity of OSW-1 analogues. Of specific interest to this dissertation, is the synthesis of biotinylated OSW-1 which is 10-times less toxic to cells than OSW-1 and may be used as a tool to identify the cellular interacting partners of OSW-1 (75).
**Figure 1.3: Structure of OSW-1.**

Chemical formula: C_{47}H_{66}O_{15}

Molecular weight: 873.03


Despite numerous studies on the chemical synthesis, bioactivity and structure activity relationship (SAR) of OSW-1 and its analogues, the mechanisms underlying the antitumor effect of this inhibitor are still not completely understood (67, 73-79). Tamura et al., demonstrated that OSW-1 directly inhibited gene expression of the ovarian steroidal limiting enzyme, cholesterol side chain cleavage cytochrome P-450 (p450scc), leading to suppression of ovarian estradiol secretion and granulosa cells proliferation in the ovary (80). Mechanistic studies from our lab revealed that OSW-1 inhibited the sodium-calcium exchanger 1 (NCX1) on the plasma membrane of leukemia cells, leading to cellular calcium overload, cytochrome c release and mitochondria dependent apoptosis (58). The mitochondrial respiration deficient human leukemia cells (HL60 C6F) were less sensitive to OSW-1 than the parental HL60 cells, suggesting that OSW-1 mediated cell death was through mitochondria dependent mechanisms. Burgett et al. (2011), identified the oxysterol binding protein (OSBP) and its closest paralog, OSBP-related protein 4L (ORP4L) as cellular binding targets of OSW-1 and cephalostatin, thus implying a role for these compounds in signal transduction, lipid transport and lipid metabolism (69). OSW-1 also induced necroptotic death in hepatocellular carcinoma through inhibition of cell invasiveness, angiogenesis, cell polarity and adhesion signaling, in addition to promoting mitochondria dependent apoptosis (81). Recently Yamada et al., identified endoplasmic reticulum (ER), golgi apparatus and to a lesser extent mitochondria as putative subcellular sites of OSW-1 localization (82).

Atomic force microscopy studies by Yokoyama et al. (2005), identified that OSW-1 distributed in the ganglioside GM3-phospholipid membrane monolayer in cells (83). Further, a strong attractive interaction between OSW-1 and GM3 was observed which seemed to be
related to the potent activity of OSW-1 against cancer cells. Specifically, treatment of B16-F0 melanoma cells with OSW-1 encapsulated in ganglioside GM3 liposomes led to a complete inhibition of cell viability within 48 h (57). Interestingly, Triptolide, a structural analogue of OSW-1, suppressed the gene expression of GD3 synthase (GD3S), the rate limiting enzyme for GD3 biosynthesis, in melanoma and breast cancer cells (24, 56). Gangliosides are glycosphingolipids containing sialic acids which have been reported to be overexpressed in carcinomas of the lung, breast, neuroblastoma and melanoma (24, 53, 54, 84-86). Ganglioside, GD3 is overexpressed in melanoma and is a well characterized therapeutic target (21).

These research findings describe a role for OSW-1 in ganglioside biology and mitochondria dependent cell signaling. Through my PhD research, I sought to investigate the effect of OSW-1 on ganglioside GM3/GD3 expression and mechanisms of mitochondria dependent cell death in melanoma cells. Further, I conducted mechanistic studies to delineate the molecular mechanisms underlying the therapeutic selectivity of OSW-1 towards melanoma cells. The results and discussion in Chapter 3 focus on the important outcomes of my research with the antitumor agent OSW-1 in melanoma.

1.2.3 Background information on PEITC

The natural product β-Phenylethyl Isothiocyanate (PEITC) is an active metabolite of cruciferous vegetables such as broccoli, cauliflower and watercress (87). It exhibits chemopreventive properties by inhibiting the activity of the transcription factor, Nuclear Factor κB (NF-κB), which plays an important role in inflammation, cancer progression and survival. NF-κB is regulated by the protein IκBα, which sequesters NF-κB in the cytosol thus preventing its nuclear translocation and activation of downstream pro-inflammatory and
oncogenic genes (88). IκBα is a well characterized target of PEITC in prostate cancer (89). PEITC stabilizes IκBα expression thus inhibiting NF-κB activation, nuclear translocation of p65, and promotes suppression of NF-κB mediated VEGF, cyclin D1, Bcl-xL, iNOS and COX-2 protein expression (89-91).

Numerous studies have reported that PEITC induces apoptosis in cancer cells, both in
in vitro and in vivo settings (63, 87, 92-94) . This apoptosis induction by PEITC was characterized by G2-M cell cycle arrest, inhibition of the anti-apoptotic proteins, Bcl-xL, Bcl-2 and Mcl1 and caspases 8 and 9 cleavage in prostate cancer and leukemia cells (95). Mi et al., identified tubulin as an important binding target of PEITC in A549 lung cancer cells (96). This study demonstrated that the covalent binding of PEITC and other isothiocyanates to the tubulin proteins leads to disruption of microtubule polymerization, cell cycle arrest, down regulation of cyclins A, D and E and cell death (96, 97). Interestingly, apoptosis initiation in HT-29 colon cancer cells by PEITC was dependent upon activation of the MAP kinase, JNK. Consequently SP600125, an anthrapyrazolone inhibitor of JNK suppressed apoptosis by PEITC (98).
Figure 1.4: Structure of PEITC

Chemical formula: C₉H₉NS

Molecular weight: 163.24

PEITC mediated cytotoxicity in cancer cells was unique because of two observations:
(a) PEITC treatment in cells led to excessive reactive oxygen species (ROS) generation (63, 64, 94, 100-102). Recent studies have attributed this ROS production to two mechanisms – glutathione depletion and inhibition of oxidative phosphorylation by PEITC. This ROS stress was of functional significance and correlated with cell death in cancer cells. In addition, the ROS induction by PEITC was instrumental in ameliorating drug resistance to clinically used anticancer agents such as Gleevec, Vorinostat and Fludarabine in leukemia cells.
(b) The cell death induced by PEITC was limited towards malignant cells with little cytotoxicity against normal cells (63, 64, 94, 103). Previous studies in our lab demonstrated that this preferential PEITC-cytotoxicity was due to altered redox biology between normal and cancer cells. Mitochondrial respiration deficient Rho-0 prostate cancer cells were also resistant to the antiproliferative activity of PEITC suggesting that the therapeutic selectivity of PEITC relies on mitochondria dependent mechanisms.

My PhD dissertation was focused on investigating the mechanisms of cytotoxicity and therapeutic selectivity of PEITC against melanoma cells. I had been particularly interested in examining if the redox differences between normal melanocytes and melanoma cells could be used as a therapeutic basis to preferentially eliminate melanoma cells. Further, mechanistic studies were undertaken to characterize the utility of PEITC to combat drug resistance to clinically used melanoma drugs such as Temozolomide and Carmustine. The results from my study on the anticancer activity of PEITC in melanoma have been discussed in chapter 4.
1.3 Research hypothesis and specific aims

The central research hypothesis for my PhD dissertation was - alterations in reactive oxygen species (ROS) and lipid metabolism in melanoma cells, as compared to normal melanocytes, may serve as a biochemical basis to preferentially kill malignant melanoma cells using proper pharmacological agents such as OSW-1 and PEITC.

To address this hypothesis I identified the following two specific aims:

1. To investigate altered lipid metabolism as the biological basis to preferentially eliminate melanoma cells using OSW-1.

2. To investigate ROS stress as a biochemical basis to selectively kill melanoma cells using PEITC.

In order to effectively achieve both of these specific aims, each specific aim was further divided into three sub aims. Experiments were designed to investigate each of these sub aims. The specific aims and the corresponding sub aims have been listed below:

Specific aim 1: To investigate altered lipid metabolism as the biological basis to preferentially eliminate melanoma cells using OSW-1.

a) To determine the effect of OSW-1 on ganglioside expression in melanoma cells.

b) To investigate the effect of OSW-1 on mitochondrial functionality in melanomas.

c) To characterize the molecular mechanisms underlying the therapeutic selectivity of OSW-1 towards melanoma cells.

Specific aim 2: To investigate ROS stress as a biochemical basis to selectively kill melanoma cells using PEITC.

a) To determine the antiproliferative effect of PEITC in melanoma cells.
b) To characterize the underlying redox mechanisms that are pivotal to PEITC’s activity in melanoma cells.

c) To elucidate therapeutic strategies to combat drug resistance in melanoma using PEITC.

The following chapters describe the project design, results obtained, key conclusions from my study and the future directions of my research.
Chapter 2: Materials and Methods

2.1 Chemicals and reagents

The compound OSW-1 was kindly provided by Dr. Zhendong Jin from the University of Iowa (Iowa City, Iowa). B-Phenylethyl Isothiocyanate (PEITC) was purchased from Sigma-Aldrich (St. Louis, MO). The original stock solutions of OSW-1 and PEITC were prepared in DMSO. For all experiments, OSW-1 and PEITC were diluted in the media at a concentration of 0.1% or less of the solvent DMSO. The fluorescent dyes Rhodamine 123, calcium green-1 AM, mitotracker red CMXRos, Dihydroethidium and mitoSOX red mitochondrial superoxide indicator were purchased from Life Technologies (Grand Island, NY). Annexin V-FITC and the 10X binding buffer were purchased from BD Pharmingen (San Jose, CA). 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), Giemsa stain, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS), Cyclosporin A (CsA), 3-Methyladenine (3-MA), Chloroquine (CQ), Triptolide, and propidium iodide (PI) were purchased from Sigma-Aldrich (St. Louis, MO).

2.2 Cell culture

The melanoma cell lines A375P, A2058, WM115 were purchased from American Type Culture Collection (ATCC, Manassas, VA). A375SM, WM35, WM46, MEWO, MEL624, SK-MEL-2, UCSD-354L, and Hs294T cell lines were purchased from the characterized cell line core at University of Texas MD Anderson Cancer Center (Houston, TX); these cell lines were validated at the core facility by STR finger printing. The normal human epithelial melanocytes (NHEM) were purchased from Promocell (Heidelberg, Germany). NHEM cells were cultured in M254CF medium (Life Technologies, Long Island, NY) supplemented with PMA-free human melanocyte growth supplement-2 (Life Technologies, Long Island, NY).
and were grown at 37 °C, 5% CO$_2$ in a cell culture incubator. MEF VDAC1-/- cells were a generous gift of Dr. William Craigen (Baylor College of Medicine, Houston, TX) and were cultured in DMEM medium supplemented with 10% heat inactivated fetal bovine serum (FBS) and sodium pyruvate. A375P, A375SM, A2058 cells cultured in DMEM medium (Mediatech, Manassas, VA) supplemented with 10% heat inactivated FBS were grown in a cell culture incubator with 5% CO$_2$ at 37 °C in humidified air. WM35, WM46, WM115, MEWO, MEL624, SK-MEL-2, UCSD-354L, and Hs294T cells were grown in RPMI 1640 medium (Mediatech, Manassas, VA) supplemented with 10% heat inactivated FBS in a cell culture incubator with 5% CO$_2$ at 37 °C in humidified air.

2.3 MTT assay for cell viability

Cell growth inhibition induced by OSW-1 and PEITC was assayed by performing the MTT assay as previously described (104). Briefly, melanoma cells, seeded in 96-well plates, were treated with log-scale serial diluted concentrations of OSW-1 and PEITC. After 72 h of incubation, 50 µl of MTT reagent per well was added for 4h. The cell media was then aspirated, formazan precipitates dissolved in DMSO and absorbance at 570 nm was measured in a Multiskan MK3 microplate-reader (Thermo Labsystem, Franklin, MA). The 50% inhibitory concentration (IC$_{50}$) values were then computed using GraphPad Prism (GraphPad Software, Inc. La Jolla, CA).

2.4 Colony formation assay

The colony formation assay was performed as described previously (105). Briefly, melanoma cells (5000 cells/well) were seeded in a 6-well plate and treated with varying concentrations of inhibitors for 2 weeks. The cells were then fixed with methanol/acetic acid
(10:1) solution, and stained with Giemsa (Sigma-Aldrich, St. Louis, MO). The cells were photographed and the colonies counted.

2.5 Mitochondrial membrane potential measurement by Rhodamine 123 staining

Rhodamine 123 assay was used to monitor the integrity of mitochondria following drug treatments as previously described (106, 107). Melanoma cells or normal melanocytes were treated with OSW-1, PEITC or other inhibitors for the indicated time points and then incubated in 0.5 μM rhodamine 123 dye during the last 1 h of drug treatment. The cells were then dissociated, washed twice with PBS and ∆Ψm measured using a BD Biosciences FACSCalibur flow cytometer (Mountain View, CA). Data analysis was performed using FlowJo (TreeStar Inc., Ashland, OR).

2.6 Annexin-V/PI analysis for apoptosis

Cell death by apoptosis/necrosis was measured by flow cytometric analysis of cells dual stained for annexin V-FITC and propidium iodide (108). Briefly, melanoma cells grown in 6-well plates were treated with inhibitors for the indicated time points, harvested and stained with annexin V-FITC for 15 minutes, washed and then stained for propidium iodide. Cell death was measured using a BD Biosciences FACSCalibur flow cytometer (Mountain View, CA) and the results were analyzed using the FlowJo software (TreeStar Inc., Ashland, OR).

2.7 Electron microscopy

The electron microscopy experiment was performed at the high resolution electron microscopy facility (core grant CA16672) at the MD Anderson cancer center, as per the core’s prescribed protocol. Briefly, melanoma cell samples were fixed with a solution containing 3% glutaraldehyde plus 2% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.3, for 1 h. After fixation, the samples were washed and treated with 0.1% Millipore-filtered
cacadylate buffered tannic acid, post-fixed with 1% buffered osmium tetroxide for 30 min, and stained en bloc with 1% Millipore-filtered uranyl acetate. The samples were dehydrated in increasing concentrations of ethanol, infiltrated, and embedded in LX-112 medium. The samples were polymerized in a 70 ºC oven for 2 days. Ultrathin sections were cut in a Leica Ultracut microtome (Leica, Deerfield, IL), stained with uranyl acetate and lead citrate in a Leica EM Stainer, and examined in a JEM 1010 transmission electron microscope (JEOL, USA, Inc., Peabody, MA) at an accelerating voltage of 80 kV. Digital images were obtained using the AMT Imaging System (Advanced Microscopy Techniques Corp, Danvers, MA).

2.8 Immunoblotting

Immunoblotting was performed to assess protein expression in drug treated and control cell lysates (109). Proteins were separated on 10-15% SDS-PAGE gels, transferred onto nitrocellulose membranes and immunoblotted using the following commercially available antibodies: β-actin (Calbiochem, San Diego, CA), LC3B (Novus Biologicals, Littleton, CO), VDAC1 (Abcam, Cambridge, MA), phosphorylated and total ERK1/2, and Caveolin (Cell Signaling Technology, Inc., Danvers, MA). Membranes were then incubated with appropriate horseradish peroxidase conjugated secondary antibodies and the signal visualized using supersignal enhanced chemiluminescence kit (Thermo Scientific, Rockford, IL).

2.9 Detection of ganglioside expression by flow cytometry

Assessment of ganglioside expression changes following drug treatment was performed by flow cytometry as previously reported (24). Melanoma cells were treated with OSW-1 or other inhibitors for the indicated time durations, harvested, washed twice in PBS and stained for 30 min, 4 ºC, with the following commercially available antibodies: PE-anti human ganglioside GD2 (Biolegend, San Diego, CA), disialoganglioside GD3 (BD Biosciences, San
Jose, CA) and anti GM3 (NeuAc) antibody (Cosmo Bio USA, Inc., Carlsbad, CA). Samples stained with PE-conjugated GD2 antibody were washed and directly assayed using the BD Biosciences FACSCalibur flow cytometer (Mountain View, CA). Samples probed with GM3 and GD3 antibodies were washed, stained with a FITC-conjugated secondary antibody for another 30 min, 4 °C and the GM3/GD3 expression was measured by flow cytometry. All data analysis was performed using FlowJo (TreeStar Inc., Ashland, OR).

2.10 Lipid raft / ganglioside enriched microdomain (GEM) isolation

The lipid raft fractions were prepared using a detergent extraction protocol described by Mitsuda et al (110). Two T-175 flasks of 80-90% melanoma cells were used for each preparation. Briefly, cells were harvested, washed twice in ice-cold PBS and suspended in 1 mL of TNE buffer (1% Triton X-100, 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EGTA). The cells were then dounce homogenized 24 times, mixed with equal volume of 80% sucrose and placed at the bottom of ultra-clear centrifuge tubes (Beckman Coulter, Brea, CA). 2 mL of 30% sucrose solution in TNE buffer without Triton X-100 and 1 mL of 5% sucrose solution in TNE buffer without Triton X-100 were carefully laid on top of the preparation. The samples were then centrifuged at 105,000 × g in a Beckman Coulter Optima XPN-100 ultracentrifuge (Brea, CA) for 16 h at 4°C. From the top of the gradient, 9 fractions of 0.5 mL sample were collected and assayed by SDS-PAGE or dot blot.

