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.

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.

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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

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- **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*)

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PRIZES

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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

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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.

Chapter 1

Targeting the p53 pathway in Ewing sarcoma

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STATEMENT OF AUTHORSHIP

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Wrote manuscript and acted as corresponding author.

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David F Callen

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PRELUDE

This review paper published in *Sarcoma* (2011) summarizes the functional capabilities and regulation of p53 in sarcomas; in particular Ewing sarcoma. The prognostic significance of *TP53* alterations and the potential of small targeted agents to restore p53 function are also discussed.

ABSTRACT

The p53 tumour suppressor plays a pivotal role in the prevention of oncogenic transformation. Cancers frequently evade the potent antitumour surveillance mechanisms of p53 through mutation of the *TP53* gene, with approximately 50% of all human malignancies expressing dysfunctional, mutated p53 proteins. Interestingly, genetic lesions in the *TP53* gene are only observed in 10% of Ewing sarcomas, with the majority of these sarcomas expressing a functional wild-type p53. In addition, the p53 downstream signaling pathways and DNA-damage cell cycle checkpoints remain functionally intact in these sarcomas. This review 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 development of several activators of p53 is discussed, with recent evidence demonstrating the potential of small molecule p53 activators as a promising systemic therapeutic approach for the treatment of Ewing sarcomas with wild-type p53.

INTRODUCTION

The p53 protein, known as the "guardian of the genome" [1] and voted Science magazine's "Molecule of the Year" in 1993, plays a pivotal role in the cellular defense against transformation of cells in the presence of oncogenic or genotoxic stress [2]. This is achieved through the ability of the p53 transcription factor to drive the expression of downstream target genes to evoke cellular responses such as cell cycle arrest, apoptosis, DNA damage repair and senescence [3]. The development of a malignant neoplasm generally requires attenuation of these p53 responses and this can occur via mutation of the p53 protein. *TP53* is the most frequently altered gene in cancer, with p53 mutations observed in approximately half of all tumours [4]. In contrast, *TP53* mutations are infrequent in the Ewing sarcoma Family of Tumours (ESFTs) with the majority of these sarcomas expressing a functional wild-type p53 [5-14]. Such features are rarely seen in cancers, and are suggestive that ESFTs will be sensitive to p53-based targeted therapeutic strategies.

Genomic integrity of *TP53* is preserved in Ewing sarcomas

Ewing sarcomas arise in the bones of children and young adolescents and are the most lethal of all bone tumours [15,16]. These sarcomas are infrequent neoplasms with international incidence rates in the pediatric population averaging less than two cases per million children. Such low incidence rates have limited the detection of *TP53* mutations to relatively small cohorts from independent clinical centres, with these studies reporting *TP53* mutation frequencies ranging from 4% to 20%. A meta-analysis of all primary or metastatic ESFTs involving *TP53* point mutations confirmed through direct sequencing that *TP53* mutations are observed in approximately 10% of cases (Table 1).

TABLE 1: *TP53* mutations in Ewing sarcoma confirmed through DNA sequencing

Sarcoma type	Study	Method	Exons sequenced	Mutation frequency	*TP53* mutation
Ewing Sarcoma	Kovar et al., 1993 [5]	PCR-SSCP Sequencing	4–8	2/37	C277Y, **R273C**
	Komuro et al., 1993 [6]	PCR-SSCP Sequencing	5–9	2/14	152(FS), G154V
	Hamelin et al., 1994 [7]	PCR-DGGE Sequencing	5–8	2/12	**R175H**, **R248W**
	Patiño-García and Sierrasesúmaga, 1997 [8]	PCR-DDGE Sequencing	5–8	1/5	**R273H**
	Radig et al., 1998 [9]	IHC PCR-SSCP Sequencing	4–8	1/24	Not Specified
	Tsuchiya et al., 2000 [10]	PCR-SCCP Sequencing	5–9	1/24	G154V
	López-Guerrero et al., 2001 [11]	Sequencing	5–8	3/19	C135F, A138D, P151R
	Park et al., 2001 [13]	PCR-SSCP Sequencing	4–9	3/35	K132M, C135S, Q287V
	Huang et al., 2005 [12]	IHC p53 GeneChip Sequencing	—	8/60	W146(STOP), M160L N239A, G244I, **R248Q R273F**, A276S, R342P
	Schaefer et al., 2008 [14]	Sequencing	5–8	2/17	C141Y, **R248W**
Total *TP53* mutations in Ewing Sarcoma					25/247 (10.1%)

DDGE: Denaturing Gradient Gel Electrophoresis; IHC: Immunohistochemistry; PCR-SSCP: Polymerase Chain Reaction Single-Strand Conformational Polymorphism; FS: Frameshift; Mutations in bold indicate p53 "hotspot mutations."

A similar analysis of the available literature suggests that the majority of other sarcoma types are also associated with low frequencies of *TP53* mutations, ranging from approximately 6% in well-differentiated/de-differentiated liposarcomas to 23% in osteosarcomas (Table 2). Although malignant fibrous histiocytomas are listed, they are a discredited entity and will soon be removed from the World Health Organisation (WHO) sarcoma atlas [17]. Collectively, Ewing sarcoma was associated with the lowest frequencies of *TP53* mutation across all sarcoma types.

TABLE 2: *TP53* mutations in sarcomas other than ESFTs confirmed through DNA sequencing

Sarcoma type	Study	Method	Exons sequenced	Mutation frequency	*TP53* mutation
Liposarcoma					
Well-differentiated/de-differentiated liposarcoma (WD/DDLPS)	Pilotti et al., 1997 [18]	IHC PCR-SSCP Sequencing	5–9	4/13	H179Y, R213(STOP) **R282W**, Gg > Gc (SS)
	Dei Tos et al., 1997 [19]	IHC PCR-SSCP Sequencing	5–8	1/14	S127F
	Schneider-Stock et al., 1998 [20]	PCR-SSCP Sequencing	4–8	0/8	—
	Schneider-Stock et al., 1999 [21]	IHC PCR-SSCP Sequencing	5–8	0/13	—
	Barretina et al., 2010 [22]	Sequencing, mass spectrometry-based genotyping	—	0/50	—
				Total *TP53* mutations	5/98 (5.1%)
Myxoid/Round cell liposarcoma	Pilotti et al., 1997 [18]	IHC PCR-SSCP Sequencing	5–9	1/6	Del nts 1506-1507 (STOP)
	Schneider-Stock et al., 1998 [20]	PCR-SSCP Sequencing	4–8	1/12	P128S
	Schneider-Stock et al., 1999 [21]	IHC PCR-SSCP Sequencing	5–8	3/19	H214L, P250T, **G245S**
	Oda et al., 2005 [23]	IHC PCR-SSCP Sequencing	5–9	5/77	Q167(STOP), H214Y V225A, C238Y, C242Y
	Barretina et al., 2010 [22]	Sequencing, mass spectrometry-based genotyping	—	0/21	—
				Total *TP53* Mutations	10/135 (7.4%)
Pleomorphic liposarcoma	Schneider-Stock et al., 1998 [20]	PCR-SSCP Sequencing	4–8	3/6	**R248Q**, E271(STOP) **R273C**
	Schneider-Stock et al., 1999 [21]	IHC PCR-SSCP Sequencing	5–8	2/9	**R248Q**, **R273C**
	Barretina et al., 2010 [22]	Mass spectrometry-based genotyping	—	4/24	C135F, T155I C > TT (SS), C > CT (SS)
				Total *TP53* mutations	9/39 (23.1%)
Undefined liposarcomas	Toguchida et al., 1992 [24]	PCR-SSCP Sequencing	2–11	1/4	AGgt > AGtt (SS)
	Leach et al., 1993 [38]	IHC Sequencing	5–8	3/13	Q144(STOP), N239S GGT > GAT (SS)
	Latres et al., 1994 [25]	PCR-SSCP Sequencing	2–9	5/25	H168R, H193Y, M246V **R248W**, 344(STOP)
	Nawa et al., 1999 [27]	PCR-SSCP Sequencing	5–8	1/9	T253A
	Das et al., 2007 [28]	IHC Sequencing	2–11	1/3	377(FS)
				Total *TP53* mutations	12/58 (20.7%)
Total *TP53* mutations in liposarcomas					36/330 (10.9%)

TABLE 2: Continued

Sarcoma type	Study	Method	Exons sequenced	Mutation frequency	*TP53* mutation
Rhabdomyosarcoma	Felix et al., 1992 [29]	PCR-SSCP Sequencing	4–8	1/6	R213P
	Toguchida et al., 1992 [24]	PCR-SSCP Sequencing	2–11	0/4	—
	Latres et al., 1994 [25]	PCR-SSCP Sequencing	2–9	0/2	—
	Castresana et al., 1995 [26]	PCR-SSCP Sequencing	5–8	1/1	V218L
	Kusafuka et al., 1997 [30]	PCR-SSCP Sequencing	5–8	1/10	**R273H**
	Nawa et al., 1999 [27]	PCR-SSCP Sequencing	5–8	0/2	—
	Taylor et al., 2000 [31]	PCR-SSCP Sequencing	5–9	1/20	Del nt 1004-1017
	Takahashi et al., 2004 [32]	PCR-SSCP Sequencing	5–9	9/45	E204G, R209T, P223R M243T, **G245C**, N247D, R249G, C291Q, P295H
	Das et al., 2007 [28]	IHC Sequencing	2–11	1/4	D393N
Total *TP53* mutations in rhabdomyosarcomas					14/94 (14.9%)
Synovial Sarcoma	Toguchida et al., 1992 [24]	PCR-SSCP Sequencing	2–11	0/5	—
	Latres et al., 1994 [25]	PCR-SSCP Sequencing	2–9	0/8	—
	Schneider-Stock et al., 1997 [33]	IHC PCR-SSCP Sequencing	5–8	0/2	—
	Dei Tos et al., 1999 [34]	PCR-SSCP Sequencing	—	4/20	Not Specified
	Nawa et al., 1999 [27]	PCR-SSCP Sequencing	5–8	1/7	L194F
	Schneider-Stock et al., 1999 [35]	IHC PCR-SSCP Sequencing	5–8	2/19	P128L, **R248W**
	Oda et al., 2000 [36]	IHC PCR-SSCP Sequencing	5–9	9/49	C141Y, A159T,V173M I195F, R196Q, G199R R213(STOP), N235D C238Y
	Das et al., 2007 [28]	IHC Sequencing	2–11	5/7	9(STOP), A63P, S96C, P250T, P250T
	Barretina et al., 2010 [22]	Sequencing, mass spectrometry-based genotyping	—	0/23	—
Total *TP53* mutations in Synovial Sarcomas					21/140 (15.0%)

TABLE 2: Continued

Sarcoma type	Study	Method	Exons sequenced	Mutation frequency	*TP53* mutation
Malignant Fibrous Histiocytoma	Toguchida et al., 1992 [24]	PCR-SSCP Sequencing	2–11	2/13	R196(STOP), **R273H**
	Andreassen et al., 1993 [37]	CDGE Sequencing	5,7,8	3/12	V143M, Y163C, G244D
	Leach et al., 1993 [38]	IHC Sequencing	5–8	1/11	R158H
	Latres et al., 1994[25]	PCR-SSCP Sequencing	2–9	0/9	—
	Castresana et al., 1995 [26]	PCR-SSCP Sequencing	5–8	3/12	Not Specified
	Schneider-Stock et al., 1997 [33]	IHC PCR-SSCP Sequencing	5–8	2/15	Y220C, C277(STOP)
	Nawa et al., 1999[27]	PCR-SSCP Sequencing	5–8	5/15	Y126F, **R175H** R213(STOP), S241T **R248Q**
	Das et al., 2007 [28]	IHC Sequencing	2–11	2/11	P77Q, 213(FS)
Total *TP53* mutations in malignant fibrous histiocytoma					18/98 (18.4%)
Leiomyosarcoma	Andreassen et al., 1993 [37]	CDGE Sequencing	5,7,8	2/6	K132M, **R248W**
	Latres et al., 1994[25]	PCR-SSCP Sequencing	2–9	5/13	Y163C, Y163C, H214R, G266E, ATgg > ATag (SS Intron 5)
	Patterson et al., 1994 [39]	PCR-SSCP Sequencing	4–9	6/29	P151H, P152L, R158H V216M, C238F, V272M
	Castresana et al., 1995 [26]	PCR-SSCP Sequencing	5–8	1/1	Not Specified
	Miller et al., 1996 [40]	PCR-SSCP Sequencing	2–11	1/8	Q165(STOP)
	Hall et al., 1997 [41]	IHC PCR-SSCP Sequencing	5–8	3/21	K163E, T211I A nt Del codon 246
	Schneider-Stock et al., 1997 [33]	IHC PCR-SSCP Sequencing	5–8	0/7	—
	Nawa et al., 1999[27]	PCR-SSCP Sequencing	5–8	0/3	—
	Zhai et al., 1999 [42]	IHC Sequencing	5–8	9/21	V173M, Y205C, S215R, **R248Q**, **R249W**, **R273H**, A276D, E285D, S303I
	Miyajima et al., 2001 [43]	IHC PCR-SSCP Sequencing	5–9	8/13	A161T, D184N, T220C T220C, C238S, C238C **R273H**, G279V
	Das et al., 2007 [28]	IHC Sequencing	2–11	0/5	—
	Barretina et al., 2010 [22]	Sequencing, mass spectrometry-based genotyping	—	0/27	—
Total *TP53* mutations in leiomyosarcomas					35/154 (22.7%)

