SENSITISING HUMAN MELANOMA CELLS
TO TRAIL-INDUCED APOPTOSIS

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B. BiomedSci (Hons)

Thesis submitted in fulfilment of the requirements
for obtaining the degree of

DOCTOR OF PHILOSOPHY in
Surgical Science

School of Medicine and Public Health

University of Newcastle

December 2012
STATEMENT OF ORIGINALITY

The thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to this copy of my thesis, when deposited in the University Library**, being made available for loan and photocopying subject to the provisions of the Copyright Act 1968.

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I hereby certify that the work embodied in this thesis contains a published paper/s/scholarly work of which I am a joint author. I have included as part of the thesis a written statement, endorsed by each co-author, attesting to my contribution to the joint publication/s/scholarly work.

___________________
Hsin-Yi Tseng
ACKNOWLEDGEMENT OF COLLABORATION

I hereby certify that the work embodied in this thesis has been done in collaboration with other researchers. I have included as part of the thesis a statement clearly outlining the extent of collaboration, with whom and under what auspices at the beginning of each research chapters.

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Hsin-Yi Tseng
THESIS BY PUBLICATION

I hereby certify that this thesis is in the form of a series of published papers of which I am a joint author. I have included as part of the thesis a written statement from each co-author, endorsed by the Faculty Assistant Dean (Research Training), attesting to my contribution to the joint publications.

___________________
Hsin-Yi Tseng
DEDICATION

I dedicate this thesis to all my family members and relatives, especially my parents Tai Yuan Tseng and Shu Hui Wang, my siblings, Hsin-Hui Tseng and Hsuan Chih Tseng, who have been so supportive throughout my PhD candidature. I also dedicate this thesis to my partner Kwang Hong Tay who keeps me going during depressed and stressful moments.
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Thanks must go to my housemates and taekwondo club buddies!

Finally, deepest thanks to my parents, Tai Yuan Tseng and Shu Hui Wang, and my siblings, Hsin-Hui Tseng and Hsuan Chih Tseng. Without their moral support, this thesis would not have been possible.
Publications Arising from Work in this Thesis


Other Publications during Candidature for PhD


Conference Publications during Candidature for PhD

Tseng, H.Y., Zhang, X.D., Hersey, P. Nutlin-3 Enhances TRAIL-Induced Apoptosis in Human Melanoma Cells.
   ➔ Poster Presentation (HMRI Cancer Research Program 2009)

   ➔ Poster Presentation (HMRI Cancer Research Program 2009)

Yang, F., Tay, K.H., Dong, L., Thorne, R.F., Jiang, C.C., Yang, E., Tseng, H.Y., Liu, H., Christopherson, R., Hersey, P., and Zhang, X.D., Cystatin B inhibition of TRAIL-induced apoptosis is associated with the protection of FLIP(L) from degradation by the E3 ligase itch in human melanoma cells.
   ➔ Poster Presentation (HMRI Cancer Research Program 2009)
   ➔ Poster Presentation (SMR (Society for Melanoma Research) 2010)

   ➔ Poster Presentation (HMRI Cancer Research Program 2009)
   ➔ Poster Presentation (Lorne Cancer Conference 2010)

Tseng, H.Y., Liu, H., Tay, K.H., Jiang, C.C., Yang, F., Hersey, P., Zhang, X.D. Nutlin-3 Sensitises Human Melanoma Cells to TRAIL-Induced Apoptosis by Up-regulation of TRAIL-R2 and Down-regulation of XIAP.
   ➔ Poster Presentation (Lorne Cancer Conference 2010)
   ➔ Poster Presentation (10 Best Research Showcase, Faculty of Health, University of Newcastle 2010)
Tseng, H.Y.,  *Contrasting Effects of Nutlin-3 on TRAIL- and Docetaxel-Induced Apoptosis due to Up-regulation of TRAIL-R2 and Mcl-1 in Human Melanoma Cells.*

- Oral Presentation (10 Best Research Showcase, Faculty of Health, University of Newcastle 2010)
- Oral Presentation (HMRI Cancer Research Program 2010)


- Poster Presentation (SMR 2010)
- Poster Presentation (HMRI Cancer Research Program 2010)
- Poster Presentation (Lorne Cancer Conference 2011)
- Oral Presentation (FEBS Translational Cancer Research Course 2011)


- Poster Presentation (SMR 2010)

Tay, K.H., Jiang C.C., Tseng, H.Y., Hersey, P., Zhang, X.D.  *Dysregulation of the CHOP-BIM Pathway contributes to Resistance of Melanoma Cells to ER Stress-Induced Apoptosis.*