2.11 Real time RT-PCR

To analyze mRNA expression of GD3S, GD2S, FASN and HMGCR, real time RT-PCR was performed as previously described (111). Briefly, total RNA was extracted from melanoma cells using the RNeasy mini kit (Qiagen, Valencia, CA). 2 µg of RNA was subjected to reverse transcription with random hexamer primers utilizing the revertaid first strand cDNA
synthesis kit (Thermo Scientific, Pittsburg, PA). Real time PCR was performed on an Applied Biosystems Viia7 machine using SYBR green with the following cDNA primers: FASN forward: 5’ – CGCTCGGCATGGCTATCT-3’; FASN reverse: 5’-CTCGTTGAAGAACGCATCCA-3’; HMGCR forward: 5’-GGCCCAGTTGTGCCTTCTT-3’; HMGCR reverse: 5’-CGAGCCAGGCTTTCACTTCTCTT-3’; GD3S forward: 5’-TGTGGTCCAGAAAGACATTTGTGGACA-3’; GD3S reverse: 5’-TGGAGTGAGGTATCTTCACATGGGTCC-3’; GD2S forward: 5’-GACAAGCCAGAGCGCGTTA-3’ and GD2S reverse: 5’-TACTTGAGACACGGCCAGGTT-3’. All experiments were performed with three biological replicates and three technical replicates and the results have been reported as the mean of the biological replicates plus or minus standard error.

2.12 Immunofluorescence staining and confocal microscopy

Immunofluorescence staining and confocal microscopic analysis were performed based on previously reported protocols (10, 112). Melanoma cells were cultured on sterilized glass coverslips, treated with OSW-1 and incubated in 50 nM mitotracker red for 25 min. Cells were washed twice with PBS, fixed with 3.7% paraformaldehyde in medium at 37 °C for 10 min, permeabilized using 0.3% Triton X-100 for 10 minutes, washed and then blocked with 4% FBS for 1 h. The samples were incubated overnight with the disialoganglioside GD3 (BD Biosciences, San Jose, CA) and/or VDAC1 (Proteintech, Chicago, IL) antibodies, washed and incubated in FITC- and rhodamine red X-conjugated secondary antibodies (Jackson Immunoresearch Laboratories, Inc., West grove, PA) for 1 h. The samples were then washed and mounted onto glass slides using the Ultracruz mounting medium (Santa
Cruz Biotechnology, Dallas, TX). Images were taken by NIKON Eclipse TE2000 confocal microscope and analyzed using the Nikon EZ-C1 software.

2.13 Cytosolic calcium detection

Cytosolic calcium levels were measured as previously described (109). Briefly, melanoma cells were treated with OSW-1 or other inhibitors for the indicated time durations, harvested, washed twice in PBS and stained with calcium green fluorescent dye for 30 min at 4 °C. Cytosolic calcium levels were detected in the FL-1 channel of the BD Biosciences FACSCalibur flow cytometer (Mountain View, CA).

2.14 Detection of cellular free radical expression by staining with the fluorescent dyes DCFDA, DAF-FM, Dihydroethidium and mitoSOX mitochondrial superoxide indicator

Flow cytometric measurements using the CM-H2-DCFDA, DAF-FM or Dihydroethidium fluorescent probes was performed to detect free radical levels in cells (63, 113). Briefly, melanoma cells were treated with OSW-1, PEITC or other inhibitors for the indicated time points and then incubated in 1 µM mitosox red or CM-H2-DCFDA or DAF-FM or Dihydroethidium during the last 1 h of drug treatment. The cells were then dissociated, washed twice with PBS and the free radical expression levels were measured using a BD Biosciences FACSCalibur flow cytometer (Mountain View, CA). Data analysis was performed using FlowJo (TreeStar Inc., Ashland, OR).

2.15 siRNA transfection

Tranfection of VDAC1 and control siRNAs was performed as previously reported (114). Briefly, A375SM melanoma cells were plated in a 6-well plate and cultured until 50% confluency, at which point, they were transfected with 50 nM silencer select siVDAC1 (ID: s14769) or the negative control siRNA (Life Technologies, Long Island, NY) by the
Lipofectamine RNAiMAX reagent (Life Technologies, Long Island, NY). Western blot was performed to determine VDAC1 expression 48 h post-transfection.

2.16 Glutathione assay

A glutathione assay kit (Cayman Chemicals, Ann Arbor, MI) was used to detect cellular glutathione levels (63, 94, 113). Cell extracts were obtained by sonication (power setting - 4, 10 s 3X) and deproteination as per the manufacturer’s guidelines. The reaction product of glutathionylated 5, 5’-dithiobis-(2-nitrobenzoic acid) was detected spectrophotometrically using the Multiskan MK3 microplate-reader (Thermo Labsystem, Franklin, MA) at 450 nm. A standard curve for glutathione was generated and the glutathione concentration in each sample was calculated. The Bicinchoninic Acid (BCA) assay was performed to determine sample protein concentration and to normalize the glutathione concentration across samples. GraphPad Prism (GraphPad Software, Inc. La Jolla, CA) was used to perform all statistical analysis.

2.17 Reverse phase protein array (RPPA) analysis

The RPPA experiments were performed at the functional proteomics reverse phase protein array core (NCI # CA16672) at the MD Anderson cancer center, following the core’s experimental protocols (115). Briefly, melanoma cells lysates were extracted in the following lysis buffer: 1% Triton X-100, 50mM HEPES, pH 7.4, 150mM NaCl, 1.5mM MgCl₂, 1mM EGTA, 100mM NaF, 10mM Na pyrophosphate, 1mM Na₃VO₄, 10% glycerol, containing freshly added protease and phosphatase inhibitors. Cellular proteins were denatured by 1% SDS (with beta-mercaptoethanol) and diluted in five 2-fold serial dilutions in dilution buffer (lysis buffer containing 1% SDS). Serial diluted lysates were arrayed on nitrocellulose-coated slides (Grace Biolab) by Aushon 2470 Arrayer (Aushon BioSystems). Sample slides were
probed with 217 primary antibodies and the corresponding biotin-conjugated secondary antibody. The signal obtained was amplified using a Dako Cytomation-catalyzed system (Dako) and visualized by DAB colorimetric reaction. The slides were scanned, analyzed, and quantified using a customerized-software Microvigene (VigeneTech Inc.) to generate spot intensity. Each dilution curve was fitted with a logistic model (“Supercurve Fitting” developed by the Department of Bioinformatics and Computational Biology in MD Anderson Cancer Center, “http://bioinformatics.mdanderson.org/OOMPA”). The protein concentration of each set of slides was then normalized by median polish, which was corrected across samples by the linear expression values using the median expression levels of all antibody experiments to calculate a loading correction factor for each sample. Heat maps were generated in Cluster 3.0 (http://www.eisenlab.org/eisen/) as a hierarchical cluster using Pearson Correlation and a center metric. The resulting heatmap was visualized in Treeview (http://www.eisenlab.org/eisen/).

2.18 Statistical analysis

Statistical analysis was computed using GraphPad Prism version 6 (La Jolla, CA). Data were graphed as mean ± standard error (SEM) for experiments performed in triplicates unless noted differently. A standard student’s two tailed t-test was performed to determine statistically significant difference between samples at a 95% confidence interval. In all cases, **** denotes p < 0.0001, *** denotes p < 0.001, ** denotes p < 0.01 and * denotes p < 0.05.
Chapter 3: The anti-tumor agent, OSW-1, effectively eliminates melanoma cells through ganglioside GD3 and mitochondrial VDAC1 dependent mechanisms

3.1 Introduction

The natural product OSW-1 is a highly potent anticancer agent. Previous studies both from our lab and others demonstrated that OSW-1 inhibited cancer cell viability at sub-nanomolar concentrations with minimal toxicity towards normal cells (67, 81). Further, it was effective in eliminating various cancer cells including leukemia, glioblastoma and pancreatic cancers that were resistant to other clinically used agents such as taxol, adriamycin and fludarabine (59, 67).

Previous research efforts from our lab revealed that OSW-1 inhibited the sodium-calcium exchanger 1 (NCX1) on the cell membrane of HL-60 leukemia cells, leading to cytosolic calcium elevations, caspase 3 activation and mitochondria dependent apoptosis (58). However, the mechanisms of OSW-1 induced cytotoxicity in solid tumors still remain to be elucidated. Recent studies demonstrated that steroidal compounds such as Estradiol and Triptolide inhibited ganglioside 3 synthase (GD3S) expression in cancer cells, thus causing cell death (56, 116). Further, a strong interaction between OSW-1 and GM3 gangliosides in melanoma cells has been noted (83).

In this study, we investigated the effect of OSW-1 on ganglioside expression in melanoma. Effect of OSW-1 on the ganglioside synthesis regulatory enzyme, GD3 synthase (GD3S) was also examined. Our results indicate that OSW-1 suppresses GD3 synthase gene expression in melanoma cells, leading to inhibition of gangliosides, GD3, GD2 and subsequent cell death by autophagy. Further, OSW-1 promoted interaction between GD3 and mitochondrial VDAC1 in melanoma cells. Significantly, OSW-1 upregulated VDAC1 expression in
melanoma, leading to loss of calcium homeostasis and cell death. These OSW-1 induced alterations in ganglioside and VDAC1 expression play a critical role in mediating the selectivity of OSW-1 to malignant melanoma cells.

3.2 Results

3.2.1 Cytotoxic effect of OSW-1 in melanoma

Previous findings, both from our lab and others, demonstrate that OSW-1 inhibits cell viability in multiple tumor cell lines including Leukemia, brain, colorectal, hepatic and pancreatic cancers at nanomolar concentrations (59, 69). To investigate the effect of OSW-1 on melanoma cell proliferation, we first performed an MTT assay with multiple melanoma cell lines. As illustrated in figure 3.1, OSW-1 is highly potent in inhibiting melanoma cell growth with an average half-maximal inhibitory concentration (IC$_{50}$) of 0.178 nM. A subsequent colony formation assay of WM35 cells treated with increasing concentrations of OSW-1 indicated that only 36% of cells formed colonies at a concentration of 0.1 nM, with a complete inhibition of colony formation at higher concentrations (figure 3.2 A, B). Because many of the metastatic melanoma cells did not form colonies on 6-well plates, we adapted the MTT assay for long term (figure 3.2 C, D). Melanoma cells were incubated in log-scale increasing concentrations of OSW-1 for 8 days. MTT assay was performed in these cells to elucidate the effect of OSW-1 on cell proliferation (figure 3.2 C). As indicated in figure 3.2 D, OSW-1 inhibited melanoma cell growth at sub-nanomolar concentrations with an average IC$_{50}$ of 0.058 nM.
Figure 3.1:

(A) Melanoma cells were treated with log-scale serial diluted concentrations of OSW-1 for 72 h, and the antiproliferative effect was determined by performing the MTT assay. The cell viability curves were plotted. Each data point represents mean ± SEM of three independent measurements. (B) The 50% cell inhibitory concentration (IC$_{50}$) ± SEM for each cell line is indicated.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC$_{50}$ value (nM)</th>
<th>Mean IC$_{50}$ ± SEM (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A375P</td>
<td>0.164 ± 0.045</td>
<td></td>
</tr>
<tr>
<td>A375SM</td>
<td>0.105 ± 0.024</td>
<td></td>
</tr>
<tr>
<td>HS294T</td>
<td>0.027 ± 0.007</td>
<td></td>
</tr>
<tr>
<td>MEL624</td>
<td>0.072 ± 0.010</td>
<td></td>
</tr>
<tr>
<td>SK-MEL-2</td>
<td>0.421 ± 0.218</td>
<td></td>
</tr>
<tr>
<td>UCSD-354L</td>
<td>0.218 ± 0.050</td>
<td></td>
</tr>
<tr>
<td>WM35</td>
<td>0.156 ± 0.024</td>
<td></td>
</tr>
<tr>
<td>WM46</td>
<td>0.097 ± 0.001</td>
<td></td>
</tr>
<tr>
<td>MEWO</td>
<td>0.343 ± 0.068</td>
<td>0.178 ± 0.043</td>
</tr>
</tbody>
</table>

Figure 3.1: Effect of OSW-1 on melanoma cell viability
Figure 3.2:

A

DMSO
0.1 nM OSW-1
0.3 nM OSW-1
1 nM OSW-1

B

Number of colonies

DMSO
0.1 nM OSW-1
0.3 nM OSW-1
1 nM OSW-1

C

\% Cell Survival

OSW1 Concentration (pM)

MEWO
WM46
WM35

D

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC₅₀ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A375P</td>
<td>0.052 nM</td>
</tr>
<tr>
<td>A375SM</td>
<td>0.045 nM</td>
</tr>
<tr>
<td>UCSID354L</td>
<td>0.039 nM</td>
</tr>
<tr>
<td>SK-MEL-2</td>
<td>0.056 nM</td>
</tr>
<tr>
<td>MEWO</td>
<td>0.037 nM</td>
</tr>
<tr>
<td>WM46</td>
<td>0.041 nM</td>
</tr>
<tr>
<td>WM35</td>
<td>0.137 nM</td>
</tr>
</tbody>
</table>
Figure 3.2: OSW-1 inhibits colony formation in melanoma cells.

(A) and (B) WM35 cells treated with 0.1, 0.3 and 1 nM OSW-1 for 2 weeks were fixed, stained with Giemsa and the number of colonies formed were counted. **** denotes p < 0.0001. (C) Melanoma cells were treated with log-scale serial diluted concentrations of OSW-1 for 8 days, and the antiproliferative effect of OSW-1 was determined by performing the MTT assay. The cell viability curves were plotted. Each data point represents mean ± SEM of three independent measurements. (D) The 50% cell inhibitory concentration (IC\textsubscript{50}) of each cell line in the long-term MTT assay is indicated.
Figure 3.3: Effect of OSW-1 on mitochondrial membrane potential ($\Delta \Psi_m$) in melanoma cells.

Melanoma cells were treated with either DMSO control or 1 nM OSW-1 for 72 h and the effect of OSW-1 on mitochondrial membrane potential was determined by rhodamine 123 staining and flow cytometric analysis. OSW-1 induced collapse of membrane potential in melanoma cells with an average DMSO vs. OSW-1 p-value = 0.007. **** denotes p < 0.0001, *** denotes p < 0.001, ** denotes p < 0.01 and * denotes p < 0.05.
Mitochondrial dysfunction has been implicated in cell death signaling in cells. Maintenance of proper mitochondrial membrane potential ($\Delta \Psi_m$) is required for cellular homeostasis and normal functioning of cells as it drives ATP synthesis and oxidative phosphorylation (117). However, an acute induction of mitochondrial membrane permeabilization (MMP) due to calcium overload, oxidative stress, inhibitors such as etoposide, arsenite, lonidamine etc. can lead to cell death (118-122). Hence we sought to investigate the effect of OSW-1 on mitochondrial transmembrane potential ($\Delta \Psi_m$) across a panel of eleven melanoma cell lines (figure 3.3). OSW-1 caused an average decrease in mitochondrial membrane potential in melanoma cells by 48%, as compared to the DMSO treated control cells (control vs. OSW-1, p-value = 0.007, n = 11). Interestingly, SK-MEL-2 cells, which have a slightly higher IC$_{50}$ (0.421 nM, figure 3.1 B) compared to other melanoma cells, also exhibited decreased sensitivity to OSW-1 in the mitochondrial membrane potential measurement assay, thus pointing towards the heterogeneity of drug response to OSW-1 in melanomas.

3.2.2 OSW-1 triggered cell death in melanoma is associated with autophagy

Autophagy is an evolutionarily conserved lysosomal degradation pathway that is essential for cellular homeostasis and response to stress conditions (123, 124). Previous studies have classified autophagy as a non-apoptotic cell death characterized by enhanced autophagic flux and accumulation of autophagic vacuoles in cells (125, 126). Transmission electron microscopy results showed formation of numerous autophagic vacuoles in WM35 cells treated with OSW-1 (figure 3.4 A). This autophagic vacuole formation increased with time with about a 9- and 21-fold increase in the observed number of autophagic vacuole per cell at 24 h and 48 h of 1 nM OSW-1 treatment (figure 3.4 B). The autophagosome-
associated lipidated form of LC3B (LC3B-II) can be differentiated from the cytosolic free isoform (LC3B-I) by immunoblotting (127). Consistent with the morphological findings, a time dependent increased expression of the lipidated LC3B (LC3B-II) isoform was observed in cells treated with OSW-1 (figure 3.5).
Figure 3.4: OSW-1 promotes autophagic vacuole formation in melanoma cells.

(A) Transmission electron microscopy images of WM35 cells treated with 1 nM OSW-1 for 24 h and 48 h. The black arrows indicate the autophagic vacuole formation induced by OSW-1. (B) The number of autophagic vacuoles formed per cell for each sample were counted and the mean ± SEM of three independent measurements are plotted as bar graphs. ** denotes p < 0.01.
Figure 3.5: Effect of OSW-1 on the autophagy marker, LC3B expression.

WM35 cells were treated with 1 nM OSW-1 for 12, 24 and 48 h and immunoblotting was performed to examine lipidation of the autophagy marker protein LC3B.
Figure 3.6: Autophagy induction is crucial to cell death induction by OSW-1.