TABLE 2: Continued

Sarcoma type	Study	Method	Exons sequenced	Mutation frequency	*TP53* mutation
Osteosarcoma	Toguchida et al., 1992 [24]	PCR-SSCP Sequencing	2–11	14/76	46 (STOP), 112 (STOP), **R175H** H193Q, E221(STOP) 227(STOP), S241Y G244V, P250L, D259V **R273H**, D281H, D281N aaTG > ggTCG (SS)
	Andreassen et al., 1993 [37]	CDGE Sequencing	5, 7, 8	2/11	D281E, E286K
	Castresana et al., 1995 [26]	PCR-SSCP Sequencing	5–8	2/7	169(STOP), D281Y
	Miller et al., 1996 [40]	PCR-SSCP Sequencing	2–11	13/42	H179Y, E224D, 239(FS), **G245D**, **R248W**, **R248**, 248(FS), **R273H** **R273H**, A276P **R282Q**, **R282Q** **R282H**
	Patiño-García and Sierrasesúmaga, 1997 [8]	PCR-DDGE Sequencing	5–8	6/37	**R175H**, R196(STOP) P250F, N268S, **R273H** **R2735**
	Gokgoz et al., 2001 [44]	PCR-SCCP Sequencing	4–10	60/272	agTCC > aaTCC (SS) L43(STOP), L43(STOP), P47L, 73(FS), 73(FS), 83(FS), 83(FS) In-Frame Ins (GGT) Codon 107/108 ACGgt/ACGtt(SS), agTAC/aaTAC (SS), V172D, **R175H** ATGgt/ATGat(SS), R181P, R196(STOP) V197G, Del codon 202–206, E204(STOP), Y205C R213(STOP), Y220C Y220C, E221(STOP) 31bp Del (FS) Intron 6 to Exon 7 229(FS), M237I, M237I, M237I, C238GC238G, C238G 241(FS), C242Y, C242Y, **G245S**, **G245S** **R248W**, **R248Q**, **R248Q**, **R248Q**, **R248Q**, P250L, T256S 15 bp In-frame Del (codon 265) **R273H**, **R273H**, **R273H**, R280H, D281H, D281H D281N, D281N E285K, Del codon 296–303 298(FS) GAGgt/GAGct (SS) R337C, R342(STOP) E343(STOP)

TABLE 2: Continued

Sarcoma type	Study	Method	Exons sequenced	Mutation frequency	*TP53* mutation
	Overholtzer et al., 2003 [45]	PCR-SCCP PCR-LDR	5–8	12/32	V173G, V173M, **R175H**, Del codon 175, Y220C, E224D, V272M, **R273H**, **R273C**, **D281H** FS (Exon 6), FS (Del 17nt) Exon 5
Total *TP53* mutations in osteosarcomas					109/477 (22.9%)

CDGE: Constant Denaturant Gel Electrophoresis; DDGE: Denaturing Gradient Gel Electrophoresis; IHC: Immunohistochemistry; PCR-SSCP: Polymerase Chain Reaction Single-Strand Conformational Polymorphism; PCR-LDR: Polymerase Chain Reaction Ligase-Detection Reaction; FS: Frame shift; Del: Deletion; Ins: Insertion; SS: Splice Site. Mutations in bold indicate p53 "hotspot mutations". Recurrences with *TP53* mutations have been omitted in studies that reported both the primary tumour and recurrence with the same mutation.

The International Agency for Research on Cancer (IARC) has recently released recommendations for the detection of *TP53* mutations, and advise direct sequencing of exons 4 to 10 of the *TP53* gene [46]. Studies listed in Tables 1 and 2 rarely fulfilled these recommendations. Furthermore, the reported frequencies of *TP53* mutations may be marginally underestimated as the majority of these studies sequenced *TP53* in patients where an initial screen detected over-expressed p53 protein by immunohistochemistry, rather than performing an unbiased sequencing of all cases. Although mutant p53 is typically stabilized in cancer cells, and over-expression of p53 protein is predictive of *TP53* mutation [47], this indirect approach cannot detect heterozygous truncating mutations of *TP53* [46]. Nevertheless, frequencies of p53 mutation reported by the studies in Table 1 and 2 are consistent with publicly available sequencing data from the IARC *TP53* database in which *TP53* point mutations were observed in 373 out of 2145 (17.4%) tumours from bone or soft tissue origins[46].

The integrity of the p53 pathway in Ewing sarcomas is further supported by studies suggesting that gross chromosomal alterations involving the *TP53* locus on chromosome 17p are relatively infrequent in ESFT samples [48]. This is in contrast to other bone malignancies, such as osteosarcomas, where chromosome alterations of the *TP53* gene are frequently observed [24,44,49,50].

Genetic alterations in regulators of the p53 pathway

Typically, cancers that retain wild-type p53 have been shown to indirectly suppress the p53 regulatory and signaling pathways. One of the most common oncogenic defects observed involves amplification or over-expression of MDM2 [51]. The stability and activity of p53 are constitutively regulated by MDM2 using an auto-regulatory feedback loop which in normal cells prevents inappropriate activation of p53 [52]. MDM2 is an E3 ubiquitin ligase that antagonizes the tumour suppressor function of p53 by silencing the ability of p53 to trans-activate target genes or promoting its degradation or nuclear exportation [53,54]. However, *MDM2* amplification is a rare event in Ewing sarcomas and is only observed in approximately 2% of ESFT cases (Table 3). In contrast, virtually all well differentiated and dedifferentiated liposarcomas contain complex marker chromosomes with multiple copies of the *MDM2* locus [55].

Cancers can also attenuate p53 function through deletion of *CDKN2A*, the gene encoding $p14^{ARF}$ [48]. The $p14^{ARF}$ tumour suppressor is a positive regulator of p53 in response to specific stimuli such as oncogenic stress. The stability of p53 is enhanced by $p14^{ARF}$ through its ability to sequester MDM2, thus releasing p53 to activate downstream pathways of growth suppression or apoptosis [56,57]. A summary of the available literature related to *CDKN2A* chromosomal alterations in ESFT cases revealed that either homozygous or hemizygous deletion of the *CDKN2A* locus are also relatively infrequent events, occurring in less than 20% of all cases (Table 3). *CDKN2A* alterations and *TP53* mutations are mutually exclusive events in the majority of ESFT cases, suggesting either genetic insult is sufficient to inactivate the p53 pathway in these cancers [11,48].

TABLE 3: *MDM2* amplification or *CDKN2A* deletion in Ewing sarcomas

Study	*MDM2* amplification	Study	*CDKN2A* deletion
Kovar et al., 1993 [5]	0/17	Kovar et al., 1997 [5]	7/27#
Ladanyi et al., 1995 [58]	3/30	Wei et al., 2000 [59]	7/39#
Tsuchiya et al., 2000 [10]	0/24	López-Guerrero et al., 2001 [11]	4/19#
Park et al., 2001 [13]	0/35	Brownhill et al., 2007 [60]	6/42*
López-Guerrero et al., 2001 [11]	0/19	López-Guerrero et al., 2010 [48]	34/169*
Total MDM Amplifications	3/125 (2.4%)	Total CDKN2A Deletions	58/296 (19.6%)

#Homozygous deletion of *CDKN2A*
*Includes both homozygous and hemizygous deletions of *CDKN2A*.

MDM4 is another key negative regulator of the p53 pathway [61-63]. This oncoprotein is closely related to MDM2 with significant homology between their DNA binding domains, however MDM4 has a more specific role in the negative regulation of p53 transcriptional activity. Amplification of the *MDM4* gene has also been reported in several tumour types, with *MDM4* amplification observed in 65% of retinoblastomas [64]. *MDM2* and *MDM4* amplification are rarely observed within the same tumour, suggesting that either event is sufficient to inactivate the p53 pathway. *MDM4* amplification is a possible mechanism for functional inactivation of the p53 pathway in Ewing sarcoma given the infrequent occurrences of *MDM2* amplification, *CDKN2A* deletion or *TP53* mutation in these cancers. Unfortunately, previous cytogenetic studies are restricted to investigating allelic imbalance of the *TP53*, *MDM2* and *CDKN2A* loci in ESFT patient material, hence the frequency of *MDM4* amplifications in Ewing sarcoma is currently unknown.

The small proportion of ESFT cases with either *MDM2* amplification or $p14^{ARF}$ deletion cannot collectively account for the ability of Ewing sarcomas to develop in a cellular context with wild-type p53. Further investigations of Ewing sarcoma are warranted to conclusively determine if the mechanisms that attenuate the p53 response during sarcomagenesis occur at the genetic or post-translational level. The presence of a *EWS-ETS* translocation event is a universal feature of Ewing sarcoma and represents another possible

genetic alteration responsible for the regulation of p53 in ESFT. Recent insights into the functional characterization of the resulting oncogenic gene product suggest a potential role of this ubiquitous translocation event in silencing p53 activity in ESFTs (see later discussion).

The p53 signaling pathways are functionally intact in Ewing sarcoma

Abrogation of the p53 pathway through *TP53* mutation is typically associated with enhanced tumour invasive and metastatic capabilities, and poorer patient survival rates [46,65]. Ewing sarcoma is an aggressive malignancy with the lowest patient survival rates of all primary musculoskeletal tumours, traits rarely possessed by cancers that retain wild-type p53. Despite observations that *TP53* alterations and *MDM2* amplifications are infrequent events in primary Ewing sarcomas, it has been speculated that the downstream signaling pathways of p53 may be inactive in these sarcomas. In order to test the functional intactness of these p53 signaling pathways in Ewing sarcoma, Heinrich Kovar and colleagues investigated the response of several ESFT cell lines with varying p53 status to ectopic p53 expression [66]. A prolonged apoptotic or growth arrest phenotype was observed upon ectopic expression of wild-type p53 in the cell lines. The sensitivity of Ewing sarcoma cell lines to X-irradiation was also dependent on the expression of an endogenous wild-type p53. These findings confirm the intactness of the p53 signaling pathways in Ewing sarcoma.

The frequent normal functioning of the p53 signaling pathway in ESFTs is also demonstrated by the observation that almost all Ewing sarcoma cell lines have acquired either *TP53* mutations or *CDKN2A* deletions, suggesting selective pressure for these genetic alterations to permit *in vitro* growth [67]. Similar observations have been made in the clinic, with p53 mutation and *CDKN2A* deletion defining a lethal subset of ESFTs associated with

poor response to chemotherapy [12]. In conclusion, the *in vitro* and *in vivo* evidence suggests the p53 signaling pathways are intact in a significant proportion of Ewing sarcomas.