- Poster Presentation (Lorne Cancer Conference 2011)

Tseng, H.Y., Yan, Y., Hersey, P., Zhang X.D.  *Phosphatidylinositol 4,5-Biphosphate 5-Phosphatase A (PIB5PA) Regulates PI3K/Akt Signalling in Human Melanoma Cells.*

- Oral Presentation (Melanoma Institute Australia Research Retreat 2011)


- Oral Presentation (AACBS (Australian Association of Chinese Biomedical Scientist) 2011)
- Poster Presentation (HMRI Cancer Research Program 2011)

Poster Presentation (Lorne Cancer Conference 2012)
Poster Presentation (AACR 2012)
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SYNOPSIS

Melanoma is a skin cancer that remains a major public health problem in Australia because of its high incidence and the high morbidity and high mortality associated with the disease. Melanoma has proven largely resistant to many chemotherapeutic and biological agents. The introduction of a member of Tumour Necrosis Factor (TNF) family named TNF-Related Apoptosis Inducing Ligand (TRAIL) seemed to be a promising candidate due to its differential sensitivity to cancer and normal cells. Although many studies have reported approaches for sensitising cancer cells to TRAIL-induced apoptosis via up-regulation of its death receptors, TRAIL-R1 and TRAIL-R2, little was known about the regulation of these receptors. Previously, reports from our laboratory have shown that the sensitivity of melanoma cells to TRAIL-induced apoptosis is in general correlated with the levels of the cell surface expression of TRAIL-R2. Therefore, the general aim of this thesis was to understand the underlying mechanism by which TRAIL-R2 is regulated and to provide more information in identifying new therapeutic approaches for increasing the sensitivity of melanoma cells to apoptosis by TRAIL.

In Chapter Three, we identified the Murine Double Minute 2 (MDM2) antagonist, Nutlin-3, could enhance TRAIL-induced apoptosis as a result of p53-mediated up-regulation of TRAIL-R2. Unexpectedly, Nutlin-3 up-regulated Myeloid-Cell Leukaemia Sequence 1 (Mcl-1) and inhibited apoptosis induced by the microtubule-targeting drug docetaxel. The contrasting effects of Nutlin-3 on TRAIL- and docetaxel-induced apoptosis demonstrated that Nutlin-3 may be a useful agent in improving the therapeutic efficacy of TRAIL in melanoma but could have unexpected adverse effects in combination with other chemotherapeutic drugs such as docetaxel.

The MAGE proteins have been demonstrated to impinge on cell survival, proliferation and apoptosis in cancer. Studies in Chapter Four demonstrated that one of the MAGE proteins, MAGE-D2, plays an important role in protecting melanoma cells from TRAIL-induced apoptosis by suppressing TRAIL-R2 expression. We determined that MAGE-D2 is generally expressed at high levels in melanoma cells compared to melanocytes. Although its inhibition by small interfering RNA (siRNA) did not cause
cell death, it rendered melanoma cells more sensitive to TRAIL-induced apoptosis which was associated with enhanced formation of Death-Inducing Signalling Complexes (DISC) and up-regulation of TRAIL-R2. Regulation of TRAIL-R2 by Melanoma-associated Antigen D2 (MAGE-D2) also appeared to be mediated by p53. We have shown that MAGE-D2 plays a role in repressing p53 expression in melanoma cells, as knockdown of MAGE-D2 resulted in up-regulation of p53 activity which in turn leads to the up-regulation of TRAIL-R2 protein expression. This up-regulation is not observed in p53-null or mutant p53 melanoma cells with MAGE-D2 knocked down, suggesting the dependency of p53 in regulating TRAIL-R2. Altogether, this suggests that targeting MAGE-D2 may be a useful strategy in improving the therapeutic efficacy of TRAIL in melanoma.

Although it is well-known that TRAIL-R2 can be up-regulated by p53, the study in Chapter Five showed that up-regulation of TRAIL-R2 by 2-Deoxy-D-Glucose (2-DG) was independent of p53. Instead, X-box Binding Protein 1 (XBP1) in the endoplasmic reticulum (ER) stress pathway was responsible for this up-regulation. Results in this chapter demonstrated that p53-null and mutant p53 melanoma cells displayed increased levels of TRAIL-R2 expression upon 2-DG treatment and that inhibiting p53 expression in p53 wild-type melanoma cell lines did not impact on the up-regulation of TRAIL-R2 by 2-DG.

