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

Kathleen Irene Pishas

Bachelor of Biomedical Science
Bachelor of Health Science (Honours)

Sarcoma Research Group & Centre for Personalised Cancer Medicine
Faculty of Health Sciences,
Discipline of Medicine,
The University of Adelaide, South Australia

Thesis submitted to The University of Adelaide
for the Degree of Doctor of Philosophy

March 2013

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.

TABLE OF CONTENTS

TABLE OF CONTENTS

CHAPTER 2

TABLE OF CONTENTS

CHAPTER 3

TABLE OF CONTENTS

CHAPTER 4

TABLE OF CONTENTS

CHAPTER 5

TABLE OF CONTENTS

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.

THESIS STRUCTURE

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-depedent 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.

I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photography, subject to the provisions of the Copyright Act 1968. I acknowledge that copyright of published works contained within this thesis resides with the copyright holder(s) of those works.

I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library catalogue, the Australasian Digital Thesis Program (ADTP) and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

Signed: …….. Date: 28th March 2013

PUBLICATIONS

- **Pishas KI**, Al-Ejeh F, Zinonos I, Kumar R, Evdokiou A, Brown MP, Callen DF, and Neilsen PM. Nutlin-3a is a potential therapeutic for Ewing sarcoma. *Clinical Cancer Research* 17: 494-504, 2011

- Neilsen PM, **Pishas KI**, Callen DF, and Thomas DM. Targeting the p53 Pathway in Ewing sarcoma. *Sarcoma*, 746939, 2011

- Suetani RJ, Ho K, Jindal S, Manavis J, Neilsen PM, **Pishas KI**, Rippy E, Bochner M, Kollias J, Gill PG, Morris HA, and Callen DF. A comparison of vitamin D activity in paired non-malignant and malignant human breast tissues. *Molecular Cell Endocrinology* 362: 202-210, 2012

- Neilsen PM, Pehere AD, **Pishas KI**, Callen DF, and Abell AD. New 26S Proteasome Inhibitors with High Selectivity for Chymotrypsin-Like Activity and p53-Dependent Cytotoxicity. *ACS Chemical Biology* 8(2):353-9, 2013

- **Pishas KI**, Neuhaus SJ, Clayer MT, Adwal A, Brown MP, Evdokiou A, Callen DF and Neilsen PM. Pre-activation of the p53 pathway through Nutlin-3a sensitizes sarcomas to Drozitumab therapy. *Oncology Reports* 30(1):471-7, 2013

PUBLICATIONS

- Centenera MM, Hickey TE, Jindal S, Ryan NK, Ravindranathan P, Kapur P, **Pishas KI,** Neilsen PM, Comstock C, Schiewer M, Robinson J, Carroll J, Callen DF, Knudsen KE, Raj GV, Butler LM and Tilley WD. Leveraging *ex vivo* culture of solid tumour tissues for molecular research and preclinical drug discovery. *Science Translational Medicine*, 2013 (*Manuscript under review*)

- **Pishas KI**, Neuhaus SJ, Clayer MT, Perugini M, Farshid G, Manavis J, Chryssidis SK, Mayo BJ, Haycox RC, Ho K, Brown MP, D'Andrea RJ, Evdokiou A, Callen DF and Neilsen PM. The role of p53 pathway alterations and downstream targets in Nutlin-3a sarcoma sensitivity. *Journal of Experimental Medicine*, 2013 (*Manuscript under review*)

SCHOLARSHIPS & GRANTS

ꕥ	SAHMRI Beat Cancer Project Travel Grant	2013
ꕥ	Florey Medical Research Foundation Postdoctoral Clinical Cancer Research Fellowship	2012
ꕥ	Channel 7 Children's Research Foundation Early Career Grant	2012
ꕥ	Freemasons Foundation Trevor Prescott Memorial Scholarship	2012
ꕥ	European Science Foundation and EMBO, Molecular Biology & Innovative Therapies in Sarcoma Conference Travel Grant	2012
ꕥ	University of Adelaide, Discipline of Medicine Travel Grant	2012
ꕥ	University of Adelaide, Florey Medical Research Foundation Postgraduate Travel Grant	2012
ꕥ	Florey Medical Research Foundation Postgraduate Top Up Cancer Research Scholarship	2011
ꕥ	Inaugural Australasian Sarcoma Study Group and Rainbows for Kate Sarcoma Research Grant	2010
ꕥ	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

ACKNOWLEDGEMENTS

A major research project like this is never the work of anyone alone. The contributions of many different people, in their different ways, has made this thesis possible. I would like to extend my gratitude and appreciation to the following.

Firstly, I sincerely wish to thank David and Kati Wood. Without the inaugural ASSG Sarcoma Scholarship in memory of your son's battle with Ewing sarcoma, findings presented in this thesis would not have been possible. I commend you for all your efforts in raising awareness for sarcoma, and thank you from the bottom of my heart for all the support and well wishes over the past three years. I know that Tom has been right beside me through every success and failure and he will forever oversee all the work that our research team undertakes. I hope Tom is proud of what we have achieved over the past few years, and as such I dedicate this thesis to him.

To my supervisor Professor David Callen. Thank you for taking a chance and allowing me to undertake my undergraduate practical placement 4½ years ago. Who would have thought that I would have gone on to complete an Honours Degree and now a PhD. Thank you for all your support and academic guidance over the years.

I am extremely grateful and indebted to my supervisor Dr Paul Neilsen. Without his daily tutelage, encouragement, and above all unequivocal patience, I certainly would have struggled throughout my Honours and PhD candidature. His unsurpassed knowledge and unwavering enthusiasm for sarcoma research constantly inspired me to push myself just that

little bit harder. Most importantly his support and belief that I could conquer any challenge presented has been invaluable and will be remembered throughout the rest of my career. Every award and scholarship that I have received is a testament to all of your hard work. I doubt that I will ever be able to convey my appreciation fully, but I owe Dr Neilsen my eternal gratitude and could not have asked for a better mentor to guide me through this amazing journey. It has been an absolute privilege and honour to have worked with him and I look forward to what 'team sarcoma' can achieve over the next few years.

My appreciation also extends to my laboratory colleagues past and present, in particular Renee Schulz, our laboratory manager extraordinaire. Your friendship and support has been indispensable. Thank you also to all of my friends for never complaining that I could not make the majority of our functions because of my work and 'kids' (a.k.a my laboratory mice and rats). In particular thank you to Bronwen Mayo, who supported me through every hurdle and taught me everything concerning IHC and animal work.

I would like to thank my parents, for their patience and unconditional support, both financially and emotionally throughout my candidature. I only hope that you can now see that working back late and 'living' in the lab on weekends and public holidays has enabled me to complete a thesis which I am very proud of.

Finally I would like to express my gratitude to all of the patients who generously donated material during their difficult journey with sarcoma. Although our findings cannot directly aid in your cancer management, I know that work presented in this thesis will make significant inroads towards more efficient treatment strategies for future sarcoma patients.