The prognostic significance of *TP53* alterations

ESFT patients with point mutation of *TP53* are associated with a poor prognosis [12,68,69]. Logically, one would expect a genetic event that confers a growth advantage to be highly represented across a tumour type through selective pressure. This is not the case in Ewing sarcoma, as *TP53* mutations rarely occur, yet define a high-risk population of patients. Huang and colleagues have provided the most compelling evidence thus far for *TP53* mutation as an independent prognostic marker using a combined immunohistochemistry, Genechip and sequencing approach to detect *TP53* mutations in 60 ESFTs [12]. *TP53* mutations were identified in 8 of these 60 cases (13.3%), and all eight patients expressing mutant p53 ESFTs died within 21 months of diagnosis with a mean survival of 11 months, as compared to a mean survival of 99 months for patients with wild-type p53 ESFTs. Multivariate analysis identified *TP53* mutation as the strongest independent prognostic factor [12]. This is the largest prognostic study to date that involves DNA sequencing of *TP53* in ESFT patient material.

The prognostic involvement of p53 mutation in Ewing sarcoma was recently challenged by findings from a retrospective study involving 308 ESFT cases collected from 1971 to 2007 [48]. Although over-expression of p53 protein was detected in 25% of these cases, this study restricted the classification of "p53 mutation" to these cases which did not express $p21^{Waf1/Cip1}$, the strongest canonical p53 target. Subsequently, 15% of ESFT cases where deemed to express mutant p53 upon application of this criteria. This study showed that "mutant p53" expression was more frequent in disseminated disease than in primary localized

tumors, indicating a role in the progression and metastasis of Ewing sarcoma. However, there was no association between "mutant p53" expression with patient survival. The conclusions from this study are limited by the absence of actual *TP53* sequencing of ESFT samples to confirm the presence of *TP53* mutations. These observations need to be attested by further investigation into the prognostic value of *TP53* mutations using unbiased direct sequencing approaches.

Gross chromosome rearrangements involving *TP53* have been recently reported to influence the prognosis of Ewing sarcoma [48,70]. Alteration of 17p (the chromosomal arm containing the *TP53* locus) was observed in 16.7% of ESFT samples and was associated with significantly poorer survival rates [70]. Lopez-Guerrero and colleagues recently showed that alteration the *TP53* locus alone was a prognostic marker for poor patient outcome [48]. Gross alteration of *TP53* gene was detected in 32 of 191 (17%) ESFT cases. Interestingly, the strongest prognostic information from these studies was observed upon loss of heterozygosity (LOH) of 16q, which occurred in 20.8% of cases and was the most significant indicator of poor outcome ($p = 0.0006$) [70]. A recent study that used a combined comparative genomic hybridization (CGH) and expression microarray analysis identified the *ANKRD11* locus at 16q24.3 as one of the most frequently deleted and down-regulated genes in Ewing sarcoma [71]. It is noteworthy that ANKRD11 was recently reported as a p53 co-activator [72], suggesting that the loss of p53 activators may contribute towards the ability of Ewing sarcomas to develop and progress in the presence of a wild-type p53.

Studies to date are consistent with the presence of *TP53* point mutations in defining a high-risk population of ESFT patients. However, more detailed studies are warranted to

conclusively evaluate the prognostic potential of *TP53* point mutation. Collectively, these findings indicate that the prognostic potential of mutant p53 will be fully realized through the application of definitive approaches to detect *TP53* mutations.

Oncogenic EWS-ETS translocations

ESFTs are cytogenetically diagnosed through specific genetic rearrangement involving the *EWS* gene (official symbol *EWSR1*) and a member of the *ETS* transcription factor gene family. This chimeric fusion protein is present in over 90% of ESFTs [73,74] and is widely considered to be causative of this malignancy. The *EWS-FLI1* translocation, t(11;22)(q24;12), is a chromosomal aberration specific to ESFTs and accounts for 85% of translocation events in Ewing sarcoma. This reciprocal translocation generates fusion of the 5' segment of *EWS* on chromosome 22 with the 3' segment of *FLI-1* on chromosome 11. Antisense DNA studies have confirmed that continuous EWS-FLI1 expression is required for the *in vitro* proliferation and *in vivo* tumorigenic capacity of Ewing sarcoma cells [75-78]. The second most common EWS translocation described involves an in-frame fusion of the *EWS* and *ERG* genes as a result of the t(21;22)(q22;q12) translocation, accounting for 5% of translocations in Ewing sarcoma [74].

Suppression of p53 activity by EWS-FLI1

Attenuation of the p53 tumour surveillance mechanisms during the development and progression of Ewing sarcomas may be explained through the ability of the EWS-FLI1 oncoprotein to silence p53 activity. Two independent studies have shown that silencing of EWS-FLI1 expression in Ewing sarcoma cell lines increases p53 activity [79,80], suggesting that

the EWS-FLI1 fusion protein plays a role in the constitutive silencing of p53 tumour suppressor activity. It appears that EWS-FLI1 can achieve this through either an indirect mechanism, involving the Notch signaling pathway [79], or through the formation of a protein complex involving EWS-FLI1 and p53 [80]. This study suggests that EWS-FLI1 attenuates p53 activity through physically sequestration facilitated by the EWS region of the fusion protein [80]. However, it is unclear whether interaction between p53 and EWS-FLI1 occurs directly or is mediated through other oncogenic binding partners. It is of great interest that the amino region of EWS enables the recruitment of p53 to EWS-FLI1, as this p53-binding region is present in almost all Ewing sarcoma gene translocation events and numerous other translocation-based cancers (Table 4). Such observations suggest that these malignancies share a common mechanism involving EWS that may potentially involve the functional inactivation of p53.

TABLE 4: Involvement of the *EWS* gene in translocation-based malignancies

Tumour type	Fusion gene	Translocation	Reference
Ewing Sarcoma	*EWS-FLI1*	t(11;22)(q24;q12)	[73]
	EWS-ERG	t(21;22)(q22;q12)	[81]
	EWS-ETV1	t(7;22)(p22;q12)	[82]
	EWS-ETV4	t(17;22)(q12;q12)	[83]
	EWS-FEV	t(2;22)(q33;q12)	[84]
Acute Leukemia	*EWS-CIZ1*	t(12;22)(p13;q12)	[85]
Angiomatoid Fibrous Histiocytoma	*EWS-ATF1*	t(12;22)(q13;q12)	[86]
	EWS-CREB1	t(2;22)(q33;q12)	[87]
Clear-cell Sarcoma	*EWS-ATF1*	t(12;22)(q13;q12)	[88]
	EWS-CREB1	t(2;22)(q33;q12)	[89]
Desmoplastic Small Round Cell Tumour	*EWS-WT1*	t(11;22)(p13;q12)	[90]
Extraskeletal Myxoid Chondrosarcoma	*EWS-CHN1*	t(9;22)(q22–31;q11-12)	[91]
	EWS-NR4A3	t(9;22)(q22;q12)	[92]
Myxoid Liposarcoma	*EWS-DDIT3*	t(12;22)(q13;q12)	[93]

The ability of EWS-FLI1 to suppress p53 activity in Ewing sarcoma is reminiscent of the functional role of the oncogenic translocation product in synovial sarcomas. The presence of the SS18-SSX fusion protein as a result of the t(X:18)(p11.2;q11.2) translocation is a universal feature of synovial sarcoma [94]. These sarcomas abrogate p53 protein levels through its enhanced proteasomal degradation facilitated by SS18-SSX [95]. Reminiscent of Ewing sarcoma, *TP53* mutations are rare events in synovial sarcomas (Table 1), suggesting that these cancers rely on the ability of the SS18-SSX fusion protein to abrogate the p53 response, facilitating oncogenic transformation in the presence of a functional, wild-type p53.

EWS-FLI1 expression stimulates the p53 pathway in normal cells, fibroblasts or non-mesenchymal cells

The development of an animal model to investigate the oncogenic properties of EWS-FLI1 has been limited by the toxic effects associated with the expression of this potent fusion protein in primary cells [96]. Due to the absence of an adequate transgenic animal model for Ewing sarcoma, experimental approaches have been restricted to forced expression of EWS-FLI1 in various cell lines. Introduction of EWS-FLI1 into primary human fibroblasts resulted in a growth arrest through stimulation of the p53 pathway [96]. Subsequent specific inhibition of p53 activity in these fibroblasts rescued the growth arrest phenotype, allowing EWS-FLI1 to promote anchorage-independent growth of these fibroblasts. Similar effects have been observed in mouse embryonic fibroblasts (MEFs) in which expression of EWS-FLI1 induced the p53-dependent growth arrest or apoptosis [97]. This apoptotic or growth arrest response was considered to be p53-dependent, as MEFs null for p53, $p19^{ARF}$ or p16 were unaffected in response to EWS-FLI1 expression. Normal cells retaliate this aberrant oncogene expression by mounting a p53-based defense mechanism resulting in cellular apoptosis or

senescence [98,99]. Consideration of the responses elicited by EWS-FLI1 in normal cells therefore suggests this fusion protein in functioning as a potent oncogene and is reminiscent of the responses associated with elevated levels of the *MYC* or *RAS* oncogenes.

The cellular response to ectopically expressed EWS-FLI1 varies in primary cells of different origin. Ewing sarcomas are derived from mesenchymal progenitor cells (MPCs) [100], and forced expression of EWS-FLI1 in MPCs was shown to be stably maintained without growth arrest or apoptosis whilst inducing an gene expression profile similar to that of a Ewing sarcoma; all in the presence of a functional, wild-type p53 [101-103]. Such findings raise questions surrounding the ability of primary MPCs to tolerate forced EWS-FLI1 expression without engaging a p53-dependent response to the oncogenic stress. Nevertheless, the mechanisms used by MPCs to abide the expression of EWS-FLI1 in the presence of wild-type p53, whilst such expression in other primary cell lines triggers a p53 response, remain largely unknown.

Attempts have been made to investigate the function of EWS-FLI1 in a transgenic mouse model. While the conditional expression of EWS-FLI1 in mouse MPCs did not induce the formation of Ewing sarcomas, this expression of EWS-FLI1 in mice was able to influence sarcoma development in the absence of p53 [104]. Conditional *TP53* deletion in mouse MPCs led to the development osteosarcomas with a median tumour onset time of 50 weeks from birth. However, when EWS-FLI1 was conditionally expressed in these p53-null MPCs, an accelerated tumour growth from a median time of 50 to 21 weeks was observed, with the histological phenotype of these malignancies shifting towards a more poorly differentiated sarcoma [104]. These data provides *in vivo* evidence to further support the cross-talk between

p53 and EWS-FLI1 which is essential in primary MPCs for the development of Ewing sarcomas.

IGF1R and p53 signaling pathways

The activity of insulin-like growth factor 1 receptor (IGF1R) is essential for tumour development and progression through the signaling of anti-apoptotic and pro-survival pathways [105-107]. IGF1R is also often over-expressed at the cell surface of malignant cells and thus has emerged as attractive therapeutic target in cancer. A role of the IGF signaling pathway in the development of Ewing sarcoma is evident through the finding that silencing of the EWS-FLI1 oncoprotein showed upregulation and activation of IGFBP genes [108]. Ewing sarcoma cell lines are highly sensitive to IGF1R inhibitors, especially in combination with conventional chemotherapy [16,109]. The IGF1R antagonist AMG 479, has shown promising results in the treatment of ESFTs in a phase I clinical trial, indicating that Ewing sarcomas may be particularly sensitive to intervention of the IGF1R signaling pathway [110,111]. However these studies raise vital questions as to why striking responses are seen in some, but not all, patients treated with these agents.