(A) WT and Atg5-/− MEF cells were treated with varying concentrations of OSW-1 for 72 h and the cytotoxic effect was determined by the MTT assay, as described in materials and methods. Percent cell survival corresponding to each OSW-1 concentration was plotted. (B) WM35 cells preincubated with 1mM 3-methyl adenine for 1 h were treated 1nM OSW-1 for 48 h. Effect of OSW-1 ± 3-MA combination on cell viability was evaluated by annexin-V/PI analysis and is represented as bar graphs normalized to the DMSO control. ** denotes p < 0.01.
The autophagy related protein 5, Atg5, is an essential autophagy protein, which conjugates with Atg12 to form a multimeric structure that is crucial for autophagosome formation (128, 129). Hence, we employed Atg5 knock out (Atg5-/-) MEF cells to characterize the role of autophagy in mediating the cell inhibitory effect of OSW-1. MTT results from WT and Atg5-/- MEF cells treated with log-scale incremental concentrations of OSW-1 for 72 h demonstrated that the Atg5-/- cells exhibited decreased sensitivity to OSW-1 (figure 3.6 A). 1 nM OSW-1 effectively inhibited cell viability by approximately 80% in MEF WT cells while an approximate 75% of MEF Atg5-/- cells were still viable at this concentration. Nevertheless, higher concentrations of OSW-1 killed both MEF WT and Atg5-/- cells. In complementation to this observation, the autophagy inhibitor, 3-Methyl Adenine (3-MA), partially rescued WM35 cells from the cytotoxic effect of OSW-1 (130, 131). As shown in figure 3.6 B, OSW-1 treatment for 48 h abolished cell survival to 30%. However, combination of OSW-1 with 3-MA reversed cell survival to 60.76%. These findings not only delineate the significance of autophagy in OSW-1 induced elimination of melanoma cells, but also allude to the fact that there might also be simultaneous alternate cell death mechanisms facilitating OSW-1’s activity.

Interestingly, combination of OSW-1 with the lysosomotrophic agent Chloroquine led to rapid apoptotic cell death. Chloroquine inhibits late phase autophagy by preventing the fusion of the autophagosomes to lysosomes (132). Because of this mechanism of action, chloroquine has found utility in sensitizing multiple cancer cells including hepatocellular and cervical cancers to radiation and drugs like cisplatin and oxaliplatin (133, 134). Combination of OSW-1 with chloroquine produced approximately 48% (normalized to control) increased cell death in WM35 cells as compared to OSW-1 treatment alone (figure 3.7 A). To evaluate
the type of drug interaction, we performed combination index (CI) analysis of different combination regimens of OSW-1 (0.1, 0.3 and 1 nM, 48 h) and chloroquine (5, 10 and 20 µM, 48 h) in melanoma cells (135, 136). As described in figure 3.7 B, the combination regimen between OSW-1 and chloroquine was synergistic (CI <1) for seven of the nine combination treatments with 1 nM OSW-1 and 5 µM chloroquine showing the strongest effect (CI = 0.111). Interestingly, an antagonistic combination effect (CI >1) was observed on combining 20 µM chloroquine with 1 nM OSW-1 in WM35 cells.
Figure 3.7:

**A**

DMSO

1nM OSW-1

OSW-1 + CQ

**B**

<table>
<thead>
<tr>
<th>Chloroquine (µM)</th>
<th>OSW-1 (nM)</th>
<th>Combination Index (CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.1</td>
<td>1.067</td>
</tr>
<tr>
<td>5</td>
<td>0.3</td>
<td>0.494</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>0.111</td>
</tr>
<tr>
<td>10</td>
<td>0.1</td>
<td>0.768</td>
</tr>
<tr>
<td>10</td>
<td>0.3</td>
<td>0.533</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>0.184</td>
</tr>
<tr>
<td>20</td>
<td>0.1</td>
<td>0.714</td>
</tr>
<tr>
<td>20</td>
<td>0.3</td>
<td>1.039</td>
</tr>
<tr>
<td>20</td>
<td>1</td>
<td>1.349</td>
</tr>
</tbody>
</table>
Figure 3.7: Cytotoxic effect of OSW-1 ± Chloroquine drug combination in melanoma cells.

(A) 1 nM OSW-1 was added to WM35 cells pretreated with 10 µM chloroquine for 20 min. After 72 h incubation, cell viability was determined by annexin v/PI staining and flow cytometric analysis. The percentage of viable cells (negative for annexin V and PI) is indicated. (B) WM35 cells were treated with the indicated concentrations of OSW-1 ± CQ drug combination for 48 h and combination index analysis was performed using Compusyn software. The combination index (CI) values were computed. The following quantitative definitions, based on the Chou-Talalay theorem, were employed to define combination effect: CI < 1 - synergism, CI = 1 - additive effect and CI > 1 - antagonism.
Figure 3.8:
**Figure 3.8: Cytotoxic effect of OSW-1 ± Rapamycin drug combination in melanoma cells.**

(A) A375SM cells were treated with 1 nM OSW-1 ± 100 nM Rapamycin for 48 h and the effect on mitochondrial membrane potential was determined by rhodamine 123 analysis. (B) Bar graphs represent the mitochondrial membrane potential changes induced by A375SM and WM35 cells treated with 1 nM OSW-1 ± 100 nM Rapamycin for 48 h. (C) Effect of 1 nM OSW-1 ± 100 nM Rapamycin 48 h treatment on the viability of A375SM and WM35 cells was determined by annexin-V/PI analysis and is represented as bar graphs. ** denotes p < 0.01.
The mammalian target of rapamycin (mTOR) serves as a central regulator of autophagy by inhibiting phosphorylation of the autophagy proteins Atg13 and Unc51-like kinase (ULK), and preventing autophagosome formation (137-139). The antibiotic, Rapamycin inhibits mTOR thus acting as a potent autophagy inducer in multiple cell lines (140, 141). In our study, we found that rapamycin ameliorated the cytotoxic effect of OSW-1 in melanoma cells (figure 3.8). A375SM and WM35 cells were co-treated with 1 nM OSW-1 and 100 nM rapamycin for 48 h and the effect on mitochondrial transmembrane potential ($\Delta \Psi_m$) and cell death was assessed through flow cytometry by staining the cells with rhodamine 123 (figure 3.8 A, B) and annexin-FITC/PI (figure 3.8 C). As illustrated in figure 3.8 A and B, rapamycin partially reversed the mitochondrial membrane permeabilization induced by OSW-1. 1 nM OSW-1 caused a loss of membrane potential by 56.33% and 60.3% in A375SM and WM35 cells. However, rapamycin + OSW-1 treatment led to a reversal of this effect to 16.2% and 34.07% in A375SM and WM35 cells. Similarly, rapamycin also partially rescued A375SM and WM35 cells from the antiproliferation effect of OSW-1 (figure 3.8 C). These drug combination (OSW ± chloroquine/rapamycin) results elucidate the dual roles of autophagy in maintaining cellular homeostasis wherein though autophagy might start-off as a cell protective mechanism, sustained autophagy mediated by OSW-1 leads to melanoma cell killing.

3.2.3 Normal melanocytes are resistant to OSW-1 induced cytotoxicity

The promising antineoplastic effect of OSW-1 against melanoma cells led us to examine the effect of this compound in normal melanocytes. As shown in figure 3.9, OSW-1 induced a time dependent loss of mitochondrial potential in A375SM melanoma cells, while normal melanocytes were resistant to this effect of OSW-1. 1 nM OSW-1, 72 h treatment, did
not trigger significant mitochondrial membrane potential loss in normal melanocytes (17.4%; figure 3.9 A) compared to melanoma cells (n = 11, 52%; figure 3.3).

Figure 3.9:

Figure 3.9: Effect of OSW-1 on mitochondrial membrane potential in normal melanocytes vs. melanoma cells.

Mitochondrial membrane potential in normal melanocytes and A375SM cells treated with 1 nM OSW-1 for 48 h and 72 h was evaluated by rhodamine 123 staining as described in materials and methods.
Figure 3.10:

A

![Graph showing cell viability over time]

- Control
- 0.5 nM OSW-1
- 1 nM OSW-1
- 2 nM OSW-1

B

![Flow cytometry plots]

- A375SM
- A375P
- SKMel-2
- WM35
- MEWO
- UCSD 354L
- WM46
- NHEM

- DMSO
- OSW-1 48 h
- OSW-1 72h
- OSW-1 96 h

- Cell viability (normalized to control)
- 90.0 %
- 6.87 %
- 3.86 %
- 3.51 %
- 81.2 %
- 80.6 %
- 76.7 %
- 78.8 %
Figure 3.10: Selective killing of melanoma cells by OSW-1.

(A) Melanoma cells and normal melanocytes were treated with 0.5, 1 and 2 nM OSW-1 for 72 h and the effect on cell viability was determined by annexin V/PI analysis. Each data point represents the mean of three independent measurements. (B) Comparison of viability of A375SM cells and normal melanocytes treated with 1 nM OSW-1 for indicated time duration. Cell viability was determined by annexin V-FITC/PI staining. The numbers indicate the percentage of viable cells (negative for annexin V and PI).
We designed a follow-up time course experiment with 1 nM OSW-1 and performed an annexin-V/PI staining assay to determine the effect of OSW-1 on cell death. Normal melanocytes were resistant to the cytotoxic effect of OSW-1; 97.04% of normal melanocytes were still viable after OSW-1 treatment for 96 h as compared to only 3.9% of A375SM melanoma cells (figure 3.10 B). Figure 3.10 A represents the cell inhibitory effect of OSW-1 against multiple melanoma cell lines (n = 7) and normal melanocytes treated with increasing concentrations of OSW-1 for 72 h. The scatter plot while delineating the heterogeneous response of melanoma cells to OSW-1, also highlights the therapeutic selectivity of OSW-1 towards melanoma cells (1 nM OSW-1, average melanoma (n = 7) cell viability = 46.88%) with limited toxicity to normal melanocytes (1 nM OSW-1, average cell viability = 93.30%).

3.2.4 OSW-1 inhibits ganglioside GD3/GD2 expression in melanoma cells

Recent studies by Kwon et al and Battula et al demonstrated that Triptolide, a diterpenoid triepoxide, inhibits ganglioside GD3 and GD2 expression by repressing GD3 synthase (ST8Sia I) expression (24, 56). Since Triptolide bears structural similarity to OSW-1, we hypothesized that OSW-1 would inhibit gangliosides GD3 and GD2 expression by suppressing GD3 synthase (GD3S) expression. To test this hypothesis we assessed alterations in ganglioside expression after OSW-1 treatment of melanoma cells (figure 3.11). Flow cytometric analysis of WM35 and A375SM treated with OSW-1 and stained with ganglioside antibodies revealed that OSW-1 caused a moderate decrease in ganglioside GD3 (figure 3.11 A, B) and a more striking inhibition of GD2 (figure 3.11 C, D). This inhibition of ganglioside expression was time dependent, with GD2 inhibition starting as early as 3 h post OSW-1 treatment (figure 3.11 C, D). Figure 3.12 depicts the effect of OSW-1 on GD2 expression against a panel of melanoma cell lines. On average, a 24 h OSW-1 treatment of melanoma
cells reduced GD2 expression to 66.86% (DMSO vs. OSW-1, p-value = 0.0029). Further, this GD3 and GD2 inhibition correlated with an accumulation of ganglioside GM3 (figure 3.11 E, F). A 24 h 1nM OSW-1 treatment led to an increased expression of ganglioside GM3 by 178.2% and 127.4% in A375SM and WM35 melanoma cells (figure 3.11 F).
Figure 3.11:

A

GD3

24 h

12 h

6 h

3 h

DMSO

B

GD3-FITC fluorescence (% control)

A375SM

WM35

D

GD2

24 h

12

3 h

6 h

DMSO

DMSO

GM3

24 h

DMSO

GM3-FITC fluorescence (% control)

A375SM

WM35

P = 0.06
Figure 3.11: Effect of OSW-1 on ganglioside expression in melanoma.

(A), (C) and (E) A75SM cells treated with 1 nM OSW-1 for the indicated time points were analyzed for ganglioside GD3, GD2 and GM3 expression by flow cytometry as described in materials and methods. (B), (D) and (F) GD3, GD2 and GM3 ganglioside expression in A375SM and WM35 cells treated with 1 nM OSW-1 for the indicated time points was determined by flow cytometric measurements. Bar graphs represent mean ± SEM of percent ganglioside (GD3/GD2/GM3) inhibition from three independent experimental measurements. ** denotes p < 0.01 and * denotes p< 0.05.
Figure 3.12: Effect of OSW-1 on ganglioside GD2 expression in melanoma cells.

Melanoma cells were treated with either DMSO control or 1 nM OSW-1 for 24 h and the effect of OSW-1 on ganglioside GD2 expression was quantified. Bar graphs represent mean ± SEM from three independent experiments. OSW-1 induced inhibited GD2 expression in melanoma cells with an average DMSO vs. OSW-1 p-value = 0.0029. **** denotes p < 0.0001, *** denotes p < 0.001, ** denotes p < 0.01 and * denotes p< 0.05.
Real time PCR was performed in A375SM cells treated with 1 nM OSW-1 for 12h and 24 h. The effect of OSW-1 on the expression of GD3S, FASN and HMGCR genes was determined. Each experiment was performed with three technical replicates. The bar graphs represent the mean result from three biological replicates. **** denotes p < 0.0001, *** denotes p < 0.001, ** denotes p < 0.01 and * denotes p < 0.05.
Figure 3.14: Effect of OSW-1 on GD2 Synthase gene expression.

Real time PCR was performed in A375SM cells treated with 1 nM OSW-1 for 12h and 24 h. The effect of OSW-1 on the expression of GD2S gene was determined. * denotes p < 0.05.
Figure 3.15: Schematics of OSW-1 mediated modulation of ganglioside expression.

The model summarizes the effect of OSW-1 on ganglioside expression. OSW-1 suppresses the expression of GD3 synthase gene leading to inhibition of GD3, GD2 and accumulation of ganglioside GM3.
Figure 3.16: Combination of OSW-1 with Triptolide partially reverses the OSW-1 induced mitochondrial membrane potential loss in melanoma.

A375SM cells were treated with 1nM OSW-1 ± 20 nM Triptolide for 48 h and the effect on mitochondrial membrane potential was determined by rhodamine 123 analyses.
As shown in figure 3.15, ganglioside GD3 is produced from its precursor GM3 through a reaction catalyzed the enzyme GD3 synthase. Results from real-time PCR, performed to ascertain the effect of OSW-1 on GD3S mRNA expression, indicated that OSW-1 represses GD3S gene expression in a time dependent fashion (figure 3.13). Expression of GD2 synthase (GD2S) gene though initially suppressed by OSW-1, increased after 24 h of OSW-1 treatment (figure 3.14). Interestingly, combination of Triptolide with OSW-1 partially reversed the mitochondrial membrane collapse induced by OSW-1 (figure 3.16), suggesting that both OSW-1 and Triptolide elicit their antiproliferation activity probably by acting on similar cellular targets. Yamauchi et al., (2011) demonstrated that elevated GD3 levels correlated with increased expression of key lipogenic enzymes in melanoma cell lines (10). Based on this, we hypothesized that inhibition of GD3 by OSW-1 would lead to suppressed expression of enzymes mediating the lipogenesis pathway. Accordingly, 1 nM OSW-1 treatment for 24 h caused a 3.24- and 5.73-fold decrease in fatty acid Synthase (FASN) and 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR) expression in melanoma cells (figure 3.13). Thus we conclude that OSW-1 suppresses GD3S expression in melanoma leading to inhibition of gangliosides GD3 and GD2 and the downstream lipogenic enzymes, FASN and HMGCR.

### 3.2.5 OSW-1 promotes interaction of ganglioside GD3 with VDAC1 in the mitochondria

Raft-like microdomains on the mitochondrial membrane are enriched with gangliosides GD3 and GM3, and act as potential catalytic sites for autophagic and apoptotic reactions (60, 142, 143). Activation of cell death receptor signaling via CD95/Fas or tumor necrosis factor-α (TNF-α) promotes intracellular redistribution and vesicular transport of ganglioside GD3 to mitochondria, wherein GD3 promotes apoptosis by acting on the
mitochondrial transition pore and causing cytochrome c release (144-146). Garofalo et al. (2005) also demonstrated that during CD95/Fas mediated apoptosis GD3 interacts with voltage dependent anion channel 1 (VDAC1) and the fission protein hFis1 in the mitochondrial lipid microdomains of lymphoblastoid CEM cells (143). Furthermore, prior results from our lab suggest that OSW-1 has a mitochondrial mechanism of action (59).