In Chapter Six, we further demonstrated that conditional induction of p53 expression did not regulate TRAIL-R2 protein expression in melanoma cells and that other mechanisms may be involved. In particular, enhancing p53 levels in p53-inducible cell lines did not impact on the level of TRAIL-R2 expression or sensitise melanoma cells to TRAIL-induced apoptosis. Interestingly, we determined that Cisplatin (CDDP), a DNA-damaging drug that activates p53, could up-regulate TRAIL-R2 mRNA but did not up-regulate TRAIL-R2 protein levels. This evidence pointed to regulation by translational mechanisms. The results were further supported by studies in TRAIL-selected cells where it was found that exposure to TRAIL for a prolonged period of time resulted in the down-regulation of cell surface TRAIL-R2 but not its mRNA. The precise mechanism of translational control remains to be defined but appears to involve cap-independent mechanisms.
**List of Abbreviations**

<table>
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<th>Abbreviation</th>
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<tr>
<td>2-DG</td>
<td>2-Deoxy-D-Glucose</td>
</tr>
<tr>
<td>2-ME</td>
<td>β-Mercaptoethanol</td>
</tr>
<tr>
<td>4E-BP</td>
<td>eIF4E-Binding Protein</td>
</tr>
<tr>
<td>5-FU</td>
<td>Fluorouracil</td>
</tr>
<tr>
<td>AJCC</td>
<td>American Joint Commission on Cancer</td>
</tr>
<tr>
<td>α-MSH</td>
<td>α-Melanocyte-Stimulating Hormone</td>
</tr>
<tr>
<td>APP</td>
<td>Amyloid Precursor Protein</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium Persulphate</td>
</tr>
<tr>
<td>ATM</td>
<td>Ataxia Telangiectasia Mutated</td>
</tr>
<tr>
<td>BAD</td>
<td>Bcl-2-Antagonist of Cell Death</td>
</tr>
<tr>
<td>BAK</td>
<td>Bcl-2 Antagonist/Killer</td>
</tr>
<tr>
<td>BAX</td>
<td>Bcl-2-Associated X Protein</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B Cell Lymphoma Gene 2</td>
</tr>
<tr>
<td>B-CLL</td>
<td>B Chronic Lymphocytic Leukaemia</td>
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<tr>
<td>BH</td>
<td>Bcl-2 Homology</td>
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<tr>
<td>BID</td>
<td>BH3-Interacting-Domain Death Agonist</td>
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<tr>
<td>BIK</td>
<td>Bcl-2-Interacting Killer</td>
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<tr>
<td>BIM</td>
<td>Bcl-2-Interacting Mediator of Cell Death</td>
</tr>
<tr>
<td>BMF</td>
<td>Bcl-2 Modifying Factor</td>
</tr>
<tr>
<td>BOK</td>
<td>Bcl-2-Related Ovarian Killer</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CARD</td>
<td>Caspase-Recruitment Domain</td>
</tr>
<tr>
<td>CCCP</td>
<td>Carboxyl Cyanide 3-Chlorophenylhydrazone</td>
</tr>
<tr>
<td>CDDP</td>
<td>Cisplatin</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin Dependent Kinase</td>
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<td>CDS</td>
<td>Coding Sequence</td>
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<tr>
<td>Chk</td>
<td>Checkpoint Kinase</td>
</tr>
<tr>
<td>CHX</td>
<td>Cycloheximide</td>
</tr>
<tr>
<td>CLL</td>
<td>Chronic Lymphocytic Leukaemia</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T Lymphocyte</td>
</tr>
<tr>
<td>DAXX</td>
<td>Death Domain-Associated Protein</td>
</tr>
<tr>
<td>DcR</td>
<td>Decoy Receptor</td>
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</table>
DD: Death Domain
DED: Death Effector Domain
DISC: Death-Inducing Signalling Complexes
DMEM: Dulbecco’s Modified Eagle’s Medium
DNA: Deoxyribonucleic Acid
DR: Death Receptor
E1: Ubiquitin-Activating Enzyme
E2: Ubiquitin-Conjugating Enzyme
E3: Ubiquitin Ligase
EDAR: Ectodysplasin A Receptor
EDTA: Ethylenediaminetetraacetic Acid
EGTA: Ethylene Glycol-bis(β-amino-ethyl ether)N, N’-tetraacetic Acid
EF: Elongation Factor
EGF: Epidermal Growth Factor
eIF: Eukaryotic Initiation Factor
ER: Endoplasmic Reticulum
ERK: Extracellular Signal-Regulated Kinase