IGF1R and p53 drive distinctly opposing biological outcomes, with a significant level of molecular cross-talk occurring between these two signaling pathways. Initial studies suggested that p53 retorts the anti-apoptotic signaling of IGF1R through repression of *IGF1R* expression [112]. Further antagonism of IGF1R activity by p53 was demonstrated though the identification of insulin-like growth factor binding protein 3 *(IGF-BP3)* as a novel p53-regulated target gene [113]. Induction of *IGF-BP3* gene expression by p53 enhanced secretion of an active form of IGF-BP3 capable of inhibiting IGF1R mitogenic signalling. Thus, the

IGF1R signalling pathway is functionally antagonised by wild-type p53. Recent evidence has also shown that IGF1R is degraded by MDM2 [114,115]. Sequestration of MDM2 in the nucleus by high levels of mutant p53 may be a possible explanation for the high levels of IGF1R observed in some cancers [115]. Interestingly, pharmacological inhibition of IGF1R signalling reduces MDM2 translational synthesis, which in turn stabilizes p53 [116]. IGF1R signalling therefore regulates the p53 pathway. Hence, the over-expression of IGF1R and frequent retention of a functional, wild-type p53 presents an opportunity of combined use of specific IGF1R inhibitors with activators of the p53 pathway.

Pharmacological p53 activation as a systemic therapy for Ewing sarcoma

Ewing sarcomas provide a unique tumor type in which the majority of cases retain the functionally intact p53 pathways that are kept in check by either EWS-FLI1 or through another unknown mechanism. At present there is no evidence of permanent suppression of the p53 pathway by specific mutation of critical components. Therefore, the most likely scenario involves abrogation of p53 function via a reversible, post-translational mechanism. This provides unique therapeutic opportunities through intervention with small molecules that directly stabilize and activate endogenous intracellular p53. This concept was first demonstrated using Nutlin-3a, a small molecule antagonist of MDM2, which has shown antitumour activity *in vitro* and *in vivo* through activation the p53 pathway in tumour cells that retain wild-type p53 [117]. Nutlin-3a antagonizes the p53-MDM2 interaction by blocking the p53-binding pocket of MDM2 and as a consequence there is rapid stabilization and accumulation of p53 protein levels [117]. Promising results from several preclinical studies have clearly demonstrated the therapeutic potential of Nutlin-3a in a variety of tumour types

expressing wild-type p53, including liposarcoma [118], rhabdomyosarcoma [119], osteosarcoma [117], synovial sarcoma [120], neuroblastoma [121], retinoblastoma [64] and leukemia [122-124].

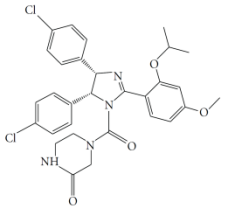
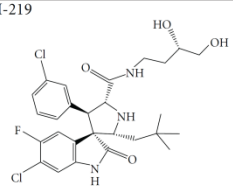
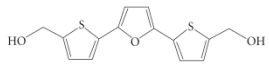
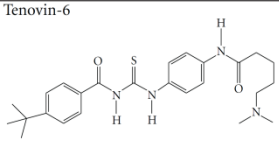
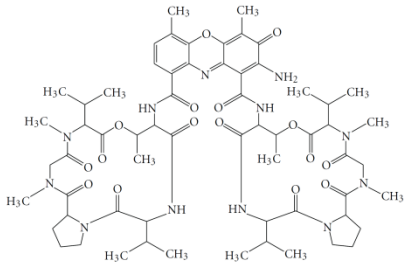
We have recently investigated the potential of a p53-targeted therapeutic approach for the treatment of Ewing sarcoma using Nutlin-3a. Interestingly, exposure of Ewing sarcoma cell lines to Nutlin-3a resulted in a robust apoptotic phenotype [125]. Nutlin-3a induced apoptosis required the presence of a wild-type p53 and did not influence the growth of ESFT cell lines expressing mutant p53. These findings provide confirmation of the functionality of downstream p53 pathways in ESFTs retaining wild-type p53 and suggest that p53 activators will provide a novel molecular-based therapeutic for the majority of ESFTs.

Due to its aggressive nature and early systemic spread, treatment of ESFT is highly challenging. Current treatment protocols for ESFT patients involve multi-agent chemotherapy [15]. Prior to the introduction of combinational chemotherapy, 5-year survival for patients diagnosed with ESFT was less than 10% [126]. Since the introduction of intensive VACD-IE (vincristine, actinomycin D, cyclophosphamide, doxorubicin, etoposide and ifosfamide) chemotherapy regimens, the current 5-year survival rates for patients with localised disease ranging from 60 to 70% [15,127]. Nevertheless, Ewing sarcoma still has the lowest survival rates of any of the muscloskeletal tumours, with minimal improvements to patient outcomes observed over the last decade. Hence, there is an urgent need to develop targeted therapeutic approaches to augment the action of these cytotoxic agents. The integral role of p53 in the DNA damage response pathways stimulated by these genotoxic agents suggests that p53 activators such as Nutlin-3a may provide a novel approach to augment intensive chemotherapies. Indeed, Nutlin-3a demonstrated synergistic activity with numerous

chemotherapies from the VACD-IE protocol in Ewing sarcoma [125] and in rhabdomyosarcoma [119]. Such encouraging observations will require further evaluation by the conduct of clinical trials using p53 activators as systemic therapies in combination with the current chemotherapy regimens for the treatment of wild-type p53 sarcomas.

The identification of additional drugs that work on the same principle as Nutlin-3a is an area of active research within the p53 community. The majority of reported p53 activators (Table 5) have been shown to rapidly stabilize and activate p53 protein levels through inhibition of the MDM2-p53 interaction. One such example is the recently identified MI-219, a highly selective MDM2 antagonist [128]. MI-219 interacts with the p53 binding pocket of MDM2 with a higher affinity and selectivity than Nutlin-3a, and hence attains a more potent stimulation of the p53 pathway. MI-219 was observed to achieve p53-dependent antitumor activity without causing visible signs of toxicity or gross abnormalities in mice [128]. In addition, MI-219 exhibits highly desirable pharmacokinetic properties and is currently in early-phase clinical trials. Small molecule p53 activators can also function through direct interaction with p53, as demonstrated with RITA (Reactivation of p53 and induction of tumour cell apoptosis) [129]. Preclinical studies show that RITA can induce a non-genotoxic activation of p53 through inhibition of the MDM2-p53 interaction via direct interaction with N-terminal domain of p53.

TABLE 5: Chemical structures and proposed mechanisms of small molecule p53 activators

Compound	Molecular formula	MWT	Mechanism	Stage in clinical testing
Nutlin-3a 	$C_{30}H_{30}C_{l2}N_4O_4$	581.5	Binds to MDM2 and inhibits p53-MDM2 interaction	Phase I
MI-219 	$C_{27}H_{32}Cl_2FN_3O_4$	552.5	Binds to MDM2 and inhibits p53-MDM2 interaction	Phase I
RITA 	$C_{14}H_{12}O_3S_2$	292.4	Binds to p53 and inhibits p53-MDM2 interaction	Preclinical
Tenovin-6 	$C_{25}H_{34}N_4O_2S$	454.6	SIRT1 and SIRT2 inhibition	Preclinical
Actinomycin D 	$C_{62}H_{86}N_{12}O_{16}$	1255.5	RPL11 and RPL5 release	Clinically approved

MDM2 antagonists have been demonstrated to elicit their most potent effects in cell lines where *MDM2* is amplified or over-expressed [118]. Since this genetic event is not observed in all tumour types, small molecules that activate p53 through alternative pathways have been developed. Sonia Lain and colleagues identified the Tenovins, a class of p53 activators that enhance the acetylation of p53 [130]. The mechanism of action of Tenovin-1 and the water soluble analog Tenovin-6 involve the direct inhibition of SIRT1 and SIRT2, two members of the sirtuin family of class III histone deacetylases responsible for the deacetylation of p53 [131-133]. It is widely accepted that acetylation is an indispensible modification of p53 that occurs during specific activation of the p53 pathway [134,135]. Interestingly, Tenovin-6 was shown to repress the growth of cancer cells using *in vitro* and *in vivo* models through hyperacetylation of p53 [130]. These studies imply that pharmacological inhibition of sitrins is an effective approach for p53 activation.

Although specific p53 activation in tumours is an attractive therapeutic approach, these recently developed small molecules are under investigation in preclinical or early-phase clinical trials. In an attempt to accelerate the implementation of p53 activators in the clinic, a study led by Sir David Lane screened a library of clinically approved drugs and successfully identified actinomycin D as a compound which mimics the action of Nutlin-3a when administered at specific dosages [136]. Surprisingly, low doses of actinomycin D induced specific activation of the p53 pathway with cellular responses remarkably similar to that of Nutlin-3a. These concentrations of actinomycin D were also demonstrated to augment the cytotoxic actions of chemotherapeutic drugs in cancer cells with wild-type p53 [136]. As actinomycin D is an FDA-approved drug, these findings catalyze the immediate application of a p53-based targeted therapeutic approach in the clinic.

High doses of actinomycin D are associated with intercalation into the DNA and subsequent double-strand DNA breaks [137]. It is presently used in the clinic as a chemotherapeutic agent and is a component of the highly successful combination treatment for Wilm's tumour. The inclusion of actinomycin D in current multi-agent chemotherapy regimens for Ewing sarcoma is variable amongst different clinical centers and is often dependent on the age of the patient. Since the pharmacokinetic data available for actinomycin D is limited [138,139], comparison between the required *in vivo* dosages of this drug with the low concentrations used *in vitro* which elicit specific activation of p53 remains a formidable challenge. Interestingly, Ewing sarcoma cells are highly sensitive to actinomycin D *in vitro,* with potent antitumour activity observed within the ranges described as 'low dose' specifically in Ewing sarcoma cell line that retain wild-type p53 [125]. Further studies are warranted to evaluate to potential of incorporation of low dose actinomycin D with the current standard of care for the treatment of patients with wild-type p53 ESFTs.

CONCLUSION

Ewing sarcomas share common genetic features including the universal presence of the *EWS-ETS* translocation and frequent retention of the wild-type p53 and its associated functional downstream pathways. Targeted exploitation of the p53 pathway holds great promise to enhance the activity of current ESFT treatment regimens and improve the currently poor survival rates associated with Ewing sarcoma. Recent identification of the first clinically approved drug, actinomycin D, as a p53 activator has facilitated the translation of these targeted therapies into current ESFT treatment regimens. Low dose actinomycin D holds an exciting potential as a directed molecular based approach to specifically activate wild-type p53 in ESFTs, and the organization of clinical trials is currently in progress to attest the potential of this approach. In addition, complementation of these studies with direct *TP53* sequencing of ESFT material would identify either patients with wild-type p53 tumours most likely to benefit from p53-based therapies, or the less frequent 'high risk' population of ESFTs containing point mutations in the *TP53* gene.

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Chapter 2

Nutlin-3a is a potential therapeutic for Ewing sarcoma

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Clinical Cancer Research (2011) 17:494-504

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Nutlin-3a is a potential therapeutic for Ewing sarcoma

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PRELUDE

This study published in *Clinical Cancer Research* (2011) determined whether Nutlin-3a (p53-MDM2 antagonist) can restore the tumour suppressive properties of wild-type p53 in sarcomas *in vitro*. Our findings suggest that Nutlin-3a induces p53 dependent apoptosis in Ewing sarcoma cell lines and can enhance the cytotoxic effects of current chemotherapeutic agents used in Ewing sarcoma treatment protocols. Furthermore, as this study was the first to report MDM4 overexpression in a high proportion of Ewing sarcoma cell lines we also investigated whether Nutlin-3a can synergise with the MDM4 antagonist SJ-172550.

STATEMENT OF TRANSLATIONAL RELEVANCE

Ewing sarcomas have the most unfavorable prognosis of all primary musculoskeletal tumors despite aggressive multimodality treatment, strongly suggesting the urgent need of systemic and targeted therapies to treat this aggressive pediatric malignancy. Although mutations in the *TP53* gene occur in half of all cancers, approximately 90% of Ewing sarcomas retain a functional wild-type p53. This study has demonstrated that Nutlin-3a, a recently discovered p53 activator, can induce apoptosis in Ewing sarcoma cell lines with wild-type p53. Our findings also suggest that Nutlin-3a can synergize with the current Ewing sarcoma chemotherapy protocols, and we have defined the dosages of these agents necessary to achieve the most potent synergistic effects. This study provides comprehensive evidence to support the rational use of p53 activators as a novel systemic therapeutic approach for Ewing sarcomas with wild-type p53.