Hence we investigated the effect of OSW-1 on mitochondrial targeting of GD3. To characterize the distribution of GD3 and VDAC1 in the membrane microdomains, we isolated lipid rafts fractions by performing a linear 5-80% sucrose gradient of the DMSO control and OSW-1 treated A375SM cell lysates (figure 3.17). Majority of the lipid raft resident protein caveolin-1 was recovered in fractions 2, 3 and 4. Ganglioside GD3 was also concentrated in fractions 2, 3 (highest expression) and 4 of the DMSO control sample. Nevertheless, OSW-1 administration promoted redistribution of GD3 expression to fractions 2, 3, 4, and 5. More significantly, OSW-1 stimulated elevated VDAC1 expression in the corresponding fractions (2, 3, 4, and 5) whereas a diminished expression of VDAC1 was observed in these caveolin enriched lipid raft fractions of the control sample. To confirm these results, A375SM cells were treated with OSW-1 for 24 h and the cellular co-localization of VDAC1 and GD3 was examined by confocal microscopy. As shown in figure 3.18, OSW-1 potentiated increased co-localization of ganglioside GD3 with VDAC1 in the mitochondria, thus corroborating previous findings that cell death signaling triggered intracellular redistribution and mitochondrial trafficking of GD3 (143, 145).
Figure 3.17: Effect of OSW-1 on GD3 and VDAC1 co-expression in lipid rafts.

Glycosphingolipid enriched microdomain/lipid raft fractions from DMSO and 1 nM OSW-1 24 h treated A375SM samples were prepared and equal amounts of the fractions were subjected to an immunoblot for VDAC1 and the lipid raft resident protein caveolin-1. Simultaneously, a dot blot was performed to determine GD3 expression in the lipid raft fractions. OSW-1 potentiates redistribution of GD3 and VDAC1 expression in lipid rafts.
Figure 3.18: OSW-1 promotes co-localization of VDAC1 with ganglioside GD3.

A375SM cells were grown on coverslips and treated with 1 nM OSW-1 for 24 h. The cells were stained with VDAC1 and GD3 antibodies as per the procedure described in materials and methods and confocal microscopic images were taken.
Figure 3.19: Correlation between OSW-1 induced mitochondrial membrane potential loss and ganglioside GD2 inhibition.

The fold change in mitochondrial membrane potential induced by OSW-1 was plotted as a function of fold change in GD2 expression. Linear regression analysis was performed and a Pearson’s correlation coefficient of 0.7100 with a p value of 0.0144 was obtained.
The relationship between inhibition of ganglioside GD3/GD2 expression and sensitivity of melanoma cells to OSW-1 was evaluated quantitatively by plotting fold change in mitochondrial membrane potential (ΔΨm) as a function of ganglioside GD2 expression change upon OSW-1 treatment (figure 3.19). Our results indicate that there was a positive correlation between these two factors (r = 0.7100, p = 0.0144), thus suggesting that ganglioside inhibition sensitizes melanoma cells to the cytotoxic effect of OSW-1.

3.2.6 OSW-1 eliminates melanoma cells through mitochondrial VDAC1 dependent mechanisms

OSW-1 stimulated increased interaction between GD3 and mitochondrial VDAC1 (figure 3.18). VDAC1 plays an important role in the transport of adenine nucleotides, Ca^{2+}, and other ions and metabolites across the mitochondria in addition to mediating apoptotic and autophagic signaling in mitochondria (147-152). Therefore, we studied the role of VDAC1 in OSW-1 induced cell death. Towards this end, we first examined melanoma cells for VDAC1 expression after OSW-1 treatment. VDAC1 expression was markedly increased by OSW-1 in A375SM and WM35 cells but was slightly decreased in normal melanocytes which were resistant to OSW-1 (figure 3.20 A, B). On average, OSW-1 incubation for 36 h triggered a 4.8- and 3.2-fold increase in VDAC1 expression in A375SM and WM35 cells while a 1.7-fold decrease was observed in normal melanocytes (figure 3.20 C). Further, the de novo protein synthesis inhibitor, cycloheximide reduced this VDAC1 elevation in melanoma cells, indicating that OSW-1 caused a translational upregulation of VDAC1 (figure 3.21). Further, cycloheximide treatment for 2 h resulted in inhibition of 50% of VDAC1 expression compared to the untreated control suggesting that the half-life of VDAC1 was around 2 h (figure 3.21). Significantly, Triptolide, which represses GD3 synthase, also
led to VDAC1 induction by 1.4-fold in A375SM cells (figure 3.22). Collectively, these data indicate that GD3S inhibition by OSW-1 may lead to VDAC1 activation and this VDAC1 increase positively correlates with cellular sensitivity to OSW-1 in melanoma cells.
Figure 3.20: OSW-1 induces VDAC1 upregulation in melanoma cells.

Immunoblots of A375SM, WM35 and NHEM cells treated with 1 nM OSW-1 for 0.5, 12, 24 and 36 h (A) and 24 h only (B). (C) Densitometric measurement of the VDAC1 band intensity relative to β-actin is shown. The bar graphs represent mean ± SEM of normalized VDAC1 expression from three independent immunoblot assessments. * denotes p < 0.05.
**Figure 3.21:**

A375SM cells were pre-incubated in 1 nM OSW-1 for 24 h, followed by 2 μM cycloheximide treatments for 0.5, 1, 2, 4 and 8 h. Simultaneously A375SM cells were treated with 2 μM cycloheximide as a single agent for 0.5, 1, 2, 4 and 8 h. Immunoblot was performed to examine changes in VDAC1 expression.
Figure 3.22: Effect of Triptolide on VDAC1 expression in melanoma cells.

A375SM cells treated with 20 nM Triptolide for 24 h and VDAC1 protein expression was detected by immunoblot analysis.
**Figure 3.23: Induction of mitochondrial superoxide release by OSW-1 in melanoma.**

A375SM and WM35 cells treated with 1 nM OSW-1 for the indicated time points were probed with the fluorescent mitochondrial superoxide indicator, mitosox red and the mitochondrial superoxide release was determined by flow cytometric analysis. **** denotes p < 0.0001, *** denotes p < 0.001, ** denotes p < 0.01 and * denotes p < 0.05.
Figure 3.24: OSW-1 induces cytosolic calcium elevation in melanoma cells.

A375SM and WM35 cells were treated with OSW-1 for the indicated time points and the changes in cytosolic calcium levels were quantified using the calcium green fluorescent probe. (A) A representative histogram of the calcium changes induced by OSW-1. (B) Bar graph indicates the mean ± SEM of percent calcium increase in A375SM and WM35 cells from three independent experiments. * denotes p < 0.05.
Apart from VDAC1 induction, OSW-1 also stimulated mitochondrial superoxide release (figure 3.23) and increase of cytosolic calcium (figure 3.24) in a time-dependent fashion. The immunosuppressant cyclosporin A (CsA) inhibits cyclophilin D, a mitochondrial matrix peptidyl-prolyl isomerase known to modulate opening of the mitochondrial transition pore (153, 154). In addition, CsA, through pharmacological inhibition of the serine/threonine phosphatase, calcineurin, promotes cell death in melanoma and other solid tumors (155-157). Therefore it was conceivable to evaluate the effect of CsA on OSW-1 mediated cytotoxicity in melanoma. Pre-incubation of WM35 cells with CsA increased the antiproliferation effect of 0.3 nM OSW-1 from 22.0% to 73.3% (figure 3.25 A). Similarly, combination of OSW-1 with CsA caused a 10.25-fold decrease in mitochondrial membrane potential as compared to the DMSO control while 0.3 nM OSW-1 single agent treatment caused a slight increase by 1.8 fold (figure 3.25 B). Interestingly, CsA+OSW-1 incubation for 24h promoted incremental VDAC1 expression in WM35 cells (figure 3.26 A). This VDAC1 induction by CsA+OSW-1 combination was slightly higher than the effect of OSW-1 as a single agent, while CsA by itself did not cause affect VDAC1. Further, pre-incubation of CsA with OSW-1 potentiated higher cytosolic calcium release when compared to the effect of CsA/OSW-1 as a single agent (figure 3.26 B, C). The cytosolic Ca\(^{2+}\) levels in WM35 cells treated with CsA+OSW-1 for 24 h increased to 229.5% while OSW-1 or CsA alone caused Ca\(^{2+}\) elevation to 200.7% and 156.2% respectively (figure 3.26 C). These results, together with our previous findings, demonstrate that the mitochondrial outer membrane protein, VDAC1 and other mitochondria dependent mechanisms including superoxide release and Ca\(^{2+}\) elevation play an important in leukemia cells executing the anticancer activity of OSW-1 (58, 59).
Figure 3.25: Combination of OSW-1 with Cyclosporin A (CsA) causes elevated cell death effect in melanoma cells.

(A) Melanoma cells treated with the indicated concentrations of OSW-1 and CsA for 48 h, were analyzed for cell death by annexin V/PI staining. The numbers denote percent cell viability (annexin V negative/PI negative) for each sample. (B) Mitochondrial membrane potential loss in cells treated with 0.3 nM OSW-1 ± 10 µM CsA for 48 h was detected by rhodamine 123 staining.
Figure 3.26: Effect of OSW-1 and Cyclosporin A (CsA) drug combination on VDAC1 expression and calcium homeostasis.

(A) Immunoblot was performed in WM35 cells treated with 1 nM OSW-1 ± 2 µM CsA for 24 h. (B) and (C) Cytosolic calcium changes in A375SM and WM35 cells treated with 1 nM OSW-1 ± 3 µM CsA for 24 h was determined. Figure (B) denotes a representative histogram of the calcium changes induced by OSW-1 in A375SM cells.
Figure 3.27: OSW-1 treatment causes elevated expression of phosphorylated ERK1/2 in melanoma.

(A) A375SM and WM35 cells were treated with 1 nM OSW-1 for the indicated time points and immunoblot was performed for total and phosphorylated-ERK1/2 proteins. (B) and (C) A375SM cells pre-incubated in 5 μM U0126 for 1 h were treated with 1 nM OSW-1 for 48 h. Mitochondrial membrane potential and cell viability were determined by rhodamine 123 (B) and annexin-V/PI analysis (C). Bar graphs represent the mean ± SEM measurements of three individual experiments.
Tringali et al. identified that ganglioside GM3 accumulation in K562 cells drove cell differentiation towards megakaryocytes and increased ERK1/2 phosphorylation through protein kinase C dependent cell signaling (158). Recent reports also indicate a positive correlation between GM3 accumulation and ERK1/2 activation in the brain astrocytes from a sandhoff disease mouse model (159). Further, overexpression of GD3 synthase gene inhibited PDGF-mediated ERK1/2 phosphorylation, thereby modulating cell proliferation and cell cycle progression in vascular smooth muscle cells (VSMC) cells (160). OSW-1 induced a time dependent increase in phospho-ERK1/2 expression in A375SM and WM35 cells (figure 3.27 A). We hypothesized that if ERK1/2 activation was crucial to the antitumor activity of OSW-1, then pre-incubation of melanoma cells with the MEK1/2 inhibitor, U0126 will rescue the cells from the cytotoxic effect of OSW-1. U0126 did not reverse the mitochondrial membrane potential loss triggered by OSW-1 (figure 3.27 B). Moreover, combination of OSW-1 with U0126 led to incremental inhibition of cell viability in melanoma cells (figure 3.27 C). U0126+OSW-1 combination produced 31.70% increased cell death in A375SM cells than OSW-1 single agent treatment (figure 3.27 C). Based on our results, we conclude that ERK1/2 activation is not a critical mechanism in mediating OSW-1’s anticancer effect, and is plausibly a downstream effect of the modulation of ganglioside expression pattern by OSW-1.
3.2.7 VDAC1 is a critical player in mediating the cytotoxic effect of OSW-1

To determine if VDAC1 induction is of functional significance to OSW-1 induced cytotoxicity, we used VDAC1 knock out (VDAC1-/-) mouse embryonic fibroblast (MEF) cells. We performed a 72 h MTT assay with MEF WT and VDAC1-/- cells treated with log scale serial diluted concentrations of OSW-1 (figure 3.28 A). MEF VDAC1-/- cells were significantly resistant to OSW-1 with a half-maximal inhibitory concentration approximately 44 times higher than the WT MEF cells (figure 3.28 A). Likewise MEF VDAC1-/- cells were resistant to the loss of mitochondrial membrane potential induced by OSW-1, whereas incubation of MEF WT cells with 1, 3 and 10 nM OSW-1 incubation for 72 h resulted a mitochondrial collapse to 41.85%, 29.50% and 16.85% respectively (figure 3.28 B).

To characterize the role of VDAC1 in melanoma cell sensitivity to OSW-1, we performed siRNA mediated knock down of VDAC1 expression in A375SM cells (figure 3.29 A). As shown in figure 3.29 B and C, OSW-1 induced mitochondrial membrane potential ($\Delta\Psi_m$) loss was substantially attenuated in siVDAC1 transfected A375SM cells. Further, a 48 h MTT assay in scrambled control and siVDAC1 transfected A375SM cells demonstrated that the siVDAC1 cells were 4-times less sensitive to OSW-1 compared to the scrambled control cells (figure 3.29 D). Interestingly the degree of resistance to OSW-1 observed in siVDAC1 transfected A375SM cells was lower than the MEF VDAC1-/- cells (figure 3.29 D, figure 3.28 A). This may be because of the transient nature of VDAC1 knockdown and the need for A375SM cells to get acclimatized to this down regulation of VDAC1. Also, pre-incubation of A375SM cells with the VDAC inhibitor, DIDS, did not rescue the cells from GD2 ganglioside inhibition triggered by OSW-1 (figure 3.30), thus indicating that VDAC1 does not have a regulatory function in the ganglioside expression change stimulated by
OSW-1. Collectively, our data strongly suggest that VDAC1 plays a crucial role in mediating OSW-1 induced cytotoxicity in melanoma cells.

Figure 3.28:

(A) MEF WT and VDAC1-/- cells were treated with log scale serial diluted concentrations of OSW-1 for 72 h and the MTT assay was performed. IC_{50} values were determined. (B) Mitochondrial membrane potential change induced by 72 h treatment of 1, 3 and 10 nM OSW-1 in MEF WT and VDAC1-/- cells was determined by rhodamine 123 analysis. ** denotes p < 0.01 and * denotes p < 0.05.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC_{50} (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEF WT</td>
<td>0.62</td>
</tr>
<tr>
<td>MEF VDAC1-/-</td>
<td>27.06</td>
</tr>
</tbody>
</table>
Figure 3.29: Down regulation of VDAC1 in A375SM cells confers resistance to OSW-1.

(A) Immunoblot for VDAC1 in A375SM cells transfected with siVDAC1 and negative control. (B) and (C) Mitochondrial membrane potential analysis of siVDAC1 and negative control transfected A375SM cells treated with 1 nM OSW-1 for 48 h. (D) A375SM WT and VDAC1-/- cells were treated with log scale serial diluted concentrations of OSW-1 for 48 h and the MTT assay was performed. IC_{50} values were determined. *** denotes p < 0.001.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC_{50} (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A375SM Scn</td>
<td>0.375</td>
</tr>
<tr>
<td>A375SM siVDAC1</td>
<td>1.497</td>
</tr>
</tbody>
</table>
Figure 3.30: Combination of OSW-1 with the VDAC1 inhibitor, DIDS, indicates that VDAC1 does not regulate ganglioside expression.

A375SM cells were treated with 1 nM OSW-1 ± 200 μM DIDS for 24 h and ganglioside GD2 expression was determined by flow cytometry.
3.2.8 Characterization of the mechanisms underlying the therapeutic selectivity of OSW-1

Therapeutic selectivity is a major concern while developing an anticancer agent. Our early findings indicate that OSW-1 is highly selective towards melanoma cells with limited toxicity in normal melanocytes (figure 3.9, 3.10). Hence we sought to delineate the mechanisms causal this selectivity of OSW-1 for melanoma cells. Towards this end, we first cultured WM35 cells in RPMI media supplemented with increasing concentrations of OSW-1 for a period of 2-3 months to generate “OSW-1 resistant” WM35 cells (WM35R). An aliquot of these cells were then returned back to RPMI media without OSW-1 (WM35 drug withdrawn, WM35DW). These WM35DW cells continued to display the OSW-1 resistant phenotype, despite being cultured in non-OSW-1 growth media, and thus represent the truly OSW-1 resistant melanoma cells. A preliminary MTT assay indicated that WM35DW and WM35R cells were about 680 and 332 times more resistant to OSW-1 than the parental WM35 cells (figure 3.31). Henceforth, we used this WM35 vs. WM35DW vs. WM35R cell model for our further study.
Figure 3.31: The OSW-1 resistant WM35 cell model.

Parental WM35 and the OSW-1 resistant WM35 R and WM35 DW melanoma cells were treated with log scale serial diluted concentrations of OSW-1 for 72 h and the MTT assay was performed to determine the antiproliferative effect of OSW-1 in these cells. The IC\textsubscript{50} values for OSW-1 in each cell sample were determined.
Figure 3.32: OSW-1 resistant WM35 DW cells have a decreased ganglioside GD3/GD2 expression profile compared to the parental WM35 melanoma cells.

The basal GD3 (A) and GD2 (B) expression in WM35, WM35 DW and WM35 R cells was determined by flow cytometry as described in materials and methods. * denotes p < 0.05.
Figure 3.33: OSW-1 resistant WM35 DW cells have an altered free radical expression profile compared to the parental WM35 melanoma cells.

