Treatment of Ewing Sarcoma Family of Tumours through the pharmacological activation of p53

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This thesis is dedicated to the memory of

Tom Wood

and all the sarcoma patients that generously donated material for this study.

May our findings one day alleviate the burden of sarcoma and
take one step closer towards a cure.
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OVERVIEW

Sarcomas constitute a diverse heterogeneous group of solid bone and soft tissue malignancies of mesenchymal origin. It is estimated that sarcomas account for approximately 15% of all paediatric and 1% of all adult cancers, with 200,000 new cases reported worldwide each year. To date 60 distinct histological subtypes have been described, ranging from indolent to highly invasive and metastatic. Clinical management primarily consists of wide excisional surgery in conjunction with adjuvant therapies (radiotherapy or chemotherapy) depending on the subtype. Despite significant strides in understanding the cytogenetic profiles of sarcomas, limited improvement in overall survival rates has been achieved over the past few decades for most sarcoma subtypes. The use of multi-agent schedules and dose intensification in patients with chemo-sensitive subtypes has yielded some improvement in survival but at the expense of significantly increased toxicity and risk of developing secondary malignancies. In light of the limitations of systemic chemotherapy, particularly for those sarcoma subtypes that are intrinsically chemo-resistant, new targeted therapeutic modalities are urgently required.

Tumourigenesis is a multifaceted process that requires dysregulation of several pathways that are essential for cellular growth and survival. One such pathway critical for the prevention of oncogenic transformation is mediated by the tumour suppressor p53. The TP53 gene located at 17p13 encodes a 53-kDa nuclear phosphoprotein with sequence-specific DNA-binding properties. In response to various cellular and oncogenic insults, p53 drives the expression of specific target genes required for the initiation of cell cycle arrest, apoptosis, DNA damage repair, and senescence pathways. Underscoring its pivotal role
against tumour development, the p53 gene (TP53) is mutated in at least 50% of all human malignancies. In the remaining wild-type p53 tumours, p53 function is suppressed through various mechanisms. In the quest for more effective cancer therapeutics, considerable research has been undertaken to reinstate p53 function in wild-type p53 tumour cells through the use of small targeted agents. As sarcomas are predominately of wild-type p53 status with less than 20% TP53 mutations, this unique tumour group presents an ideal model system for the pre-clinical testing of p53-based therapies.

One mechanism frequently employed by wild-type p53 tumours to circumvent the tumour surveillance function of p53 is through overexpression or amplification of MDM2 (Murine Double Minute 2) or MDM4 (structural homologue of MDM2). MDM2 is a key E3 ubiquitin ligase that targets p53 for ubiquitin-dependent degradation, thereby tightly regulating the stability and subcellular localization of p53. In contrast, MDM4 primarily regulates the transcriptional activity of p53 as it possesses no intrinsic E3 ligase activity and therefore cannot directly promote the degradation of p53. Crystallization studies of the MDM2–p53 complex revealed that three residues within the transactivation domain of p53 (Phe^{19}, Trp^{23} and Leu^{26}) were responsible for binding the hydrophobic cleft located on the N-terminal surface of MDM2. The well-defined, small interface of MDM2-p53 has led to the design of numerous small-molecule inhibitors to target the MDM2-p53 interaction. The most well-known and extensively studied MDM2-p53 antagonist is Nutlin-3a. Identified by Vassilev and colleagues (Hoffmann-La Roche), this cis-imidazoline compound effectively binds the p53-binding groove of MDM2 by mimicking the interactions of the three key p53 amino acids. Promising results from several preclinical studies have demonstrated the therapeutic potential of Nutlin-3a in various solid and haematological malignancies with
wild-type p53. As the clinical translation of MDM2 inhibitors is relatively advanced with Nutlin-3a (RG7112) entering phase II trials, the principal focus of the research detailed in this thesis was to evaluate whether pharmacological activation of the p53 pathway can provide a new therapeutic means for the targeted treatment of sarcomas, in particular Ewing sarcoma. In addition to Nutlin-3a, the ability of low dose actinomycin D and SJ-172550 (MDM4 inhibitor) to restore p53 function has also been assessed.
The primary focus of this research has been to investigate the ability of new targeted therapeutic agents to restore the tumour suppressive properties of p53 in sarcomas using *in vitro*, *in vivo* and *ex vivo* techniques. This thesis is composed of seven chapters, four of which are published papers (chapters 1, 2, 4 and 5). Chapter 3 is currently under review.

◊ **Chapter 1 (published review paper, Sarcoma-2011)**

This chapter summarizes recent insights into the functional capabilities and regulation of p53 in Ewing sarcoma, with a particular focus on the cross-talk between p53 and the *EWS-FLI1* gene rearrangement frequently associated with this disease. The potential of several p53 activators currently undergoing clinical testing is also discussed.