Pishas, K. I., Al-Ejeh, F., Zinonos, I., Kumar, R., Brown, M.P., Evdokiou, A., Callen, D.F. & Neilsen, P.M. (2011) Nutlin-3a is a potential theraputic for Ewing sarcoma.
Clinical Cancer Research, v. 17(3), pp. 494-504

Chapter 3

The role of p53 pathway alterations and downstream targets in sarcoma Nutlin-3a sensitivity

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PRELUDE

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 human sarcoma tissue 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. Furthermore, we provide conclusive evidence to show that the benchmark biomarkers, *TP53* and *MDM2* status cannot be used to predict Nutlin-3a induced apoptosis. This manuscript is currently under review in the *Journal of Experimental Medicine*.

Pishas, K.I., Neuhaus, S.J., Clayer, M.T., Perugini, M., Farshid, G., Manavis, J., Chryssidis, S., Mayo, B.J., Haycox, R.C., Ho, K., Brown, M.P., D'Andrea, R.J., Evdokiou, A., Callen, D.F. & Neilsen, P.M. (2013) The role of p53 pathway alterations and downstream targets in sarcoma Nutlin-3a sensitivity. *Journal of Experimental Medicine,* forthcoming.

Chapter 4

Pre-activation of the p53 pathway through Nutlin-3a sensitises sarcomas to Drozitumab therapy

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Oncology Reports (2013) 30(1):471-7

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PRELUDE

This study published in *Oncology Reports* (2013) 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.

Pishas, K.I., Neuhaus, S.J., Clayer, M.T., Adwal, A., Brown, M.P., Evdokiou, A., Callen, D.F. & Neilsen, P.M. (2013) Pre-activation of the p53 pathway through Nutlin-3a sensitises sarcomas to Drozitumab therapy.
Oncology Reports, v. 30(1), pp. 471-477

Chapter 5

New 26S-proteasome inhibitors with high selectivity for chymotrypsin-like activity and p53-dependent cytotoxicity

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Published: ACS Chemical Biology (2013) 8(2):353-9

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PRELUDE

Another approach to non-specifically activate the p53 pathway in sarcomas involves inhibiting the downstream ubiquitin-proteasome system. The 26S proteasome (catalyzes protein degradation) 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. This manuscript was published in *ACS Chemical Biology* in 2013.

Neilsen, P.M., Pehere, A.D., Pishas, K.I., Callen, D.F. & Abell, A.D. (2013) New 26S-proteasome inhibitors with high selectivity for chymotrypsin-like activity and p53-dependent cytotoxicity. *ACS Chemical Biology, v. 8(2), pp. 353-359*

Chapter 6

p53 dependent effects of low dose actinomycin D:

An alternative to Nutlin-3a

PRELUDE

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 *in vitro* and *in vivo* using mouse xenografts.

I would like to acknowledge Dr Sally Martin and Miss Bronwen Mayo for technical assistance with animal and immunohistochemical work.

INTRODUCTION

Currently 27 million people are living with a malignancy in which the tumour suppressive properties of p53 have been suppressed[1]. In half of these tumours, the p53 pathway is indirectly abrogated through inactivation of signalling or effector components such as overexpression MDM2 and MDM4 (negative regulators of p53). For this reason, reactivation of the tumour suppressive properties of p53 in patients harbouring wild-type p53 tumours has been a prime goal in the quest for more effective cancer therapeutics. Using mouse models of human cancer, the importance of sustained p53 inactivation for tumour maintenance was validated by Ventura and colleagues in 2007. Using a Cre-loxP-based strategy to temporally control p53 gene expression, this landmark study demonstrated that restoration of endogenous p53 expression into a p53 null background leads to regression of primary autochthonous lymphomas and sarcomas *in vivo* [2]. These findings provide proof-of-principle for the treatment of sarcoma and haematological malignancies by way of pharmacological reactivation of p53.

Over the past few decades, tremendous effort has been made towards the development of p53 based cancer therapies. X-ray crystallography studies of p53 have provided a solid foundation for the structure-based design of small agents designed to pharmacologically reactivate wild-type p53, several of which are currently undergoing clinical evaluation [3 (Chapter 1)]. The most advanced of these non-genotoxic p53 activating agents is Nutlin-3a (RG7112)[4]. Although Nutlin-3a has demonstrated preclinical efficacy in numerous models of sarcoma, its potency is only achieved when administered at high doses both *in vitro* (only active in the micromolar range) and *in vivo* [*5-8 & Chapter 2*]. Furthermore, as it's

clinical application has yet to be approved, the cost associated with treating the 200,000 patients currently diagnosed with sarcoma with an agent that is currently off-label would not be feasible. Fortunately, Sir David Lane and colleagues performed high throughput screening of natural product libraries to identify compounds with similar action to Nutlin-3a. This study identified actinomycin D as a compound that mimics Nutlin-3a in the highly specific activation of p53 dependent transcription [9]. As actinomycin D is the only p53 activator with current FDA approval, this provides a unique opportunity to expedite the implementation of p53 activating agents into existing sarcoma treatment protocols.

Introduced into clinical oncology in 1954[10], actinomycin D is an anti-tumour antibiotic routinely used in the treatment of paediatric malignancies such as Ewing sarcoma, rhabdomyosarcoma and Wilms tumour [11]. At high doses the DNA damage mechanism of action of actinomycin D is thought to involve DNA intercalation resulting in subsequent inhibition of DNA transcription and RNA synthesis [11,12]. In contrast, low concentrations actinomycin D (<10nM) selectively inhibits RNA pol I or pol II-dependent transcription thereby perturbing ribosomal biogenesis [12,13]. However the exact mechanism of action of low dose actinomycin D is an area of contention. In contrast to Nutlin-3a, which specifically antagonises the interaction between p53 and MDM2 [4], nucleolar stress instigated by low dose actinomycin D causes the release of free ribosomal proteins such as L11 (critical component of the 60s ribosomal subunit) which bind MDM2 [14,15]. The interaction between L11 and MDM2 leads to stabilisation of p53 due to impairment of MDM2 mediated p53 ubiquitination [16]. Furthermore, actinomycin has found to be non-genotoxic at low doses as its treatment does not induce Ser15 phosphorylation of p53 *in vitro,* which is synonymous with activation of DNA damage-dependent pathways [9,17].

Despite its long history of clinical use, the p53 dependent effect of actinomycin D in Ewing sarcoma has yet to be reported. As such, the primary focus of work presented herein was to define the p53 dependent concentration of actinomycin D required to induce Ewing sarcoma regression *in vivo.* The delineation of such a dose will be critical for the implementation of low dose actinomycin D as an alternative for Nutin-3a in sarcoma clinical settings.

Cell culture and reagents

Wild-type p53 Ewing sarcoma cell lines CADO-ES1, TC252, VH-64, WE-68 were kindly supplied by J. Sonnemann (Department of Pediatric Haematology and Oncology, University Children's Hospital, Jena, Germany), G. Hamilton (Department of Surgery, University of Vienna, Austria) and F. van Valen (Department of Orthopaedic Surgery, Westfälische-Wilhelms-University, Germany). Cell lines were cultured as previously described [7] (Chapter 2).

(-)-Nutlin-3a was purchased from Caymen Chemical (Ann Arbor, MI) and stored as a 10mM stock solution in DMSO. Lyophilised actinomycin D (Cosmegen) which was a kind gift from Professor Michael Brown, was reconstituted in 1.1mls of sterilised water (stock solution of 0.5mg/ml) as per manufacturer's instructions and stored at room temperature protected from light.

RNA interference

Cell lines with silenced expression of p53 were generated using the pGIPZ lentiviral shRNAmir system (Open Biosystems) as previously described [18]. Briefly, HEK-293T cells were seeded at 50% confluency and transfected with either a non-silencing scrambled control (RHS4346) or shRNA directed against human p53 (V2LHS217) using trans-lentiviral packaging mix according to the manufacturer's protocol (Thermo Scientific, Waltham, MA). Forty-eight hours post-transfection, growth medium containing lentivirus particles was filtered and added to recipient sarcoma cells seeded at 50% confluency. Polyclonal

populations of transduced cell were generated through subsequent puromycin selection. Expression of the shRNAmir was evident through GFP expression.

Cell Viability Assays

Cells were seeded in 96-well microtiter plates at a density of $3x10^4$ cells/well in the presence of Nutlin-3a or actinomycin D for 48 hours. The viability of harvested cells was determined using 7-amino-actinomycin-D staining and processed on a FACS Calibur flow cytometer (Becton Dickinson Immunocytometry Systems, New Jersey, NY) as previously described [7 (Chapter 2)].

Western Blot Analysis

Western blot analysis was performed as previously described [19]. Protein extracts were resolved by SDS polyacrylamide gel electrophoresis on 8% polyacrylamide gels and incubated with anti-p53 DO-1 (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) or $p21^{WAF1}$, Ab-3 (1:500; Neomarkers, Fremont, CA). Anti-β-actin (1:2000, Sigma Aldrich, St. Louis, MO) was used as an internal loading control.

Real-time PCR

Total RNA was extracted using RNeasy mini kit (Qiagen), using on-column RNase-free DNase digestion according to the manufacturer's instructions. cDNAs were generated and real-time PCR reactions processed and normalised as previously described [20]. Primer sequences are listed in Chapter 2.

In vivo techniques: Mouse strain and animal care

Four week old female athymic BALB/c *nu/nu* mice were purchased from the University of Adelaide Animal Facility (Adelaide, South Australia). Animals were housed at the IMVS Animal Care facility (Adelaide, South Australia) under a 12 hour light, 12 hour dark cycle (22 ± 3°C) with access to rodent lab chow and acidified water *ad libitum*. Animals were acclimated for seven days in the animal holding room before commencement of experimental schedules.

All experimental procedures were approved by the Animal Ethics Committees of the University of Adelaide and Institute of Medical and Veterinary Sciences and were conducted in accordance with the National Health and Medical Research Council (Australia) Code of Practice for Animal Care in Research and Training (2004).

Subcutaneous injection of sarcoma cells

TC252 cells expressing p53 or non-silencing shRNA were harvested at 80% confluency, washed in PBS and resuspended in 200µl ice cold, serum free RPMI 1640 medium. The cell suspension was then mixed with an equal volume of Matrigel matrix (Becton Dickinson, USA), using pre-cooled pipette tips to avoid polymerisation of the matrix. Mice were anaesthetised by 3% (v/v) isoflurane inhalation and placed on a nose-cone to maintain continuous exposure to anaesthesia throughout surgery. Using pre-cooled syringes and 26-gauge needles, the chilled mixture was subcutaneously injected into the left dorsal flank of the anaesthetised mouse. Each mouse was injected with $5x10^6$ TC252 cells. Tumour growth was measured every two days using an electronic calliper and calculated

using the formula π/6 x (length x width x depth) [21]. Mice were euthanized (cervical dislocation) once a tumour volume of 1000mm^3 was reached.

Subcutaneous implantation of Alzet Osmotic pumps

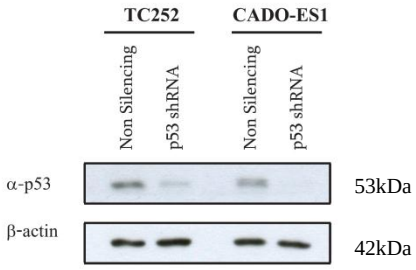
Once subcutaneous tumours developed to 50mm^3, an Alzet osmotic pump (Model 1002, DURECT Corporation, USA) containing 100µls of 250µg/ml stock solution actinomycin D was subcutaneously implanted in the upper dorsum of each mouse with the wound closed with wound clips. The pump flow modulator was positioned directly in front of the subcutaneous tumour. For control mice, osmotic pumps were filled with saline. Actinomycin D was released from the pump at a flow rate of 0.25µls/hour, delivering a total of 31.5µg of actinomycin D over a 21 day period.