(A) Basal mitochondrial superoxide levels were evaluated using the mitosox red fluorescent indicator as described in materials and methods. Bar graphs represent mean ±SEM from three independent experiments. (B) Basal level expression of reactive oxygen species, superoxides and nitric oxide was determined by flow cytometric analysis of cells stained for DCFDA, Dihydroethidium and DAF-FM fluorescent probes. * denotes p < 0.05.
A comparison of the basal GD3 and GD2 ganglioside expression indicated that WM35DW cells have a significantly decreased GD3 (p = 0.08) and GD2 (p = 0.02) expression as compared to the parental WM35 cells (figure 3.32 A, B). But no significant change in ganglioside GM3 expression between the two cell samples was observed. Further, the basal mitochondrial superoxide levels were significantly reduced in WM35DW than the parental WM35 cells (figure 3.33 A). This correlated with decreased expression of reactive oxygen species, cytosolic superoxides and nitric oxide in the WM35DW vs. WM35 cells (figure 3.33 B). Based on these results, we postulate that the basal ganglioside GD3/GD2 expression profile plays a critical role in governing the response of melanoma cells to the antitumor effect of OSW-1.

To explore other potential markers of sensitivity to OSW-1, we performed reverse phase protein array (RPPA) of 217 total- and phospho-proteins (figure 3.34). Of specific interest to us were lipogenesis (figure 3.35 A, B), and mitochondria (figure 3.35 A, C) signaling proteins, whose expression was statistically significantly different (p < 0.05) between WM35 vs. WM35DW vs. WM35 R cells. RPPA analysis results demonstrate that VDAC1 (porin), Bcl-2, Mcl-1, GSK3 and PKC-α expression was reduced in WM35DW and WM35R compared to the parental WM35 cells (figure 3.35 A, C). However cyclophilin F and phosphorylated NF-κB levels were higher in the resistant cells. Among the lipogenesis proteins, fatty acid synthase (FASN) expression was significantly increased, while caveolin-1, and focal adhesion kinase (FAK) expression was decreased in the WM35DW and WM35R resistant cells than the parental WM35 cells (figure 3.35 A, B). Interestingly, expression levels of cell survival and proliferation proteins like PI3K, Akt and phospho-MAPK and –
MEK was lower in the resistant WM35DW and WM35R cells than the parental WM35 cells (figure 3.34).

Figure 3.34: Differential protein expression in WM35 vs. WM35 R vs. WM35 DW cells. Heat map generated based on the RPPA analysis of WM35 vs. WM35 DW vs. WM35 R cells for the basal expression of the proteins indicated.

87
Figure 3.35: Differential expression of lipogenesis and mitochondria signaling proteins in WM35 vs. WM35 R vs. WM35 DW cells.

RPPA analysis was performed in WM35 vs. WM35 DW vs. WM35 R cells. The expression of proteins from the lipogenesis (figures A, B) and mitochondrial signaling (figure A, C) pathways that differed significantly in WM35 vs. WM35 DW vs. WM35 R cells is indicated as a heat map and bar graphs.
3.3 Discussion

In this study we demonstrated that OSW-1 effectively eliminates melanoma cells by causing inhibition of gangliosides GD3/GD2 and upregulation of mitochondrial VDAC1 leading to autophagic cell death.

Burgett et al identified the lipid metabolism proteins, OSBP and ORP4L as cellular binding targets of OSW-1(69). Prior findings from our lab demonstrated that OSW-1 functions by disrupting cellular calcium homeostasis through inhibition of the Na\(^+\)/Ca\(^{2+}\) exchanger 1 (NCX1) leading to apoptotic cell death in leukemia cells (58). More recently, Yamada et al., employed a fluorescent analog to investigate the cellular internalization and localization of OSW-1 in HeLa cells (82). Microscopic imaging studies revealed that OSW-1 rapidly internalized and distributed primarily in the intracellular membranes of the endoplasmic reticulum (ER) and Golgi apparatus and to a lesser extent in the mitochondria (82). Cumulatively, these findings suggest that a novel target for OSW-1 other than NCX1/OSBP may exist in these subcellular sites and merits further investigation.

Gangliosides are constituents of the glycosphingolipid-enriched microdomains (GEM or rafts) in the cell membrane, where they play a pivotal role in mediating cell proliferation, invasion and adhesion signaling (17, 19). Previous investigations provide evidence that ganglioside synthesis occurs intracellularly in the golgi complex and the ‘mitochondria-associated membrane’ (MAM) compartment of the endoplasmic reticulum through a glycosylation process regulated by glycosyltransferases, including GD3 synthase, a resident Golgi protein (161, 162). Specifically, the ganglioside GD3 plays a paradigmatic role, wherein, in contrast to its cell proliferative function, it also acts as a cell death effector
through its intracellular translocation to the mitochondria during stress conditions, leading to cytochrome c release, ROS generation and cell killing (143, 144, 146).

Our results demonstrate that OSW-1 inhibits ganglioside GD3 in melanoma cells by repressing gene expression of the enzyme, GD3 synthase (ST8SIA1 gene), which catalyzes the biosynthesis of ganglioside GD3 from its precursor GM3 by addition of a second sialic acid residue (163, 164). Consequently we observed a moderate decrease in ganglioside GD3, a more significant inhibition of its downstream product, GD2 and an accumulation of the precursor GM3 (figure 3.11). Importantly, this inhibition of ganglioside GD2 correlated with the cell death effect (represented by MMP) of OSW-1 in melanoma cells (figure 3.19). Nevertheless, it is unresolved if GD3S is a direct target for OSW-1 in melanoma. Interestingly, the OSW-1 structural analog, Triptolide, induced down regulation of human GD3 synthase (hST8SIA1) gene expression through NF-κB activation in melanoma cells (56). In addition, the transcription factor TWIST and GNE kinase are also known to positively regulate GD3S expression (165, 166). Therefore, it might be important to examine the effect of OSW-1 on signaling transduction proteins that are upstream of GD3 synthase. Another key protein that may potentially be a cellular interacting target of OSW-1 is the steroid regulatory element binding protein (SREBP), which acts as a master transcription factor regulating cholesterol and fatty acid biosynthesis (167). Prior structure-activity relationship studies demonstrated that the steroid nucleus in OSW-1’s structure was critical to its potent cytotoxic effect (66, 72). The SREBP protein, because of its ability to bind to sterol regulatory elements (168), may form a putative binding partner of OSW-1. Further, OSW-1 treatment inhibits expression of lipogenesis genes, FASN and HMGCR, which are downstream targets of SREBP. Cumulatively, these results point towards the possibility of
SREBP being a direct target of OSW-1 in melanoma cells. My future studies will be directed towards characterizing the molecular binding target(s) of OSW-1, with a strong focus on SREBP. Please refer to chapter 5 (summary and future directions) for a detailed description of my experimental plans for this aspect of the OSW-1 future study. VDAC1 is a multifunctional channel protein involved in mitochondria dependent cell death, redox metabolism, and cancer cell signaling. It has been implicated in cell death induction by stimuli such as cisplatin, curcumin, myostatin, UV irradiation and oxidative stress through either homooligomerization or interaction with Bcl-2 family proteins, adenine nucleotide translocase (ANT), regulation of Ca\(^{2+}\) and ATP flux, or ROS detoxification (169, 170). Our original results demonstrated that OSW-1 induced interaction between ganglioside GD3 and VDAC1 in the mitochondrial membranes (figure 3.17, 3.18). Hence we characterized the effect of OSW-1 on VDAC1 expression and VDAC1 dependent cell death signaling in melanoma. Our data indicated that OSW-1 caused VDAC1 upregulation, increased cytosolic calcium and mitochondrial superoxide release in melanoma cells (figures 3.20, 3.23, 3.24). Interestingly, Triptolide, which inhibits GD3S, also activated VDAC1 expression (figure 3.22). Nevertheless, combination of OSW-1 with the VDAC inhibitor, DIDS, did not affect OSW-1 mediated ganglioside GD3/GD2 inhibition (figure 3.30), thus suggesting that GD3S might regulate VDAC1 expression but not vice versa. Significantly, combination of OSW-1 with the immunosuppressant drug, cyclosporin A, not only upregulated VDAC1 expression but also led to elevated cell death (figure 3.26 A, 3.25). Considering that combination therapy with cyclosporine has been known to improve clinical efficacy in advanced malignancies, we propose that this OSW-1+CsA treatment strategy may improve the therapeutic benefit of OSW-1 in melanoma and thus merits further investigation (171, 172).
Finally, knock out or knock down of VDAC1 ameliorated the cytotoxic effect of OSW-1 in melanoma (figure 3.28, 3.29), thus corroborating our findings that VDAC1 and mitochondria dependent cell death mechanisms are crucial to the antineoplastic activity of OSW-1.

Increased expression of gangliosides GD3 and GM3 in melanoma cell lines and tissues, and a correlation between GD3 expression levels and the GD3 synthase enzymatic activity in cells has been reported (53, 54, 173). This is obvious in the comparison between melanoma cells and normal melanocytes wherein the melanocytes exhibit extremely low GD3 synthase activity and GD3 cell surface expression (16). Based on this information, we postulated that differential ganglioside expression profile may form a basis for the therapeutic selectivity of OSW-1 towards melanoma cells with limited toxicity to normal melanocytes (figure 3.9, 3.10). To test this hypothesis, we compared the basal ganglioside expression pattern between the OSW-1 resistant WM35DW and the parental WM35 cells (figure 3.32). Our results indicate WM35DW cells have a reduced GD3 and GD2 expression than the parental WM35 cells. A decreased free radical expression profile was also observed in the resistant WM35DW cells (figure 3.33). RPPA analysis revealed altered expression of multiple lipid metabolism proteins including FASN, YAP, FAK and caveolin-1 in WM35 vs. WM35DW cells (figure 3.35 A, B). Differential expression of mitochondrial signaling proteins such as VDAC1, cyclophilin and various interacting partners of VDAC1 such as Bcl-2 family members, and GSK3 was also observed (figure 3.35 A, C). Further investigation into the molecular mechanisms regulating GD3 synthase activity and the cell signaling regulating VDAC1-GD3 interaction may provide deeper insights into the therapeutic basis for the selective elimination of melanoma cells by OSW-1.
In summary, our study delineates the mechanisms essential to the highly potent anticancer activity of OSW-1 in melanoma cells. Our results demonstrate that OSW-1 induces autophagic cell death in melanoma by repressing GD3 synthase gene expression, leading to inhibition of gangliosides GD3, GD2 and accumulation of GM3. Ganglioside GD3 then interacts with the mitochondrial VDAC1 leading to mitochondrial membrane potential collapse, aberrant calcium signaling and cell death. VDAC1 is a critical player in mediating OSW-1’s activity as its down regulation confers resistance to OSW-1. A significant characteristic of OSW-1 induced cytotoxicity is its therapeutic selectivity towards melanoma cells. Our investigation reveals that elevated ganglioside expression in melanoma may form the basis for the increased sensitivity of these cells towards OSW-1. To our knowledge, this is the first study to demonstrate the antitumor effect of OSW-1 on ganglioside expression and VDAC1 mediated mitochondrial signaling in melanoma. The role of OSW-1 as an inhibitor of GD3 synthase gene expression is crucial, since in melanoma ganglioside GD3 is a well characterized therapeutic target. Therefore, further research needs to be undertaken to shed light on the mechanisms regulating this GD3 synthase inhibition by OSW-1.
Chapter 4: PEITC effectively eliminates melanoma cells through redox mechanisms

4.1: Introduction

PEITC is a highly active metabolite of cruciferous vegetables with potent chemopreventive and chemotherapeutic properties. It inhibits expression of pro-oncogenic proteins such as Bcl-2, Mcl1 and Bcl-xL, triggers cell cycle arrest, DNA and mitochondrial damage leading to caspase activation, cytochrome c release and apoptosis (95, 98).

PEITC exhibits a unique mechanism of cell death – it induces excessive ROS accumulation in cancer cells leading to massive cytotoxicity (63, 64, 94). This ROS induced toxicity by PEITC is specific to tumor cells; normal melanocytes with a lower basal oxidative stress phenotype and reserve antioxidant capacity are resistant to PEITC and other pro-oxidant compounds (63).

In this dissertation, I investigated the mechanisms of cytotoxic action and therapeutic selectivity of PEITC in melanoma cells. I aimed to characterize the differences in redox status between the normal cells and cancer cells and use this as a mechanistic basis to specifically eliminate melanoma cells. My results demonstrate the PEITC depletes cellular glutathione in melanoma cells leading to ROS stress, loss of mitochondrial membrane potential and cell death through apoptosis. Further, a positive correlation between the basal cellular ROS expression levels and sensitivity to PEITC was observed. Accordingly, normal melanocytes which have a much lower intrinsic oxidative stress compared to melanoma cells are resistant to PEITC. The glutathione depletion mediated by PEITC is an early event which occurs much before cell death and is crucial to the cytotoxic effect of PEITC in melanoma. Due to this distinctive mechanism of cell death, PEITC is effective in attenuating drug resistance to clinically used melanoma agents.
4.2 Results

4.2.1 Redox status of melanoma cells

A characteristic feature of melanoma biology is the presence of aberrant melanosomes in melanoma cells that generate free radicals (174). Chronic oxidative stress potentiated by oncogenic Akt signaling or loss of tumor suppressors such as p53 and p16, in combination with exposure to ultraviolet radiations (UV), UVA and UVB, may lead to oncogenic transformation of dysplastic nevi and development of an intrinsic drug resistant phenotype in melanoma cells (44, 62, 175-177). On the other hand, excessive reactive oxygen species (ROS) accumulation in melanoma cells leads to massive toxicity and cell death, thus making it a unique therapeutic target. Hence, I hypothesized that melanoma cells are under elevated intrinsic oxidative stress and can be preferentially eliminated by pharmacological agents such as PEITC which modulate the cellular redox homeostasis.

To investigate this hypothesis, we first characterized the basal redox status of melanoma cells. The intracellular free radical expression levels were determined across a panel of melanoma cells by flow cytometric analysis using the fluorescent reagents, CM-H$_2$-DCFDA, DAF-FM and dihydroethidium. 5-,6-Chloromethyl-2’7’-Dichlorodihydrofluorescein Diacetate (CM-H$_2$-DCFDA) is an indicator of hydroxyl radicals, peroxides and other ROS molecules in cells. DCFDA passively diffuses into the cells, where its acetate groups are cleaved by intracellular esterases and the compound is subsequently oxidized to yield the fluorescent 2’7’-Dichlorofluorescein (DCF), which can be detected by flow cytometry (178). 4-Amino-5-Methylamino-2’, 7’-Difluorofluorescein (DAF-FM) detects nitric oxide (NO) in cells. The non-fluorescent DAF-FM react with intracellular NO and is converted into the fluorescent benzotriazole molecule which can be quantified (179). Dihydroethidium (Het) is a blue fluorescent dye which is oxidized by superoxides to form
oxiethidium, which then intercalates with nucleic acids to emit a bright red fluorescence that can be detected by flow cytometry or microscopy (180).

Our results indicated that Hs294T (BRAF WT, metastatic) melanoma cells had the highest expression of basal ROS and nitric oxide (figure 4.1 A, B, D). Superoxide levels were elevated across the melanoma cells evaluated in this study. Overall, SK-MEL-2 (BRAF WT, NRAS Q617R, metastatic) cells contained relatively higher free radical expression among the melanoma cells tested (figure 4.1). The A375SM (BRAF V600E, NRAS WT, highly metastatic) cells had a moderately decreased free radical expression profile compared to the other melanoma cells (figure 4.1). Hence, SK-MEL-2 and A375SM cells were used as representative cell line models in the follow-up mechanistic studies.
Figure 4.1: Basal free radical expression profile in melanoma cells.

(A), (B), (C) Melanoma cells were plated in 6-well plates for 24 h prior to the measurement of cellular reactive oxygen species, nitric oxide and superoxides using the fluorescent probes DCFDA, DAF-FM and Dihydroethidium by flow cytometry. (D) Mean count of the basal reactive oxygen species, nitric oxide and superoxide expression levels in melanoma cells.
4.2.2 The cytotoxic effect of PEITC in melanoma

Earlier studies from our lab demonstrated that the natural product, PEITC was effective in inhibiting leukemia cells including fludarabine resistant chronic lymphocytic leukemia and Gleevec-resistant chronic myelogenous leukemia (63, 102). To investigate the effect of PEITC on melanoma cell viability, I performed an MTT assay across a panel of cell lines treated with varying concentrations of PEITC for 72 h. As illustrated in figure 4.2, PEITC was cytotoxic to melanoma cells with an average half-maximal inhibitory concentration (IC$_{50}$) of 2.21 µM. Interestingly, WM46 cells which have the lowest levels of basal ROS expression amongst all the melanoma cells tested (figure 4.1D), also have the highest IC$_{50}$ value (4.10 µM; figure 4.2B).