FADD: Fas-Associated Death Domain
FCS: Foetal Calf Serum
FDA: Food and Drug Administration
FITC: Fluorescein Isothiocyanate
FLICE: FADD-like Interleukin 1β-Converting Enzyme
FLIP: FLICE-Inhibitory Protein
GDP: Guanosine Diphosphate
GTP: Guanosine Triphosphate
HDAC: Histone Deacetylase
Hrk: Harakiri
IAP: Inhibitor of Apoptosis
ICE: Interleukin-1-β-Converting Enzyme
IF: Initiation Factor
IFN: Interferon
IL: Interleukin
IRES: Internal Ribosome Entry Site
ITAF: IRES-Transacting Factors
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>kb</td>
<td>Kilobase</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal Kinase</td>
</tr>
<tr>
<td>LARD</td>
<td>Lymphocyte-Associated Receptor of Death</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny Broth</td>
</tr>
<tr>
<td>LT-α</td>
<td>Lymphotoxin-α</td>
</tr>
<tr>
<td>m^7G</td>
<td>7-Methyl Guanosine</td>
</tr>
<tr>
<td>MAGE</td>
<td>Melanoma-Associated Antigen</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-Activating Protein Kinase</td>
</tr>
<tr>
<td>MC1R</td>
<td>Melanocortin Receptor 1</td>
</tr>
<tr>
<td>Mcl-1</td>
<td>Myeloid-Cell Leukaemia Sequence 1</td>
</tr>
<tr>
<td>MDM2</td>
<td>Mouse Double Minute 2</td>
</tr>
<tr>
<td>MEK</td>
<td>Mitogen-Activated Protein Kinase Kinase</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean Fluorescence Intensity</td>
</tr>
<tr>
<td>MHD</td>
<td>MAGE Homology Domain</td>
</tr>
<tr>
<td>miR</td>
<td>MicroRNA</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MTS</td>
<td>3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear Factor-Kappa B</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve Growth Factor</td>
</tr>
<tr>
<td>NGFR</td>
<td>Nerve Growth Factor Receptor</td>
</tr>
<tr>
<td>NHPA</td>
<td>National Health Priority Area</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer</td>
</tr>
<tr>
<td>NSCLC</td>
<td>Non-Small Cell Lung Cancer</td>
</tr>
<tr>
<td>OPG</td>
<td>Osteoprotegrin</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly(ADP-Ribose) Polymerase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<td>PCD</td>
<td>Programmed Cell Death</td>
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<td>Polymerase Chain Reaction</td>
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<td>Programmed Death Receptor 1</td>
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<td>PDK</td>
<td>Phosphoinositide-Dependent Kinase</td>
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<td>R-Phycoerythrin</td>
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<tr>
<td>PI</td>
<td>Propidium Iodide</td>
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PI3K: Phosphatidylinositol 3-Kinase
PIB5PA: Phosphatidylinositol-4,5-biphosphate 5-Phosphatase A
PKA: Protein Kinase A
PP2A: Protein Phosphatase 2A
PTEN: Phosphatase and Tensin Homologue Deleted on Chromosome 10
PTM: Post-Translational Modification
PUMA: p53-Upregulated Modulator of Apoptosis
qPCR: Quantitative Polymerase Chain Reaction
RF: Release Factor
RGP: Radial Growth Phase
RHD: Rel Homology Domain
RIP: Receptor Interacting Protein
RNA: Ribonucleic Acid
ROS: Reactive Oxygen Species
rRNA: Ribosome RNA
RTK: Receptor Tyrosine Kinase
SAPK: Stress-Activated Protein Kinase
SDS-PAGE: Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
shRNA: Short Hairpin RNA
siRNA: Small Interfering RNA
SNB: Sentinel Node Biopsy
SOC: Super-Optimal broth with Catabolite repression
SODD: Silencer of Death Domain
TAA: Tumour-Associated Antigens
TBE: Tris Borate EDTA
TBS: Tris-Buffered Saline
TBS-T: TBS-Tween 20
TEMED: N, N, N’, N’-Tetramethylethylenediamine
TGA: Therapeutic Goods Administration
TM: Tunicamycin
TNF: Tumour Necrosis Factor
TNFR: Tumour Necrosis Factor Receptor
TNFRSF: Tumour Necrosis Factor Receptor Super Family
TRADD: TNFR-Associated Death Domain Protein
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