To determine the p53 dependent effects of low dose actinomycin D in Ewing sarcoma, lentiviral-based delivery of shRNAs targeting p53 (or non-silencing control shRNAs) were delivered into a panel of Ewing sarcoma cell lines (TC252, CADO-ES1, VH-64 and WE-68). Knockdown of p53 resulted in effective ablation of p53 protein levels (Figure 1A). We then sought to compare the sensitivity of sarcoma cell lines expressing either p53 shRNA or control shRNA to actinomycin D and Nutlin-3a (Figure 1B). Following 48 hours of treatment, the concentration of actinomycin D required to induce 50% apoptosis (IC_{50}) in control (non-silencing shRNA) sarcoma cell line derivatives ranged from 2.2-15.3ng/ml. Upon silencing of p53, a maximum threefold increase in IC_{50} values was achieved following actinomycin D treatment. The concentration of Nutlin-3a required to induce 50% apoptosis in control (non-silencing shRNA) sarcoma cell line derivatives ranged from 2.9-4.5μM. Similarly, silencing of p53 significantly ablated the ability of Nutlin-3a to induce apoptosis ($p<0.001$). Even at the maximum concentrated tested (10μM), IC_{50} values could not be obtained for all cell lines (Figure 1B).

To confirm that the ability of low dose actinomycin D to drive apoptosis in Ewing sarcoma cells occurs through activation of the p53 pathway, p53 protein levels and transactivation of p53 target genes were assessed through western blot and real-time PCR analysis. Exposure of TC252 non-silencing shRNA cells to low dose actinomycin D was associated with dose and time-dependent stabilization of p53 protein levels and accumulation of established p53 target genes, p21 and MDM2, both at the protein (Figure 1C) and mRNA level (Figure 1D). Although not completely ablated, knockdown of p53 significantly reduced the transactivation of p53 target genes following actinomycin D treatment ($p<0.001$).

Figure 1

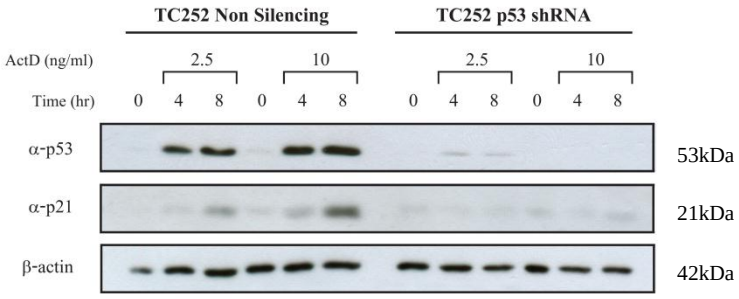
A



B

Ewing sarcoma cell line	Actinomycin D IC_{50} (ng/ml)		Nutlin-3a IC_{50} (μM)	
	Non Silencing	p53 shRNA	Non Silencing	p53 shRNA
CADO-ES1	15.3 ± 0.4	30.5 ± 1.0	2.9 ± 0.1	>10
TC252	3.5 ± 0.2	11.2 ± 0.8	3.0 ± 0.4	>10
VH-64	2.2 ± 0.5	6.2 ± 0.1	2.4 ± 0.2	>10
WE-68	3.3 ± 0.8	6.3 ± 0.6	4.5 ± 0.1	>10

C



D

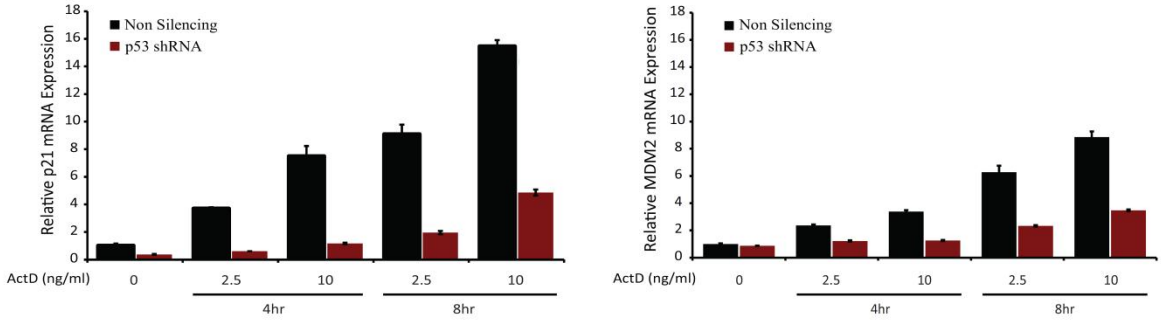


Figure 1: Low dose actinomycin D activates the p53 pathway *in vitro*

A. Western blot analysis of p53 expression in non-silencing (scrambled control) and p53 shRNA cell lines. Total β-actin levels was used as a protein loading control. The Ewing sarcoma cell lines TC252 and CADO-ES1 shRNA derivatives are shown.

B. p53 knockdown and non-silencing control cell lines were treated with increasing concentrations of Nutlin-3a (0-10μM) and actinomycin D (0-80ng/ml) for 48 hours. Cell viability was determined by 7AAD staining and analyzed by flow cytometry. IC_{50}: Concentration of agent required to induce 50% cell death.

C. TC252 non-silencing and p53 shRNA cell lines were treated with 2.5 and 10ng/ml of actinomycin D (ActD). Cell lysates were collected 4 and 8 hours after treament. Western blot analysis depicts p53 and p21 expression. Total β-actin levels was used as a protein loading control.

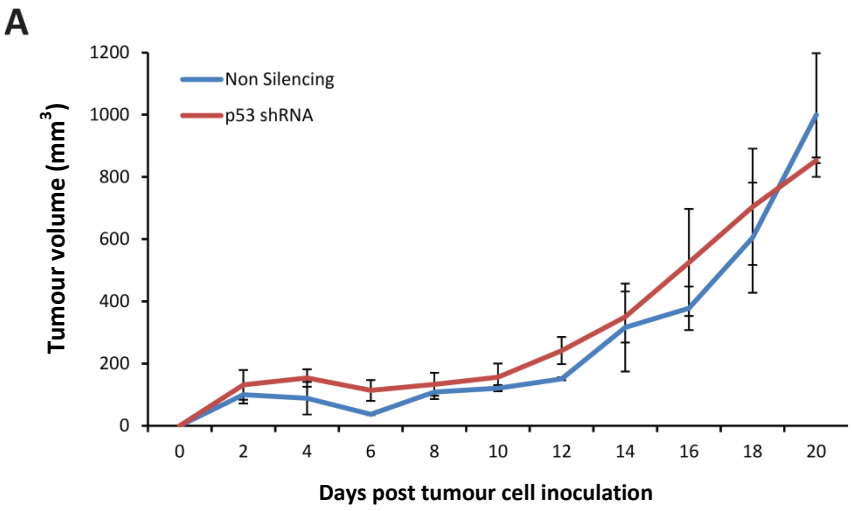
D. p21 and MDM2 mRNA expression levels (fold induction) from cells treated as in (C) was determined through real-time PCR analysis. Data represent mean ± SE from triplicate reactions

We next endeavoured to delineate the low dose of actinomycin D required to induce p53-dependent regression of sarcoma xenograft tumours. Firstly, the tumour growth kinetics of the TC252 control and p53 shRNA cell lines was assessed *in vivo*. Cell lines ($5x10^6$ cells) were subcutaneously injected into BALB/c *nu/nu* mice, with tumour growth measured every two days (n=2 mice per cell line). Knockdown of p53 had no effect on tumour growth; with tumours from both cell lines reaching volumes of $1000mm^3$ approximately 20 days post tumour cell inoculation (Figure 2A).

A pilot study was then conducted to define the dose of actinomycin D required to induce regression of TC252 control xenografts. In clinical settings, actinomycin D is administered to patients as a single bolus intravenous dose. Pharmacokinetic studies have demonstrated that actinomycin D has a high affinity for nucleated cells, highlighting its DNA intercalating characteristics *in vivo*. It penetrates poorly into red blood cells and is selectively concentrated into nucleated blood elements such as lymphocytes and granulocytes [22]. Median peak plasma concentration (C_{max}) of actinomycin D in human patients is observed 15 minutes post administration with a terminal plasma phase half-life (t ½) ranging from 14 to 43 hours, suggesting extensive extravascular distribution[11,22,23]. As actinomycin D is rapidly absorbed by nucleated cells, slow release administration through osmotic pumps was chosen as we believe that a constant low dose cannot be achieved systemically through repeat bolus injections. Once tumour volumes of $50mm^3$ were reached ALZET osmotic pumps containing either saline or actinomycin D were subcutaneously implanted into mice (n=6 mice per treatment group). Over a 21 day period, 31.5µg of actinomycin D (0.25µl/hour) can be delivered subcutaneously. Using this dose of actinomycin D, we observed a 66.2% decrease in tumour volume, 20 days post inoculation compared to control treated mice (Figure 2B).

However, severe cutaneous ulceration at the pump flow moderator site developed during actinomycin D treatment requiring premature euthanasia of mice (Figure 2C). Due to these severe side effects, we performed subsequent experiments using a reduced concentration of actinomycin D (18.9µg over 21 days). Although skin ulceration was not observed at this lower dose, actinomycin D treatment did not induce tumour regression in TC252 Ewing sarcoma xenografts (data not shown). Hence, we were unable to proceed with this *in vivo* study as we could not define a dose of actinomycin D that could induce tumour regression at doses that could be ethically used without inducing ulceration. As such, future studies investigating the effects of low dose actinomycin D will be challenging as the unfavourable pharmacokinetic properties of actinomycin D indicate that bolus intravenous dosing cannot be used to deliver a constant low dose. Further investigations into the delivery of actinomycin D are required to overcome these issues.

Figure 2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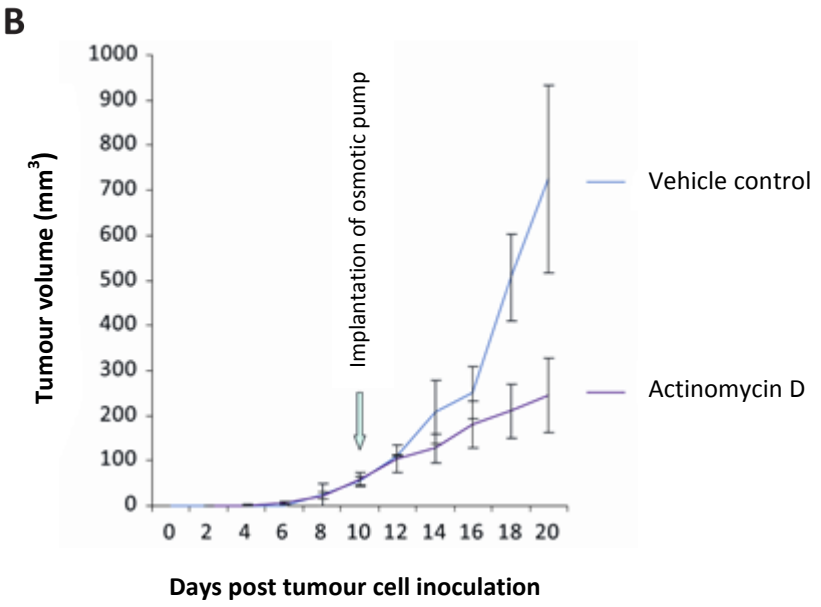
Days post tumour cell inoculation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Tumour volume (mm3)
1000
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Implantation of osmotic pump
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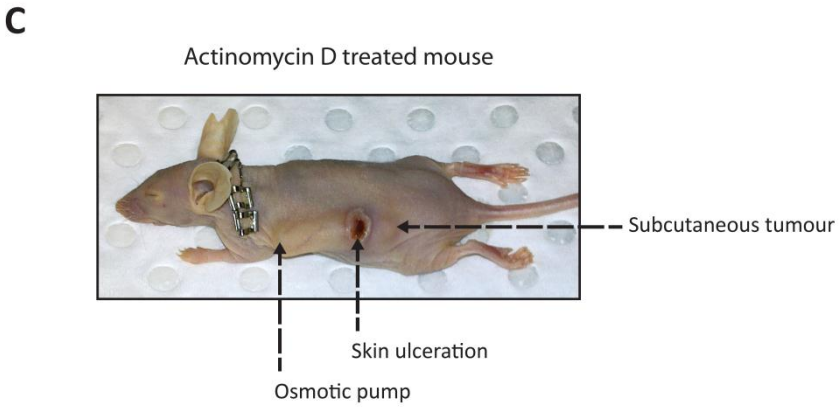
C
Actinomycin D treated mouse
Subcutaneous tumour
Skin ulceration
Osmotic pump

Figure 2: Actinomycin D induces tumour regression *in vivo*

A. Athymic BALB/c *nu/nu* mice were subcutaneoulsy injected with either $5x10^6$ TC252 non-targeting (n=2 mice) or p53 shRNA cells (n=2 mice). Tumour growth was measured every two days using digital calpers. Date represents mean tumour volume ± STDEV.