Biochemical analysis by annexin V-FITC/propidium iodide staining indicated that PEITC induced apoptosis in melanoma cells in a time dependent fashion (figure 4.3A, B). PEITC induced cell death was detectable at 12 h and reached over 70% by 24 h in A375SM and SK-MEL-2 cells (figure 4.3A). The bar graphs in figure 4.3B represent the heterogeneity of melanoma cell response to PEITC. On average, a 24 h PEITC treatment caused 55.73% (DMSO vs. PEITC 24h, p-value < 0.0001) cell death in melanoma cells. Significantly, combination of PEITC with the pan caspase inhibitor, Z-VAD-FMK, partially rescued melanoma cells from the cytotoxic effect of PEITC (figure 4.3C). Caspases are a group of intracellular proteases that play a pivotal role during apoptosis by catalyzing the disassembly of cells into membrane-enclosed vesicles (apoptotic bodies), ultimately leading to death (181). Treatment of SK-MEL-2 cells with 5 µM PEITC induced 71.74% cell killing. However, co-treatment of cells with 20 µM Z-VAD-FMK and 5 µM PEITC reduced cell
death to 30.23% (figure 4.3C). These results delineate the role of caspase activation and apoptosis in mediating the inhibitory activity of PEITC.

Figure 4.2:

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC\textsubscript{50} Value (µM)</th>
<th>Mean ± SEM (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A375SM</td>
<td>2.42 ± 0.179</td>
<td></td>
</tr>
<tr>
<td>WM35</td>
<td>1.52 ± 0.327</td>
<td></td>
</tr>
<tr>
<td>SK-MEL-2</td>
<td>2.70 ± 0.418</td>
<td></td>
</tr>
<tr>
<td>WM46</td>
<td>4.10 ± 0.618</td>
<td>2.206 ± 0.362</td>
</tr>
<tr>
<td>UCSD354L</td>
<td>2.18 ± 0.219</td>
<td></td>
</tr>
<tr>
<td>HS294T</td>
<td>1.00 ± 0.140</td>
<td></td>
</tr>
<tr>
<td>MEL624</td>
<td>2.67 ± 0.295</td>
<td></td>
</tr>
<tr>
<td>MEWO</td>
<td>1.06 ± 0.241</td>
<td></td>
</tr>
</tbody>
</table>

Figure 4.2: Effect of PEITC on melanoma cell viability

Melanoma cells were treated with log-scale serial diluted concentrations of PEITC for 72 h, and the cytotoxic effect was determined by performing the MTT assay. The cell viability curves were plotted. Each data point represents mean ± SEM for three independent measurements. (B) The 50% cell inhibitory concentrations (IC\textsubscript{50}) of PEITC for each cell line are indicated.
Figure 4.3:

A

<table>
<thead>
<tr>
<th></th>
<th>DMSO</th>
<th>6 h</th>
<th>12 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>A375SM</td>
<td>91.8%</td>
<td>84.4%</td>
<td>71%</td>
<td>23%</td>
</tr>
<tr>
<td>SK-MEL-2</td>
<td>94.2%</td>
<td>75.5%</td>
<td>60.7%</td>
<td>21.3%</td>
</tr>
</tbody>
</table>

Annexin V-FITC

B

% Cell Viability (normalized to control)

<table>
<thead>
<tr>
<th></th>
<th>DMSO</th>
<th>6 h</th>
<th>12 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>A375SM</td>
<td>91.3%</td>
<td>93.4%</td>
<td>25.8%</td>
<td>63.7%</td>
</tr>
</tbody>
</table>

C

<table>
<thead>
<tr>
<th></th>
<th>DMSO</th>
<th>20 µM Z-VAD-FMK</th>
<th>5 µM PEITC</th>
<th>PEITC + Z-VAD-FMK</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001
Figure 4.3: PEITC induced cytotoxicity is through apoptosis in melanoma cells

(A) SK-MEel-2 and A375SM cells were treated with 10 µM PEITC for the indicated time points and PEITC induced cell death induced was detected by annexin V-FITC/PI staining and flow cytometric analysis as described in materials and methods. (B) Percent cell viability of melanoma cells treated with 10 µM PEITC for the indicated time points. Bar graphs represent mean ± SEM of three independent measurements. (C) SK-MEL-2 cells incubated in 20 µM Z-VAD-FMK for 1 h were treated with 5 µM PEITC for 24 h and cell death was measured by flow cytometric analysis (annexin V-FITC/PI staining). **** denotes p < 0.0001, *** denotes p < 0.001, ** denotes p < 0.01 and * denotes p< 0.05.
Mitochondrial dysfunction constitutes an important event in the induction of cell death. Opening of the mitochondrial transition pore leading to depolarization of membrane potential, release of apoptogenic proteins and uncoupling of oxidative phosphorylation have been linked to apoptosis stimulation by agents such as tert-Butylhydroperoxide, the calcium ionophore A23187, selenite, sulforaphane etc. (182-186). To test if PEITC caused mitochondrial membrane damage in melanoma, I treated the cells with 5 µM PEITC for varying time points and the effect on mitochondrial membrane integrity was assessed by flow cytometric analysis of cells labeled with Rhodamine 123, a lipophilic, cationic fluorescent dye that is readily taken up by active mitochondria. Depolarization of the mitochondrial membrane, therefore, decreases the mitochondrial rhodamine 123 signal, which can be quantified (187). My results indicate that PEITC potentiated a time-dependent mitochondrial membrane potential collapse in SK-MEL-2 and A375SM cells (figure 4.4). Mitochondrial membrane permeabilization was first observed at 16 h and by 24 h there was a massive (over 90%) loss of membrane potential in PEITC treated cells as compared to the DMSO treated controls (DMSO vs. PEITC 24h, p-value < 0.0001).
Figure 4.4: PEITC induces mitochondrial membrane potential loss in melanoma cells.

A375SM and SK-MEL-2 cells were treated with 5 μM PEITC for the indicated time points and the effect on mitochondrial membrane potential was evaluated by flow cytometric analysis of Rhodamine 123 labelled cells.
Figure 4.5: Correlation between cellular basal ROS levels and cell death induced by PEITC.

Cellular intrinsic ROS expression levels were measured by performing the DCFDA assay. Cell death was detected by annexin V-FITC/PI assay in melanoma cells treated with 10 µM PEITC for 24 h. Linear regression analysis between these two parameters was performed using the mean ± SEM measurements of three independent experiments.
Figure 4.6: Effect of PEITC on JNK and p38 expression in melanoma.

SK-MEL-2 cells were treated with 5 µM PEITC for the indicated time points and the effect on JNK and p38 kinase expression was assessed by western blotting.
Quantitative evaluation of the relationship between basal cellular ROS levels and PEITC induced cell death revealed a strong correlation between these two parameters ($r = 0.8068$, p-value = 0.0155) (figure 4.5). ROS have been known to promote apoptotic signaling by mediating the activation of pro-apoptotic JNK and ASK1 kinases, leading to upregulation of TNFα, Fas-L and Bak, release of cytochrome C, and mitochondrial translocation of Bax and Bad apoptotic proteins (188-191). Interestingly, western blot analysis indicated that PEITC stimulated a time-dependent increase in phosphorylated-JNK expression in SK-MEL2 cells (figure 4.6). However, no significant change was observed in the expression of total JNK as well as total and phosphorylated p38 MAP kinase (figure 4.6). Collectively these results indicate that PEITC executes its anticancer effect through apoptosis and ROS mediated mechanisms in melanoma cells. Of significance is the observation that the basal ROS status of melanoma cells is proportional to its sensitivity to PEITC.

4.2.3 PEITC exhibits minimal toxicity in normal melanocytes

The promising cytotoxic activity of PEITC in melanoma cells led us to investigate the effect of this compound on normal melanocyte cell viability. As shown in figure 4.7, normal melanocytes were significantly less sensitive to PEITC compared to melanoma cells. Annexin-FITC/PI analysis demonstrated that a 24 h PEITC treatment caused 60% cell death in melanoma cells ($n = 9$), while 94.73% of normal melanocytes were still viable at this time point (figure 4.7B). Interestingly, PEITC stimulated massive cytotoxicity in HS294T (figure 4.7A) and SK-MEL-2 (figure 4.3A) melanoma cells, which have a relatively higher ROS capacity. Previous research findings have shown that PEITC mediated cell death is characterized by ROS production either though depletion of the glutathione antioxidant system or inhibition of oxidative phosphorylation in leukemia, ovarian and prostate cancers.
This led me to speculate that the anticancer effect of PEITC relied on disrupting on the cellular redox homeostasis leading to ROS accumulation and subsequent toxicity. As expected, incubation of SK-MEL-2 cells with 5 µM PEITC for 8 h resulted in substantial ROS accumulation, whereas only a slight increase in ROS levels was observed in normal melanocytes (figure 4.8). These results suggest that distinct redox regulatory mechanisms exist between melanoma cells and normal melanocytes and these may form the basis for their differential response to PEITC.
Figure 4.7: Preferential killing of melanoma cells by PEITC

(A) Cell death induced by 10 µM PEITC in melanoma cells and normal melanocytes was determined by flow cytometric analysis of annexin V-FITC/PI stained cells. Representative dot blots are shown here. (B) Bar graphs illustrate the quantitative comparison of PEITC triggered cell death (normalized to DMSO control) in melanoma (n = 9) and normal melanocyte cells at 24 h treatment. Mean ± SEM of cell death measurements from three independent experiments of each melanoma cell line and normal melanocytes is denoted here. ** denotes p < 0.01.
Figure 4.8: Induction of ROS accumulation in melanoma cells and normal melanocytes by PEITC.

SK-MEL-2 cells and normal melanocytes were treated with 5 µM PEITC for 8 h and ROS levels were detected by flow cytometric analysis of cells labelled with 1 µM DCFDA.
Figure 4.9: Comparison of ROS expression between melanoma cells and normal melanocytes.

(A) and (C) Flow cytometric analysis to detect ROS (DCFDA) and specifically superoxides (Dihydroethidium) was performed in SK-MEL-2 and normal melanocytes (NHEM) stained with 1 µM DCFDA and Dihydroethidium. (B) and (D) Bar graphs represent the mean ± SEM of ROS and superoxide measurements from three independent experiments of each melanoma cell line (n = 8) and normal melanocytes. ** denotes p < 0.01 and * denotes p < 0.05.
The preferential ROS accumulation in melanoma cells treated with PEITC suggested that these cells were under increased intrinsic oxidative stress thus making them more vulnerable to further free radical elevation and redox disturbance by PEITC. To evaluate this observation, I compared the basal ROS and specifically superoxide expression levels between melanoma cells and normal melanocytes. As illustrated in figure 4.9 A and B, melanoma cells exhibit increased ROS expression compared to normal melanocytes (P-value < 0.05). SK-MEL-2 cells had a 2.3 fold increased intrinsic ROS expression compared to normal melanocytes (figure 4.9A). Importantly, the intrinsic superoxide levels were 3-fold higher (p-value < 0.01) in melanoma cells than the normal melanocytes (figure 4.9D). A representative histogram indicating the differential superoxide expression in melanoma cells is shown in figure 4.9C. Based on these results, I concluded that the higher intrinsic oxidative stress observed in melanoma cells was important in modulating their sensitivity to PEITC.

4.2.4 The ROS mediated mechanisms of cell death by PEITC

Treatment of SK-MEL-2 and A375SM cells with 5 µM PEITC produced a time dependent increase in cellular ROS levels (figure 4.10B). A 6 h PEITC incubation of SK-MEL-2 cells increased cellular ROS levels by 3.2-fold (figure 4.10A). In contrast, PEITC (10 µM, 8 h) did not induce any significant ROS change in normal melanocytes (figure 4.8), suggesting that this ROS elevation rendered the melanoma cells susceptible to PEITC’s cytotoxic activity. Interestingly, flow cytometric analysis of melanoma cells stained with Dihydroethidium indicated that PEITC treatment did not generate any significant change in the cellular superoxides (O$_2^-$) (figure 4.10D), although the intrinsic superoxide expression was significantly increased in melanoma cells compared to normal melanocytes (figure 4.9 C, D). PEITC stimulated a time dependent increase in nitric oxide levels (figure 4.10C).
Figure 4.10:

(A), (C) and (D): SK-MEL-2 cells were treated with 5µM PEITC for the indicated time points and alterations in ROS, nitric oxide and superoxide expression in cells was assessed by DCFDA, DAF-FM and dihydroethidium staining. (B) A375SM and SK-MEL-2 cells were treated with 10µM PEITC for a varying time course and DCFDA assay was performed. *** denotes p < 0.001, ** denotes p < 0.01 and * denotes p< 0.05.
Cumulatively, these results suggest that PEITC induced ROS may include other molecules such as hydrogen peroxide, hydroxyl radical and nitric oxide but not superoxides. This data corroborates with previous findings from our lab in oncogenic Ras transformed ovarian epithelial cells (T72 Ras) where PEITC induced ROS and NO generation, without any significant changes in $\text{O}_2^-$ (113). Further, the relationship between ROS accretion and PEITC mediated toxicity was quantitatively evaluated by plotting PEITC induced cell death as a function of ROS increase (figure 4.11). A positive correlation ($r = 0.7357$, p-value < 0.05) between these two factors was observed.
Cell death was detected by annexin V-FITC/PI assay in melanoma cells treated with 10 µM PEITC for 24 h. ROS expression levels in PEITC treated melanoma cells were measured by DCFDA assay. Cell death was plotted as a function of ROS fold change and linear regression analysis was performed.
Since PEITC triggered cell death was characterized by ROS accumulation in cells, I next investigated the potential cause for this ROS stimulation by PEITC. Glutathione is a major antioxidant which has been shown to promote metastatic melanoma cell survival in the hepatic microvasculature by protecting the cells against oxidative stress induced damage (192). Importantly, glutathione depletion either by pharmacological intervention using L-Buthionine (S, R)-Sulphoximine (BSO) or by dietary modulation using an L-glutamine enriched diet reduced melanoma tumor growth and sensitized cells to combination therapy (192, 193). Further, studies by Trachootham et al., and Lam et al., indicated that PEITC scavenges glutathione in leukemia and breast cancer cells leading to massive cytotoxicity (93, 113). I postulated that the high ROS capacity in malignant melanoma cells would make them dependent upon the glutathione antioxidant system to maintain redox balance in cells, and therefore depletion of glutathione by PEITC would disrupt the redox homeostasis resulting in excessive ROS accumulation, beyond the cell’s tolerance threshold, and subsequent cell death. To examine this hypothesis, I treated SK-MEL-2 and A375SM cells with 5 µM PEITC for 3 and 6 h and detected the cellular glutathione levels (figure 4.12). PEITC induced inhibition of glutathione was detected at 3 h and by 6 h about 60% of cellular glutathione was depleted. It is important to note that PEITC treatment exhibits limited cytotoxicity at these time durations (figure 4.3B), suggesting that this glutathione depletion may be a primary event that potentiates ROS accumulation and consequent cell death. To verify this possibility, I treated SK-MEL-2 and A375SM cells pre-incubated in the glutathione precursor, N-Acetyl Cysteine (NAC) with PEITC (figure 4.13). As shown in figure 4.13 A and B, NAC reverses the ROS elevation caused by PEITC. More importantly, combination of NAC with PEITC completely abrogates the cytotoxic effect of PEITC in SK-
MEL-2 cells (figure 4.13C). Hence glutathione depletion is an important mechanism in PEITC induced ROS increase and cell death.

Figure 4.12:

Figure 4.12: PEITC causes depletion of cellular glutathione.

A375SM and SK-MEL-2 cells were treated with 5 μM PEITC for the indicated time points and cellular glutathione levels were detected by spectrophotometric analysis as described in materials and methods. ** denotes a statistical significance of p < 0.01 between the DMSO control and PEITC treated samples in each of the respective cell lines.
Figure 4.13:
Figure 4.13: N-Acetyl Cysteine (NAC) reverses the inhibitory effect of PEITC

(A) SK-MEL-2 cells incubated in 1 mM NAC for 2 h were treated with 5 µM PEITC for 6 h and ROS were detected by the DCFDA assay. (B) Bar graphs denote the mean ± SEM of three independent measurements of ROS in A375SM and SK-MEL-2 cells treated with PEITC ± NAC. (C) Annexin V-FITC/PI assay was performed to detect the effect on cell death in SK-MEL-2 cells treated with PEITC ± NAC for 24 h. **** denotes p < 0.0001 and *** denotes p < 0.001.
4.2.5 Combination of Temozolomide / BCNU with PEITC attenuates drug resistance to these agents in melanoma

Malignant melanomas are characterized by significant drug resistance to currently used chemotherapeutic agents such as Temozolomide (TMZ) and 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU). Interestingly, BCNU has been shown to inhibit glutathione reductase activity in erythrocytes from patients as well as in mouse liver, lung and heart tissues (194, 195). Further, Kohsaka et al. demonstrated that combination of TMZ with the glutathione synthesis inhibitor, BSO, potentiated the toxic effect of Temozolomide in TMZ resistant glioblastoma cells (196). Based on this information, I postulated that combination of PEITC with either BCNU or TMZ would cause incremental oxidative stress due to an enhanced depletion of cellular glutathione. This would lead to significant toxicity in melanoma cells and would be of therapeutic benefit. Towards this end, I first performed annexin V-FITC/PI assay to examine the effect of PEITC ± TMZ/BCNU combination on cell death in melanoma cells (figure 4.14). The results indicate that supplementation of PEITC with either BCNU or TMZ promoted significantly higher inhibitory effect than that of any (PEITC/TMZ/BCNU) single agent treatment. The cytotoxic effect of this drug combination was evaluated by performing the colony formation assay. As illustrated in figure 4.15, PEITC, BCNU or TMZ as a single agent moderately inhibited colony formation (figure 4.15 A, C). However, combination of TMZ/BCNU with PEITC caused a striking decrease in the number of colonies formed. For instance, PEITC+BCNU treatment potentiated 3.5-fold decrease in the number of colonies as compared to PEITC single agent treatment (figure 4.15B).