◊ **Chapter 2 (published manuscript, Clinical Cancer Research-2011)**

This study has evaluated the molecular and cellular responses of cultured Ewing sarcoma cell lines following exposure to Nutlin-3a, the recently developed MDM2 antagonist. Our findings demonstrate that Nutlin-3a induces robust p53-dependent apoptosis and can synergize with current Ewing sarcoma chemotherapy protocols. Furthermore we were the first to conclusively elucidate that MDM4 is overexpressed in a high proportion of Ewing sarcoma cell lines.
Chapter 3 (manuscript under review, Journal of Experimental Medicine)

In the age of personalised medicine, the use of biomarkers to predict patient response and resistance, will be critical for the development and optimal clinical implementation of molecularly targeted therapies. Using a novel ex vivo tissue explant system, this study has evaluated the cellular responses and molecular mechanisms underlying sensitivity of fresh human sarcoma specimens to Nutlin-3a. Detailed genomic analyses of the p53 pathway alterations in these sarcomas have identified candidate biomarkers that may prove useful in predicting response to Nutlin-3a.

Chapter 4 (published manuscript, Oncology Reports-2013)

This study has evaluated the efficacy of Drozitumab, a human monoclonal agonistic antibody directed against Death Receptor 5 (DR5), as a new therapeutic avenue for the targeted treatment of sarcomas. As DR5 is a p53 regulated gene, the anti-tumour activity of Drozitumab as a monotherapy or in combination with Nutlin-3a was evaluated in a panel of sarcoma cell lines in vitro and human sarcoma patient samples ex vivo. Our findings provide the first pre-clinical evidence that pre-activation of the p53 pathway in conjunction with Drozitumab will potentially offer an effective therapeutic means to maximise the apoptotic response from both the extrinsic and intrinsic pathway.
Chapter 5 (published manuscript, ACS Chemical Biology-2013)

The 26S proteasome has emerged over the past decade as an attractive therapeutic target in the treatment of cancers. Here, we report new tripeptide aldehydes (potent proteasome inhibitors) that demonstrate p53 dependent apoptotic activity specifically in sarcoma cell lines and not in non-malignant primary cells. Collectively, these findings suggest that p53 is a critical downstream mediator of cell death following proteasomal inhibition.

Chapter 6

Low nanomolar doses of the FDA approved chemotherapeutic agent actinomycin D have been shown to mimic Nutlin-3a in the highly specific activation of p53. This chapter examines the p53 dependent effects of low dose actinomycin D in Ewing sarcoma cell lines.

Chapter 7

p53-based cyclotherapy has emerged as a new paradigm in cancer treatment that specifically protects normal tissues from the cytotoxic effects of chemotherapy, whilst maintaining the genotoxicity of chemotherapy to tumour cells. The purpose of this dose defining study was to define the concentration of actinomycin D required to induce reversible cellular growth arrest of intestinal cells in vivo.
DECLARATION

I, Kathleen Irene Pishas, certify that this work 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. In addition, I certify that no part of this work will in the future be used in a submission for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution for the joint-award of this degree.

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Signed: ……………………………….. Date: ………………………………..

28th March 2013
PUBLICATIONS

논문 제목: Nutlin-3a is a potential therapeutic for Ewing sarcoma. 
公報名稱: Clinical Cancer Research 17: 494-504, 2011

논문 제목: Targeting the p53 Pathway in Ewing sarcoma.
저자: Neilsen PM, Pishas KI, Callen DF, and Thomas DM.
公報名稱: Sarcoma, 746939, 2011


논문 제목: New 26S Proteasome Inhibitors with High Selectivity for Chymotrypsin-Like Activity and p53-Dependent Cytotoxicity.
저자: Neilsen PM, Pehere AD, Pishas KI, Callen DF, and Abell AD.

논문 제목: Pre-activation of the p53 pathway through Nutlin-3a sensitizes sarcomas to Drozitumab therapy.
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PUBLICATIONS


SCHOLARSHIPS & GRANTS

◦ SAHMRI Beat Cancer Project Travel Grant 2013

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◦ European Science Foundation and EMBO, Molecular Biology & Innovative Therapies in Sarcoma Conference Travel Grant 2012

◦ University of Adelaide, Discipline of Medicine Travel Grant 2012

◦ University of Adelaide, Florey Medical Research Foundation Postgraduate Travel Grant 2012

◦ Florey Medical Research Foundation Postgraduate Top Up Cancer Research Scholarship 2011

◦ Inaugural Australasian Sarcoma Study Group and Rainbows for Kate Sarcoma Research Grant 2010

◦ Australian Postgraduate Award Scholarship 2010
PRIZES

 Outstanding Poster Presentation 2012
   Molecular Biology and Innovative Therapies in Sarcoma Conference,
   Pultusk, Poland

 Best Poster Presentation (School of Medicine Award) 2012
   University of Adelaide, Postgraduate Research Conference,
   Adelaide, Australia
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