B. BALB/c *nu/nu* mice were injected with $5x10^6$ TC252 non-targeting cells. Once tumours reached $50mm^3$, ALZET osmotic pumps containing either saline (n=6) or actinomycin D (n=6) were subcutaneoulsy implanted into mice. Tumour growth was measured every two days using digital calpers. Date represents mean tumour volume ± SE.

C. Actinomycin D treated mouse with severe cutaneous ulceration.

In summary, our preliminary findings suggest that the majority of the cytotoxic activity of actinomycin D at low doses is mediated through p53. In contrast, higher doses of actinomycin D (>15nM) induces death of Ewing sarcoma cell lines through a p53-independent pathway. Nevertheless, Ewing sarcoma cell lines were shown to be sensitive to the p53 dependent anti-tumour activity of low-dose actinomycin D, further supporting the notion of p53 activators as a novel therapeutic modality for the treatment of this sarcoma subtype. However the therapeutic window for non-genotoxic activation of the p53 pathway through actinomycin D is narrow, thereby highlighting a significant challenge for its clinical application in therapeutic regimens.

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Chapter 7

Cyclotherapy

Protection of normal cells from the cytotoxic effects of chemotherapeutic agents

PRELUDE

p53-based cyclotherapy has emerged as a new paradigm in cancer treatment that aims to specifically protect normal tissues from the cytotoxic and mutagenic effects of chemotherapy, whilst maintaining the genotoxicity of chemotherapy to tumour cells. The purpose of this dose defining study was to delineate the concentration of actinomycin D required to induce reversible cellular growth arrest of intestinal cells *in vivo* (rat model).

I would like to acknowledge all the members of the Mucositis Research Group, in particular Miss Bronwen Mayo for technical assistance with all animal and immunohistochemical work.

INTRODUCTION

The basis for chemotherapeutic treatment of cancer is to ablate rapidly proliferating cell populations through the induction of apoptosis. Despite its efficiency, actions of genotoxic agents are not cancer cell-specific, often resulting in undesired deleterious cytotoxic damage to normal proliferating cells such as those found in the intestinal epithelium. Chemotherapeutic induced cytotoxicity within the gastrointestinal tract, often referred to as mucositis, is one of the most severe side effects associated with chemotherapy. Mucositis, characterised by atrophy, thinning and ulceration of the mucosal epithelium [1], is experienced by approximately 40% of patients receiving standard dose chemotherapy and all patients undergoing concomitant chemotherapy and radiotherapy or stem-cell transplantation [2]. Symptoms associated with chemotherapy induced mucositis such as pain, malabsorption, nausea and diarrhoea can be so severe that it is cited by patients as the principal reason for discontinuing chemotherapy treatment [3]. For this reason development of mucositis significantly compromises the efficacy of clinical outcomes as it leads to delays in scheduled chemotherapy courses and limits the dose of chemotherapy that can be tolerated by patients. Despite the prevalence and severity of mucositis, there is currently no definitive prophylaxis or broadly effective preventative treatments available. The development of effective intervention strategies is therefore seen as a high priority in oncological supportive care. As such, this study has assessed the potential role of p53 activators such as low dose actinomycin D as a therapeutic means to protect intestinal tissues from chemotherapy-induced mucositis.

The preponderance of current chemotherapeutic agents indiscriminately target rapidly

dividing cells. Therefore, exploiting growth arrest pathways that are altered in tumour cells but not normal cells represents a new paradigm in the treatment of cancer. This requirement is fulfilled by the p53 pathway which is inactivated in approximately 50% of all human malignancies [4]. It is proposed that pre-treatment of patients with p53 activating agents would preferentially induce a reversible p53 dependent G_1-S and or G_2-M cell cycle arrest in normal tissues thereby shielding normal cells, but not cycling mutant *TP53* tumour cells from subsequent treatments with anti-mitotics [5] (Figure 1). This sequential drug treatment strategy termed 'cyclotherapy' will spare normal tissues from the non-specific targeting effects of conventional anti-cancer drugs thereby significantly improving clinical efficacy and quality of life for cancer patients.

Figure 1: Principles of cyclotherapy

Pre-activation of the p53 pathway through low dose actinomycin D will selectively induce a reversible cell cycle arrest in normal cells. Sequential drug treatment with classic S or M phase-specific cytotoxic drugs will target cycling mutant *TP53* cancer cells resulting in apoptosis. Arrested normal cells will be protected from these cytotoxic agents and will retain their proliferative capacity after drug recovery. Figure adapted from Cheok et al., 2011[6]

In vitro proof-of-concept studies have demonstrated that pharmacologic activation of p53 using Nutlin-3a can protect normal cells from mitotic agents such as the aurora kinase inhibitor VX680 [7], taxanes [8,9], and polo-like kinase inhibitors [10]. However, the clinical use of p53 activating agents such as Nutlin-3a have yet to be approved. High-throughput screening of natural product libraries by Choong et al., identified that low non-genotoxic doses of actinomycin D (<10nM) can mimic Nutlin-3a in the highly specific activation of p53 dependant transcription [11]. At low doses, actinomycin D a clinically-approved antineoplastic agent, inhibits RNA pol I/II-dependent transcription resulting in accumulation of p53 through binding of free ribosomal proteins such as L11 to MDM2 [12,13]. In addition, treatment of normal keratinocytes with low dose actinomycin D resulted in p53-dependent reversible cell-cycle arrest [11]. Therefore, it is proposed that actinomycin D could potentially be utilised as a chemo-protective agent for patients bearing tumours with mutant *TP53*. Using an *in vivo* rat model, the purpose of this dose defining study was to define the concentration of actinomycin D required to impart reversible cellular growth arrest of intestinal cells. A rat model was chosen as our collaborators have previously established reproducible models of irinotecan and 5-Fluorouracil (5-FU) induced gastrointestinal mucositis in rats [14,15].

MATERIALS & METHODS

Animals

Twenty-four male Albino Wistar rats weighing between 240 and 260 grams were purchased from Animal Resources Centre (ARC, Perth, Australia). Animals were randomly assigned to four experimental groups and housed under a 12 hour light, 12 hour dark cycle (22 ± 3°C) with access to rodent laboratory chow and acidified water *ad libitum*. Animals were acclimated for seven days in the animal holding room before commencement of experimental schedules.

Actinomycin D

Lyophilised actinomycin D (Cosmegen), was reconstituted in 1.1mls of sterilised water to a concentration of 0.5mg/ml as per manufacturer's instructions and stored at room temperature protected from light.

Experimental Procedures

All experimental procedures were approved by the Animal Ethics Committees of the University of Adelaide and Institute of Medical and Veterinary Sciences and were conducted in accordance with the National Health and Medical Research Council (Australia) Code of Practice for Animal Care in Research and Training (2004). An overview of the experimental protocol is presented in Figure 2.

On Day 1, animals were administered with a single bolus intraperitoneal (*i.p*) injection of actinomycin D (0.02, 0.06 or 0.20mg/kg). Control animals were given equivalent volumes of sterilised water (approximately 2ml). The rationale for choosing doses below 0.2mg/kg is that the LD_{50} for actinomycin D in Wistar rats (single *i.p* injection) is reported to be in the order of 0.40mg/kg [16].

Animals were weighed daily and monitored for signs of distress (dull/ruffled coat, sunken eyes, diarrhoea, reluctance to move and hunched appearance). As high doses of actinomycin D are cytotoxic, diarrhoea was assessed as a measure of gastrointestinal damage. On the basis of stool consistency, a numerical score for diarrhoea grading was assigned as follows: 0 (no diarrhoea), 1 (mild diarrhoea), 2 (moderate diarrhoea) and 3 (severe diarrhoea). Two animals per experimental group were euthanized via cervical dislocation whilst under deep anaesthesia (Isoflurane) at each time point (24, 72 and 96 hours post injection). All animals were injected (*i.p*) with 50mg/kg 5-bromo-2'deoxyuridine (BrdU) (Sigma; St Louis, MO) 1½ hours before sacrifice. The small and large intestine were surgically removed and flushed with chilled sterile saline (0.9% NaCI). Two 1cm segments of proximal duodenum, middle jejunum, distal ileum and colon were fixed in formaldehyde, processed and embedded in paraffin.

H&E Staining

Serial 4 µM transverse sections of gastrointestinal tissue were stained with haematoxylin and eosin (H&E). Briefly, sections were deparaffinised in xylene, rehydrated in ethanol and stained with Lillie–Mayer's haematoxylin for 10 minutes. After differentiating in 1% acid alcohol and bluing in Scott's tap water, sections were counterstained in eosin for 3 minutes, dehydrated in ethanol, cleared in xylene and mounted on glass slides.

Immunohistochemistry

Immunohistochemistry was performed as previously described (Chapter 3). Briefly sections were immunolabelled with the following primary antibodies: rabbit polyclonal activated-caspase 3 (ab4051, 1:100, Abcam, Cambridge, United Kingdom), and mouse monoclonal anti-bromodeoxyuridine (Bu20a, 1:250, Dako Cytomation). BrdU is incorporated into newly synthesized DNA of actively replicating cells during S-phase. All incubations were performed overnight at 4°C. Sections were subsequently incubated with the appropriate biotinylated secondary antibody, anti-rabbit IgG (1:200, Vector Laboratories, Burlingame, CA) or anti-mouse IgG (1:200, Vector Laboratories) for 30 minutes at room temperature, followed by incubation with avidin-biotin peroxidase complexes (Vector Laboratories) for an additional 30 minutes. Digital images of all sections were acquired using a Nanozoomer Digital Pathology Scanner (Hamamatsu, Japan) at a magnification of 400x. For quantitative analysis, 20 full crypts or villi per section/animal were randomly selected and scored for the number of positive/negative BrdU stained cells. Analysis was conducted in a blinded fashion.