Furthermore, combination index (CI) analysis using the Chou-Talalay algorithm was performed to characterize the type of drug interaction (figure 4.16). SK-MEL-2 cells were
incubated with varying combination regimens of PEITC (5 µM, 10 µM and 20 µM; last 24 h) and BCNU (50 µM, 100 µM and 200 µM; 48 h) or TMZ (0.3 mM, 0.6 mM and 1 mM; 48 h). As described in figure 14.6B, the combination of PEITC with TMZ was synergistic (CI<1.0). Similarly, BCNU and PEITC combination index analysis mostly produced synergistic and additive (CI=1) end points (figure 4.16D).
Figure 4.14: Effect of PEITC ± TMZ/BCNU treatment on melanoma cell viability

SK-MEL-2 and A375SM cells were incubated in varying concentrations of BCNU and TMZ for 48 h. During the last 24 h of treatment 5 mM PEITC was added and the effect of the drug combination on cell death was determined by annexin V-FITC/propidium iodide staining. Bar graphs represent the percentage of viable cells in each drug treatment sample as a function of the DMSO treated control cell viability. **** denotes p < 0.0001, *** denotes p < 0.001, ** denotes p < 0.01 and * denotes p< 0.05.
Figure 4.15: Combination of Temozolomide / BCNU with PEITC inhibits colony formation in melanoma cells

(A) and (C) A375P cells were treated with the indicated concentrations of PEITC and BCNU/TMZ for 2 weeks and the effect of the drug combination on colony formation was determined by Giemsa staining. (B) and (D) The colonies formed were counted manually and have been represented as bar graphs. *** denotes p < 0.001, ** denotes p < 0.01 and * denotes p< 0.05.
Figure 4.16:

<table>
<thead>
<tr>
<th>PEITC (µM)</th>
<th>TMZ (µM)</th>
<th>Combination Index (CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.3</td>
<td>0.678</td>
</tr>
<tr>
<td>5</td>
<td>0.6</td>
<td>0.556</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>0.485</td>
</tr>
<tr>
<td>10</td>
<td>0.3</td>
<td>0.678</td>
</tr>
<tr>
<td>10</td>
<td>0.6</td>
<td>0.456</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>0.448</td>
</tr>
<tr>
<td>20</td>
<td>0.3</td>
<td>0.991</td>
</tr>
<tr>
<td>20</td>
<td>0.6</td>
<td>0.422</td>
</tr>
<tr>
<td>20</td>
<td>1</td>
<td>0.586</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PEITC (µM)</th>
<th>BCNU (µM)</th>
<th>Combination Index (CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>50</td>
<td>0.973</td>
</tr>
<tr>
<td>5</td>
<td>100</td>
<td>1.053</td>
</tr>
<tr>
<td>5</td>
<td>100</td>
<td>0.668</td>
</tr>
<tr>
<td>10</td>
<td>50</td>
<td>0.852</td>
</tr>
<tr>
<td>10</td>
<td>100</td>
<td>0.938</td>
</tr>
<tr>
<td>10</td>
<td>200</td>
<td>0.653</td>
</tr>
<tr>
<td>20</td>
<td>50</td>
<td>0.819</td>
</tr>
<tr>
<td>20</td>
<td>100</td>
<td>0.910</td>
</tr>
<tr>
<td>20</td>
<td>200</td>
<td>0.641</td>
</tr>
</tbody>
</table>
Figure 4.16: Combination index analysis of PEITC ± TMZ/BCNU treatment

(A) and (C) Representative dot blots indicating cell death induced by TMZ ± PEITC and BCNU ± PEITC 48 h treatment in SK-MEL-2 cells. PEITC was added during the last 24 h of treatment. (B) and (D) SK-MEL-2 cells were treated with the indicated concentrations of PEITC ± TMZ/BCNU for 48 h, and combination index (CI) analysis was performed using Compusyn software. The following quantitative definitions, based on the Chou-Talalay theorem, were employed to define combination effect: CI < 1 represents synergism, CI = 1 represents an additive effect, and CI > 1 represents antagonism.
Figure 4.17: Redox mechanisms mediate the combinatorial cell death effect of TMZ/BCNU ± PEITC treatment in melanoma.

(A) Melanoma cells were treated with 0.5 mM TMZ for 24 h. 5 µM PEITC was added during the last 6 h of treatment. ROS, nitric oxide and mitochondrial superoxides were measured by DCFDA, DAF-FM and mitosox red staining and flow cytometric analysis. (B) 5 µM PEITC was added during the last 3h of a 24 h 100 µM BCNU or 0.5mM TMZ treatment. Bar graphs represent mean ± SEM of cellular glutathione measurements for the indicated drug concentrations in three independent experiments. * denotes p< 0.05.
To delineate the mechanisms that are pivotal to this increased cytotoxicity of PEITC+TMZ/BCNU drug combination in melanoma cells, we analyzed for changes in redox expression in cells treated with PEITC ± TMZ/BCNU. Co-treatment of PEITC (5 µM, 6 h) with TMZ (0.5 mM, 24 h) led to increased ROS and nitric oxide generation as compared to the effect of PEITC as a single agent (figure 4.17A, B). Also, Temozolomide, by itself, did not induce any significant changes in ROS. Further, this combination (PEITC+TMZ) also stimulated a slightly increased generation of mitochondrial superoxides as detected by the mitosox red indicator (figure 4.17C). Importantly, this ROS increase was preceded by glutathione depletion in SK-MEL-2 cells. Addition of 5 µM PEITC to melanoma cells pre-incubated in BCNU or TMZ led to inhibited cellular glutathione. The glutathione depletion was higher in combinatorial treatment of PEITC+BCNU/TMZ as compared to a single agent. Thus, glutathione depletion forms a primary event in attenuating drug resistance to TMZ or BCNU in melanoma using the redox modulating agent, PEITC.

In conclusion, the natural product PEITC exhibits significant anticancer activity in melanoma. Its inhibitory effects in melanoma are mediated by ROS accumulation and disabling of the glutathione antioxidant system.
4.3 Discussion

Reactive oxygen species have been long known to promote melanoma development, metabolism and metastasis (38). However, the utility of ROS as a therapeutic target is yet to be characterized. Earlier studies in our lab revealed that pharmacologically induced ROS accumulation effectively eliminated cancer cells (61, 113). In this study, I demonstrate that the redox modulatory agent, PEITC selectively kills melanoma cells based on their differential intrinsic oxidative stress status as compared to normal melanocytes. Melanoma cells are under an elevated ROS stress and therefore, are dependent upon antioxidants to maintain redox balance. This makes them vulnerable to therapeutic agents that impair the cellular antioxidant system, leading to ROS accumulation and massive cytotoxicity. Normal melanocytes, on the other hand, are less susceptible to this strategy since they have a lower basal ROS expression and so any additional oxidative stress induced by pharmacological agents is still within the cells’ tolerance threshold. This biochemical difference between normal and cancer cells may be used to selectively eliminate melanomas.

The key conclusions from this study are: (a) PEITC is highly cytotoxic to melanoma cells with limited toxicity in normal melanocytes (figure 4.7). (b) Melanoma cells are under higher intrinsic oxidative stress compared to normal melanocytes (figure 4.9). Basal ROS expression levels of melanoma cells positively correlate with their sensitivity to PEITC (figure 4.5). (c) The elevated oxidative stress renders melanomas sensitive to PEITC which depletes the antioxidant glutathione, leading to severe ROS accumulation, mitochondrial membrane damage, activation of JNK kinase and rapid apoptotic cell death (figure 4.3, 4.4, 4.6, 4.10, 4.12). In contrast, PEITC causes modest ROS increase in normal melanocytes, which is not significant enough to promote cell death (figure 4.8). Consequently, these cells
are resistant to PEITC. (d) Inhibition of cellular glutathione by PEITC is a critical event since the glutathione precursor, NAC, reverses ROS accumulation as well as PEITC mediated cell death (figure 4.13). Furthermore, glutathione depletion can be detected much earlier than cell death suggesting that it is a primary event that instigates ROS elevation and PEITC induced toxicity in melanoma (figure 4.12). (e) Combination of PEITC with BCNU/TMZ ameliorates drug resistance to these agents in melanoma (figure 4.14, 4.16). Higher glutathione depletion, ROS accumulation and consequent cell death is observed in the samples treated with PEITC+BCNU/TMZ as compared to single agent treated samples (figure 4.17).

Trachootham et al. and Xu et al. demonstrated that PEITC disabled glutathione in leukemia cells by two mechanisms: conjugating with glutathione, thus promoting its export from the cells and inhibiting Glutathione Peroxidase (GPX) activity (100, 113). Interestingly, incubation of cells with 5 – 10 µM PEITC caused inhibition of glutathione in the mM range (113). A possible explanation for this stochiometric discrepancy may be that PEITC is concentrated at higher concentrations in cells. Indeed, incubation of the oncogenic T72Ras cells with 5 - 10 µM PEITC for 2 h led to intracellular concentrations of 0.25 – 0.59 mM. Subsequent biochemical analysis indicated that 0.5 mM PEITC suppressed GPX activity by over 90% in a cell-free enzyme assay. These data suggest that PEITC at µM concentrations, used in cell culture assays, may deplete the cellular glutathione pool and GPX enzyme activity significantly leading to ROS accumulation and cytotoxicity. PEITC mediated inhibition of oxidative phosphorylation and mitochondrial damage leading to ROS generation in leukemia and prostate cancer has also been reported (64, 94). Furthermore, PEITC potentiated activation of JNK kinase in DU145 prostate cancer cells (197). Interestingly, this PEITC-induced apoptosis was attenuated by pharmacological inhibition of JNKs with
SP600125 suggesting that JNK activation was critical to PEITC’s activity in these cells (197). Stress induced activation of ERK1/2 was also observed. JNK proteins play a crucial role in regulating cytochrome C release and apoptosis induction following UV irradiation in MEF cells (198). JNK kinases have also been reported to activate apoptotic signaling by modulating the expression of pro-apoptotic and anti-apoptotic mitochondrial proteins (188, 199, 200). PEITC causes a time dependent increased JNK phosphorylation in melanoma cells (figure 4.6). Considering that PEITC triggers apoptosis through mitochondrial membrane permeabilization and caspase activation (figure 4.3, 4.4), it is plausible that JNK proteins may be instrumental in mediating this cell death process. Further studies, using either inhibitors or siRNAs to downregulate JNK expression need to be undertaken to delineate the role of JNK in effecting PEITC’s cytotoxic activity in melanoma cells.

PEITC potentiated ROS accumulation in cells was detected by DCFDA and DAF-FM fluorescent probes but not by HEt suggesting that the free radicals activated by PEITC were mostly peroxides, hydroxyl radicals and nitric oxide but not superoxides (figure 4.10). Nevertheless, the basal superoxide expression in melanoma cells is significantly higher than normal melanocytes (figure 4.9C, D). This may be because of the rapid conversion of superoxides to hydrogen peroxide, peroxynitrites etc (201, 202).

A recent study by Vazquez et al. (2013), demonstrated that the transcriptional co-activator, PGC1α, promoted increased mitochondrial energy metabolism and ROS detoxification capacities in melanoma (203). Consequently, depletion of PGC1α in these cells sensitized them to ROS-inducing drugs such as PEITC and Piperlongumine (203). These results suggest that redox modulation therapies might be especially useful for the subset of melanomas which are PGC1α negative. Further, combining PGC1α inhibition with
ROS accretion (using PEITC or other ROS inducing agents) might be another strategy to increase drug sensitivity in PGC1α positive tumors. The mutant BRAF inhibitor, Vemurafenib promoted mitochondrial respiration and elevated ROS stress in BRAF V600E melanoma cells irrespective of their PGC1α status (204). Further, vemurafenib-resistant cells, generated by treating the parental A375, SKMEL28 and WM9 melanoma cells with increasing concentrations of vemurafenib, exhibited high mitochondrial activity and intrinsic oxidative stress compared to the parental cells (204). Importantly, the vemurafenib-resistant cells were more sensitive to pro-oxidative drugs such as Elesclomal and PEITC than the parental cells (204). Thus redox mediated cell death strategies find increasing utility in melanoma chemotherapy either as a single agent or in combination with currently used clinical melanoma drugs including vemurafenib.

Recent studies reported that activation of oncogenes such as K-Ras and Akt in an inducible system led to an elevated intrinsic oxidative stress phenotype in cells (112, 205). Hence, cancer cells with these specific oncogene activations may be especially susceptible to ROS inducing agents. Nogueira et al., through in vitro and in vivo studies demonstrated that using PEITC (as a single agent or in combination with Rapamycin) in cancer cells with Akt activation may constitute a strategy to selectively inhibit these cells (205). This therapeutic scheme may be of significant utility in melanomas where 20% tumors exhibit PTEN loss leading to increased basal Akt activation (39).

My results indicate that combination of PEITC with either BCNU or TMZ had a synergistic cell death effect (figure 4.16). The cytotoxicity of this drug combination was mediated by redox mechanisms wherein PEITC + TMZ treatment led to significantly higher glutathione depletion and ROS accumulation compared to that of a single agent treatment of
either PEITC or TMZ (figure 4.17). Glutathione efflux pumps (GS-X) play a major role in resistance to various chemotherapeutic inhibitors such as cisplatin, temozolomide, carmustine and other alkylating agents, daunorubicin and natural products (206, 207). The drug resistance mediated by GS-X pumps directly correlates with the cellular glutathione or glutathione S- transferase (GST) levels and involves two mechanisms (a) formation of GSH-S drug conjugate (b) exclusion of this GSH-S drug conjugate from the cell by the GS-X export carrier pump (208, 209). Incidentally, the glutathione synthesis inhibitor, BSO has been reported to partially reverse this GS-X pump induced drug resistance to alkylating agents in lung tumor cell lines (210). Previous reports have indicated that PEITC conjugates with glutathione causing its export from the cell (100). Hence, I propose that PEITC mediated export of glutathione by GS-X pumps would inhibit the exclusion of BCNU and TMZ from the cells. This would sensitize the cells to the DNA alkylating effects of Temozolomide/BCNU, trigger ROS elevation and subsequently cause cell death. Further investigation needs to be undertaken to shed light on the mechanisms that direct the synergistic combinatorial effect of PEITC+TMZ/BCNU in melanoma. These studies may constitute the future perspectives of this project.

In conclusion, my research on the anticancer effects of PEITC in melanoma cells presents a novel strategy to selectively kill melanoma cells based on the differential redox biology between normal melanocytes and melanoma cells. PEITC scavenges glutathione antioxidant system leading to ROS accumulation and cytotoxicity in melanomas. This unique mechanism of action of PEITC and other glutathione inhibitors such as BSO can be used to combat resistance to other chemotherapeutic agents such as Temozolomide, BCNU and Vemurafenib.
Chapter 5: Summary and future directions

5.1 Summary

My PhD dissertation outlines two novel therapeutic strategies to effectively eliminate melanoma cells with limited toxicity to normal melanocytes.

The saponin molecule, OSW-1 is highly cytotoxic to melanoma cells with half-maximal inhibitory concentrations in the sub-nanomolar range. It induces autophagic cell death in melanoma cells by repressing the gene expression of GD3 synthase enzyme, leading to inhibition of gangliosides GD3, GD2 and accumulation of GM3. Ganglioside GD3 is overexpressed in melanoma cells and is a well characterized therapeutic target. OSW-1 also promotes co-localization of ganglioside GD3 with the mitochondrial voltage dependent anion channel 1 (VDAC1) protein. This causes VDAC1 upregulation, mitochondrial membrane permeabilization, superoxide release and loss of calcium homeostasis leading to cell death. The elevated GD3 and GD2 expression in melanoma cells may be the basis of therapeutic selectivity of OSW-1 against melanoma cells since the normal melanocytes as well as the OSW-1 resistant, WM35 DW cells exhibit a decreased basal GD3 and GD2 expression. Further studies need to be undertaken to identify the direct cellular binding target of OSW-1. Studies also need to focus on delineating the mechanisms of selectivity of OSW-1.

The natural product PEITC relies on the redox differences between normal and cancer cells to selectively eliminate cancer cells. In my study, I first characterized the basal ROS and RNOS expression status of melanoma cells and normal melanocytes. I observed that the sensitivity of melanoma cells to PEITC correlated with the basal ROS expression status of the cells. Normal melanocytes with reduced intrinsic oxidative stress were resistant to PEITC. PEITC induced ROS accumulation and glutathione depletion in melanoma cells.
Glutathione depletion occurred much before cell death. Supplementing the cells with the glutathione precursor, NAC, reversed the ROS accumulation and apoptosis mediated by PEITC. This indicates that the glutathione depletion is an important mechanism to PEITC induced cytotoxicity. Further, drug combination of PEITC with TMZ or BCNU ameliorates the chemoresistance to these agents. Future studies will focus on investigating oxidative stress based strategies to combat drug resistance in melanoma.

5.2 Future directions

5.2.1 Mechanisms of selectivity of OSW-1 and PEITC against malignant melanoma cells

Mechanisms of selectivity of OSW-1 towards melanoma cells:

An important feature of OSW-1 mediated cell death was its selectivity against melanoma cells with minimal toxicity to normal melanocytes. I generated OSW-1 resistant WM35 (WM35 DW) cells (figure 3.31) to characterize the mechanisms underlying this therapeutic selectivity of OSW-1. My results indicate that WM35DW cells have a reduced basal ganglioside GD3 and GD2 expression profile compared to the parent WM35 cells (figure 3.32). This ganglioside expression pattern is similar to normal melanocytes which express only trace amounts of the pro-oncogenic ganglioside GD3 but prominent levels of the tumor suppressive ganglioside GM3 (16). OSW-1 inhibits GD3 by repressing gene expression of the enzyme GD3 synthase. Previous reports demonstrated an elevated expression of GD3 Synthase (GD3S) in melanoma compared to other cancers (173). Further, the initial NCI-60 cell line panel in vitro screening identified that melanoma cells (exhibit ganglioside GD3 overexpression) were more sensitive to OSW-1 than the other cell lines tested (67). Hence, I speculate that the differential ganglioside GD3 expression in normal
melanocytes compared to melanoma cells may form the basis for the selectivity of OSW-1 against malignant melanoma cells.

In order to test this possibility, I would perform ganglioside GD3 overexpression experiments in normal melanocytes. I hypothesize that if GD3 mediated signaling is critical to executing the cytotoxic activity of OSW-1, then overexpressing GD3 in normal melanocytes by transfecting them with the \( GD3S \) gene would sensitize these cells to OSW-1. For this purpose, GD3 deficient SK-Mel-28 cells (referred as N1) as well as the GD3 synthase overexpressing G5 and G11 cell clones that were established from N1 cells would serve as valuable cell models (17, 211). If the GD3 expression status of the cell governs its sensitivity to OSW-1, then the N1 cells would be resistant to OSW-1 whereas the G5 and G11 SK-MEL-28 cells would exhibit increased sensitivity to OSW-1. I hope to initiate collaborations to acquire these cells and test them for the cytotoxic effect of OSW-1.

VDAC1 was a critical effector molecule in mediating the cell death effect of OSW-1. RPPA results showed that the OSW-1 resistant WM35 DW cells exhibited a lower basal expression of VDAC 1 and VDAC1 interacting proteins such as Bcl-2 and GSK-3 (figure 3.35 A, C). Further the VDAC1 knock out MEF cells (MEF VDAC1-/-) and siVDAC1 transfected A375SM melanoma cells were resistant to OSW-1 (figure 3.28, 3.29). Hence, I think it is important to compare the basal VDAC1 expression levels across melanoma cell lines and the normal melanocytes and investigate if there is a correlation between VDAC1 expression status and sensitivity to OSW-1. It would be worthwhile to perform VDAC1 transfecion experiments in normal melanocytes to examine if induced expression of VDAC1 would sensitize these cells to OSW-1.
Finally OSW-1 mediated cell death in melanoma cells is through autophagy. Inhibition of autophagy by using pharmacological inhibitors or down regulating the expression of autophagy genes abrogates OSW-1 induced cell death (figure 3.6). Hence, I would investigate normal melanocytes and melanoma cells to ascertain if differences in intrinsic autophagy flux (as indicated by expression of p62, Beclin-1, Atg5, Atg 8) and mitochondrial mass between the cells contributes to their sensitivity to OSW-1.

Mechanisms of melanoma cell selectivity of PEITC:

PEITC inhibited prostate and leukemia cancer growth by disrupting mitochondrial oxidative phosphorylation (OXPHOS) leading to ATP depletion and ROS production (64, 94). Further, ROS generation in was an early, upstream event that contributed to DNA fragmentation, collapse of mitochondrial membrane potential, cytochrome c release and apoptosis. Interestingly, this ROS production and other downstream events such as caspase activation and mitochondrial membrane damage were not observed in the OXPHOS deficient Rho-0 prostate cancer cells, indicating that oxidative stress were a crucial mechanism regulating the selectivity circuitry of PEITC (64). Similar results were observed in my study wherein ROS generation was observed much before cell death and normal melanocytes were resistant to free radical induction by PEITC. This ROS accumulation in melanoma cells was partly due to glutathione depletion. My next step would be to investigate the molecular mechanisms that promote ROS elevation in melanoma cells. Towards this end, my focus will be two-fold: investigate effect of PEITC on (a) mechanisms regulating glutathione biosynthesis and metabolism (b) mitochondrial respiration and the respiratory complex activity.
(a) Glutathione synthesis is a 2-step process: $\gamma$-glutamyl cysteinyl synthetase (GCS) enzyme first catalyzes the generation of $\gamma$-glutamyl cysteine from cysteine and glutamate. Second, the glutathione synthetase enzyme combines $\gamma$-glutamyl cysteine and glycine to produce glutathione (GSH) (212). The availability of cysteine is the rate limiting step in this process (212, 213). The balance between reduced (GSH) and oxidized (GSSG) forms of glutathione in the cell is maintained by the enzymes glutathione peroxidase (GPX) and glutathione reductase (GR). Previous research from our lab indicated that PEITC inhibited glutathione peroxidase activity thus potentiating hydrogen peroxide accumulation in cells (113). First I would perform enzyme activity assays to determine if PEITC treatment abrogates the activity of GPX, GR, GCS and glutathione synthetase enzymes. The fact that supplementation of NAC reverses the cell death effect of PEITC (figure 4.13C) suggests that PEITC may not be inhibiting the activity of enzymes that catalyze glutathione biosynthesis (i.e. GCS and glutathione synthetase). After having identified the putative target enzyme modulated by PEITC, I would then examine the effect of PEITC on this target enzyme expression and activity in melanoma cells vs. normal melanocytes.

(b) To examine the effect of PEITC on oxidative phosphorylation, I would employ the Seahorse Bioscience XF24 extracellular flux analyzer to compare oxygen consumption rate, and reserve respiration capacity in normal melanocytes and melanoma cells (both basal levels and PEITC induced changes). I would then use metabolic inhibitors such as oligomycin, 2-Deoxy glucose, rotenone and carbonyl cyanide p-trifluoromethoxyphenyl hydrazone (FCCP) to determine the effect of PEITC on each of the mitochondrial respiratory complex activity. Simultaneously, I would perform western blots to test the effect of PEITC on mitochondrial respiratory complex (es) expression in both normal melanocytes and melanoma cells.
I believe that this two-fold approach to characterizing the mechanisms that regulate PEITC induced ROS accumulation in melanoma cells vs. normal melanocytes will provide insights into the selectivity of PEITC towards melanoma cells.

5.2.2 Characterization of the direct molecular target for OSW-1: SREBP and GD3S regulatory signaling pathways

My PhD dissertation research demonstrates that OSW-1 represses the gene expression of GD3 synthase enzyme in melanoma cells. However, it remains unclear if GD3 Synthase is the direct target of OSW-1. The fact that OSW-1 inhibits GD3S gene expression points towards the possibility that OSW-1 may potentially interact/bind with a protein (such as a transcription factor) that is upstream of GD3 synthase signaling pathway and regulates its expression. Recent studies demonstrated that GD3 expression in melanoma cells correlated with activation of the steroid regulatory element binding protein (SREBP) leading to increased expression of HMGCR and elevated cholesterol biosynthesis (10). Burgett et al. (2011), through affinity chromatography and quantitative mass spectrometry approaches in HeLa-S3 cell lysate treated with the OSW-1 affinity reagent (prepared by covalently linking OSW-1 analogue to sepharose) identified the oxysterol binding protein (OSBP) as a cellular binding target of OSW-1 (69). However, the authors in this study did not explore the functional significance (such as effect on downstream proteins, signaling pathways etc.) of this interaction between OSW-1 and OSBP; nor did they prove that this interaction was imperative to mediating the cytotoxic effect of OSW-1. More importantly, shRNA mediated knockdown of OSBP expression did not exhibit a cytotoxicity profile similar to OSW-1 treatment of cells, suggesting that there may be other molecular binding targets for OSW-1. Yamada et al. (2014), using fluorescent OSW-1 demonstrated that the putative binding
targets of OSW-1 could be located in the ER and golgi apparatus since the fluorescent OSW-1 molecule localized with high affinity to these organelles in the cell (82). Based on these studies and my own research results I postulate that OSW-1 potentially interacts with a ganglioside or lipid signaling protein which is primarily located in the ER/golgi apparatus of the cell.

SREBP is a master transcription factor regulating cholesterol and fatty acid biosynthesis (167). The premature and mature forms of SREBP reside in the ER and golgi apparatus, before being cleaved by serine proteases and transported to the nucleus (214). Further, SREBP expression correlates with GD3 expression status of the cell (10). Hence, I plan to investigate if OSW-1 directly interacts with SREBP in melanoma cells. Below I outline the experimental approaches I would employ to test this hypothesis.

(a) I would perform Chromatin Immunoprecipitation (ChIP) for SREBP binding DNA elements in the control and OSW-1 treated cells. I would then perform real time-PCR experiment with the ChIP output DNA product to detect the expression of SREBP target genes including FASN and HMGCR. If OSW-1 directly interacted with SREBP, then the expression of FASN and HMGCR genes would be suppressed in the OSW-1 treated samples.

(b) Biotinylated OSW-1 (75) will be used for this experimental design. Streptavidin magnetic beads (Solulink, San Diego, CA) may be used to isolate proteins that bind to biotinylated OSW-1 in an experimental design similar to affinity chromatography. Gel electrophoresis and quantitative mass spectrometry will then be performed with the product protein sample from the streptavidin beads-biotinylated OSW-1 pull down assay to identify the interaction partners of OSW-1, including SREBP-1.
The second approach of using the streptavidin beads-biotinylated OSW-1 pull down assay (similar design as affinity chromatography) will be extremely useful in identifying the interaction protein partners of OSW-1. It would also be important to determine the effect of OSW-1 on the expression of GD3 synthase expression regulatory proteins such as TWIST, UDP-GlcNAc 2-epimerase/ManNAc 6-kinase (GNE), and amyloid precursor protein (APP) by immunoprecipitation, streptavidin beads-biotinylated OSW-1 pull down assay and western blotting (165, 166, 215).

5.2.3 Oxidative stress based strategies to combat drug resistance in melanoma using PEITC

My results demonstrate that combination of PEITC with either TMZ or BCNU induces a synergistic drug cytotoxic effect. This increased cell death due to PEITC+TMZ/BCNU co-treatment in melanoma cells was characterized by enhanced depletion of glutathione and consequent ROS accumulation in cells. Prior studies have shown BCNU inhibits glutathione reductase in blood cells (194). My results indicate that treatment of melanoma cells with 100 µM BCNU or 0.5 mM TMZ for 24 h causes modest glutathione inhibition. However, supplementation of PEITC (5 µM) during the last 3 h triggered significant glutathione depletion. Glutathione impairment occurred much earlier than cell death implying that it is an important mechanism regulating the anticancer effect of PEITC ± BCNU/TMZ drug combination in melanoma cells. I postulate that PEITC would promote increased cytotoxicity in TMZ/BCNU treated cells due to its ability to conjugate with and export GSH out of the cell thus limiting drug resistance to TMZ/BCNU by Glutathione efflux pumps. This in combination with inhibition of GR by BCNU may lead to a higher cell death.
To test this possibility, I would examine intracellular as well as culture media concentrations of glutathione and PEITC after PEITC ± TMZ/BCNU treatments in melanoma cells. Furthermore, I would also investigate the effect of TMZ/BCNU/PEITC treatment on GPX/GR enzyme activity in melanoma cells.

ROS generation and ERK1/2-mediated protective autophagy has been reported to cause resistance to Temozolomide in glioblastoma cells (216, 217). I propose that combination of PEITC with TMZ/BCNU would push the ROS generation triggered by these alkylating agents beyond the cell’s tolerance levels leading to massive toxicity. Figure 4.17 A, B and C describes that PEITC + BCNU/TMZ treatment causes elevated ROS, nitric oxide and mitochondrial superoxide generation. Of specific interest is the induction mitochondrial superoxide by this drug combination. Inhibition of oxidative phosphorylation may cause superoxide accumulation in the mitochondria. Hence I would perform western blot as well as use Seahorse Bioscience XF24 extracellular flux analyzer to investigate the effect of PEITC + TMZ/BCNU drug combination on mitochondrial respiration and oxygen consumption. Finally, I would test the efficacy of this PEITC ± TMZ/BCNU drug combination in vivo using athymic nude mice. Khor et al., demonstrated that i.p. injections of PEITC (2.5 µmol, 3X per week) and curcumin (3 µmol, 3X per week) in human PC-3 prostate xenografts in immunodeficient mice significantly reduced tumor growth (218). I propose to test the effect of PEITC ± TMZ/BCNU in immunodeficient mice inoculated with A375SM cells with optimized drug dosage and treatment times.

Recent studies have indicated that the clinical mutant BRAF inhibitor, vemurafenib, causes ROS stress in melanoma cells (204). Combination of vemurafenib with ROS inducing agents might be a worthwhile strategy to eliminate these tumors. Important PEITC may be
effective in killing vemurafenib resistant tumors since these are characterized by elevated intrinsic oxidative stress compared to the vemurafenib sensitive melanomas.

Both OSW-1 and PEITC induced cell death is characterized by elevations in ROS levels and mitochondria dependent mechanisms of action. Hence it will be worthwhile to characterize the combinatorial drug effect of OSW-1 and PEITC in melanoma cells. In this study, the effect of OSW-1 + PEITC combination on various molecular markers such as glutathione, mitochondrial VDAC1, gangliosides GD3 and GD2, nitric oxide and superoxides will be examined. It may be crucial to be compare and correlate the basal expression and drug induced changes of these markers in a particular cell line to its sensitivity to OSW-1+PEITC treatment. This type of analysis will be of clinical significance as it may lead to the identification of biomarkers that would predict patient response to this combinatorial OSW-1+PEITC drug treatment strategy.

Therapeutic selectivity is an important characteristic of any successful pharmacological agent. In this dissertation, I elucidate the mechanisms of anticancer activity and therapeutic selectivity of two novel inhibitors, OSW-1 and PEITC. OSW-1 exerts its antiproliferative effect by inhibiting ganglioside GD3 expression and causing VDAC1 activation, subsequently leading to acute autophagy. PEITC relies on the differential redox biology between the cancer cells and normal melanocytes to preferentially eliminate melanoma cells. Both OSW-1 and PEITC employ relatively novel therapeutic strategies to effectively eliminate malignant melanomas and warrant further testing in pre-clinical and clinical settings.
References


65. Kubo S MY, Terao M, Sashida Y, Nikaido T, and Ohmoto T. Acylated cholestane
glycosides from the bulbs of ornithogalum saundersiae. Phytochemistry. 1992;31(11):3969-
73.

of OSW-1 and their cytotoxic activities. Bioorg Med Chem Lett. Epub 2011/05/03.

glycosides with potent cytostatic activities on various tumor cells from Ornithogalum

68. Guo C, LaCour TG, Fuchs PL. On the relationship of OSW-1 to the cephalostatins.

69. Burgett AW, Poulsen TB, Wangkanont K, Anderson DR, Kikuchi C, Shimada K,
Okubo S, Fortner KC, Mimaki Y, Kuroda M, Murphy JP, Schwalb DJ, Petrella EC, Cornella-
Taracido I, Schirle M, Tallarico JA, Shair MD. Natural products reveal cancer cell
2011/08/09.

70. Kuroda M, Mimaki Y, Yokosuka A, Sashida Y, Beutler JA. Cytotoxic cholestane

71. Ma X, B. Yu, Y. Hui, D. Xiao, and J. Ding. Synthesis of glycosides bearing the
disaccharide of OSW-1 or its 14-linked analogue and their antitumor activities. Carbohydrate
research. 2000;329:495-505.


Vita

Kausar Begam Riaz Ahmed was born in Chennai, India on November 15, 1984, the daughter of Rahimunissa Riaz Ahmed and Riaz Ahmed. After completing her high school at Holy Family Convent Higher Secondary School, Chennai, India in 2002, she entered Anna University in Chennai, India. She received the degree of Bachelor of Technology with a major in industrial biotechnology from Anna University in May, 2006. She then travelled to the United States of America to pursue a Masters in Biotechnology at the University of Utah. In August of 2008 she entered The University of Texas Graduate School of Biomedical Sciences at Houston.

Permanent address:

No.1, Swami Malai Nagar,

Old Pallavaram, Chennai – 600 117,

India.