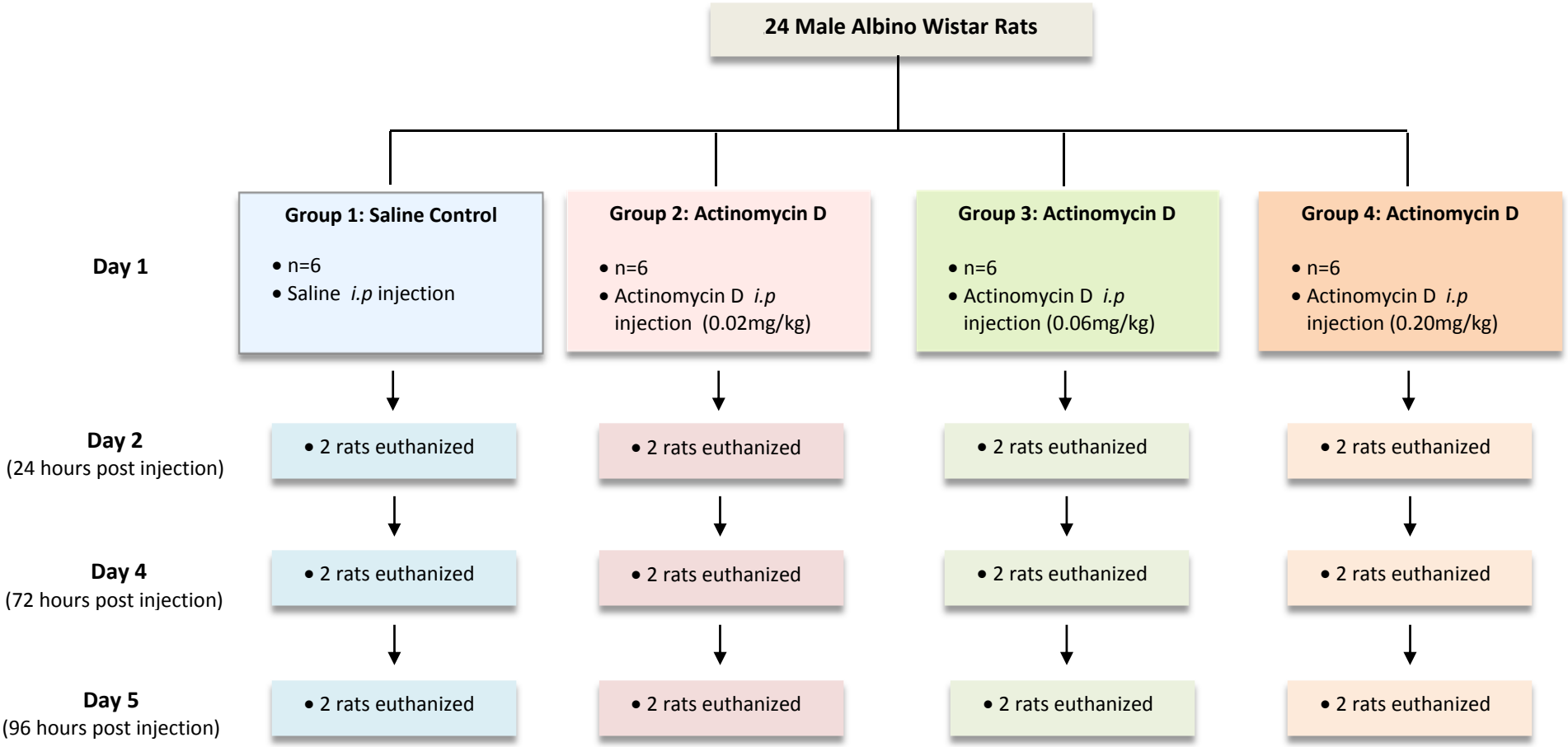


Figure 2: Experimental procedure for dose defining actinomycin D *in vivo* study.

Twenty-four Albino Wistar rats were injected (*i.p*) with either vehicle control or actinomycin D (0.02, 0.06, or 0.20mg/kg). Two rats from each treatment group were euthanized 24, 72 and 96 hours post injection. Sections of small and large intestine were removed for histological and immunohistochemical analysis. All animals were injected (*i.p*) with BrdU (50mg/kg) 1 hour prior to euthanasia.

RESULTS & DISCUSSION

General condition of animals

Single bolus administration of actinomycin D (0.02, 0.06 and 0.20mg/kg) had no effect on overall survival of animals (no mortality was observed). However, a single 0.20mg/kg dose induced an abrupt change in body weight (-3.1%) 24 hours post-administration, reaching a maximum weight loss of approximately 12.1% 96 hours post-injection (Figure 3). Grade 2 diarrhoea and ruffled coats was observed at all-time points for this treatment group. Furthermore, necropsy showed abdominal adhesions and an excess of fluid in the abdominal cavity of an unknown source.

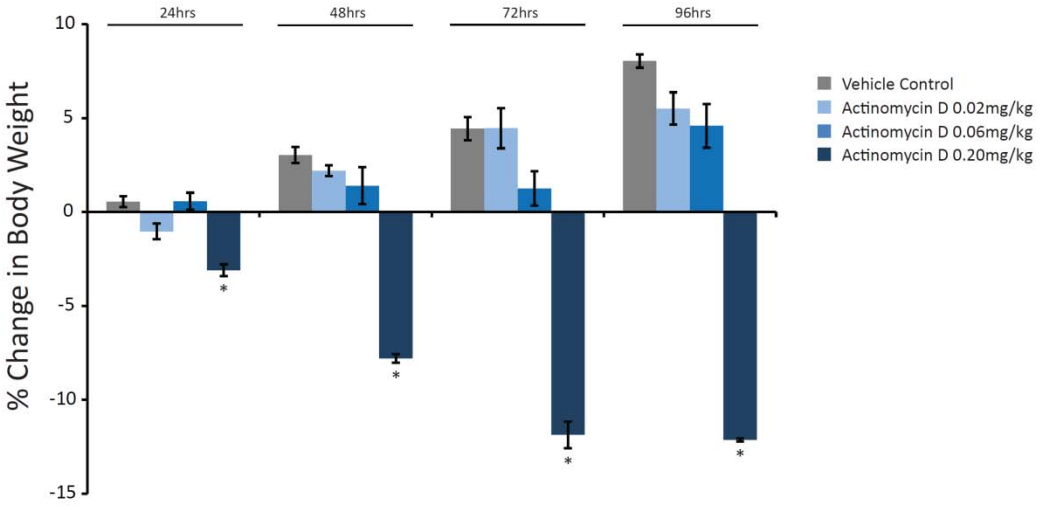


Figure 3: Effect of actinomycin D on body weight

Animals were injected (*i.p*) with either vehicle control or actinomycin D (0.02, 0.06 or 0.20mg/kg) and euthanized 24, 72 and 96 hours after injection. Values are expressed as mean ± standard deviation (n=2). * Statistical significance compared to vehicle control ($p<0.005$)

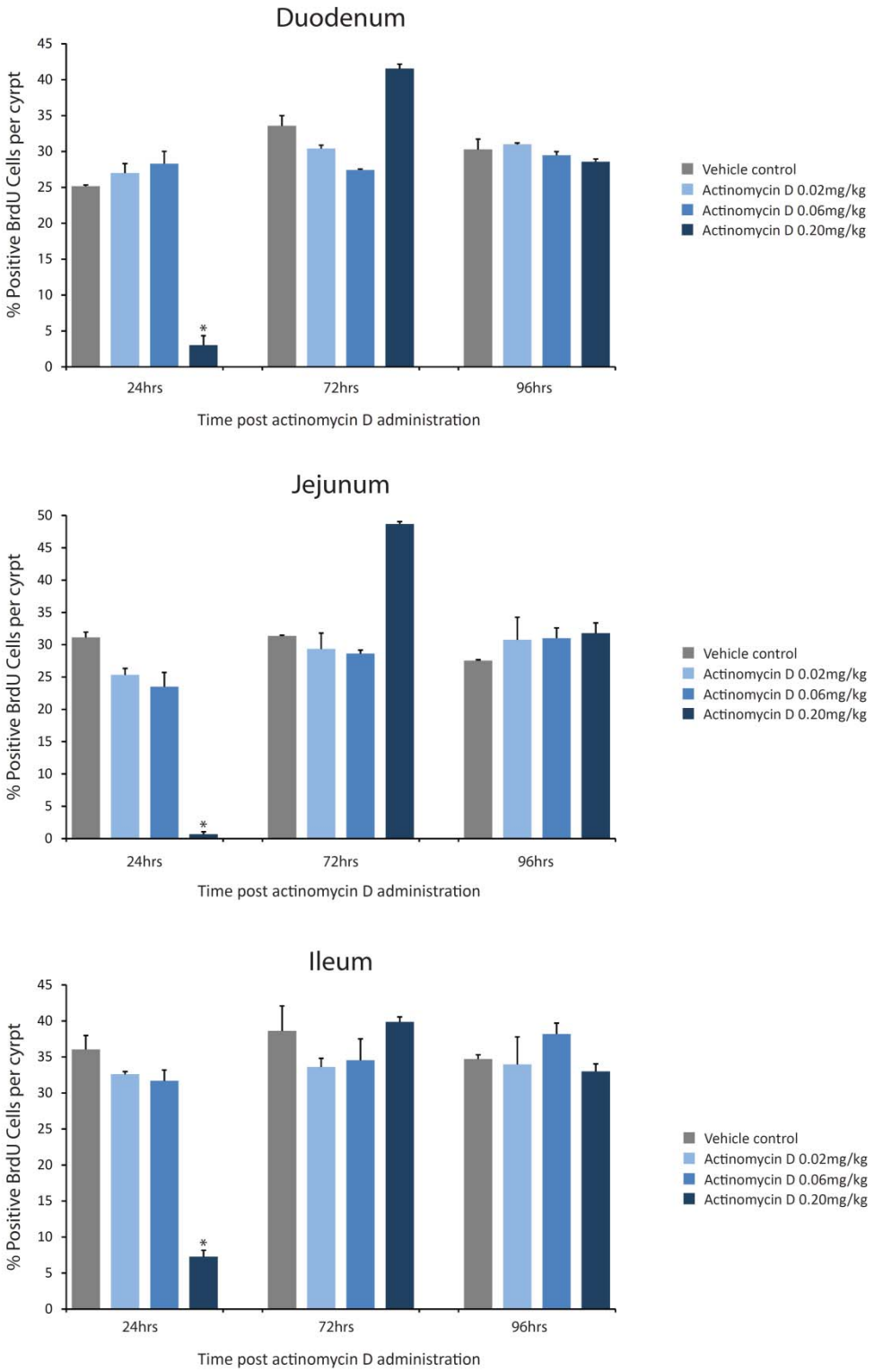
Effect of Actinomycin D treatment on intestinal cell proliferation and morphology

To ascertain whether actinomycin D treatment can induce cell cycle arrest of intestinal cells, localization of proliferation was evaluated through immunohistochemical detection of incorporated BrdU, a marker of DNA synthesis (S-phase cells). As measured by the percentage of BrdU positive cells within the crypt epithelium, actinomycin D treatment showed minimal effect on the number of cells proliferating in S-phase at all-time points in duodenum, ileum, jejunum and colon sections following injection (Figures 4 and 5), except 24 hours post-injection of the highest dose actinomycin D. Actinomycin D at this dose (0.20mg/kg) resulted in a significant decrease *($p<0.005$)* in the number BrdU positive cells 24 hours post injection throughout the intestinal tract. However by 72 hours the number of mitotic figures per crypt had returned to baseline levels.

The reduction in mitotic index could be attributed to an observed increase in apoptotic cells as determined by activated caspase-3 immunohistochemistry (Figure 6). As no cell cycle arrest was seen post actinomycin D treatment, activated caspase-3 immunohistochemistry data was not quantified. H&E staining demonstrated 0.20mg/kg of actinomycin D caused severe morphological damage in terms of considerable loss of crypt integrity and villus structure 24 hours post injection. Mucosal architecture was preserved for all other treatment groups. Consistent with reports that the mammalian small intestine epithelium is replenished every 3-4 days [17], few apoptotic bodies were observed 96 hours post administration of actinomycin D (0.20mg/kg) with villi length and crypt structure restored. Actinomycin D (0.06 and 0.20mg/kg) preferentially induced apoptosis between the 3-6 cell positions within the intestinal crypts, the putative location of intestinal stem cells which are highly

chemo/radiation sensitive [18]. This is in accordance previous reports which demonstrated that induction of apoptosis by actinomycin D (single *i.p* injection, 0.1mg per mouse) is localised to positions 6-8 of intestinal crypts (proliferative region) [19].

Figure 4


Duodenum
% Positive BrdU Cells per cyrpt
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24hrs
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96hrs
Time post actinomycin D administration
Vehicle control
Actinomycin D 0.02mg/kg
Actinomycin D 0.06mg/kg
Actinomycin D 0.20mg/kg
Jejunum
% Positive BrdU Cells per cyrpt
50
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40
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96hrs
Time post actinomycin D administration
Vehicle control
Actinomycin D 0.02mg/kg
Actinomycin D 0.06mg/kg
Actinomycin D 0.20mg/kg
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Time post actinomycin D administration
Vehicle control
Actinomycin D 0.02mg/kg
Actinomycin D 0.06mg/kg
Actinomycin D 0.20mg/kg

Figure 4: Actinomycin D does not affect intestinal cellular proliferation levels

Animals were injected (*i.p*) with either vehicle control or actinomycin D (0.02, 0.06 or 0.20mg/kg) and euthanized 24, 72 and 96 hours after injection. Cellular proliferation was determined via immunohistochemical staining for BrdU. Values are expressed as mean ± standard deviation (n=2). * Statistical significance compared to vehicle control ($P<0.005$)

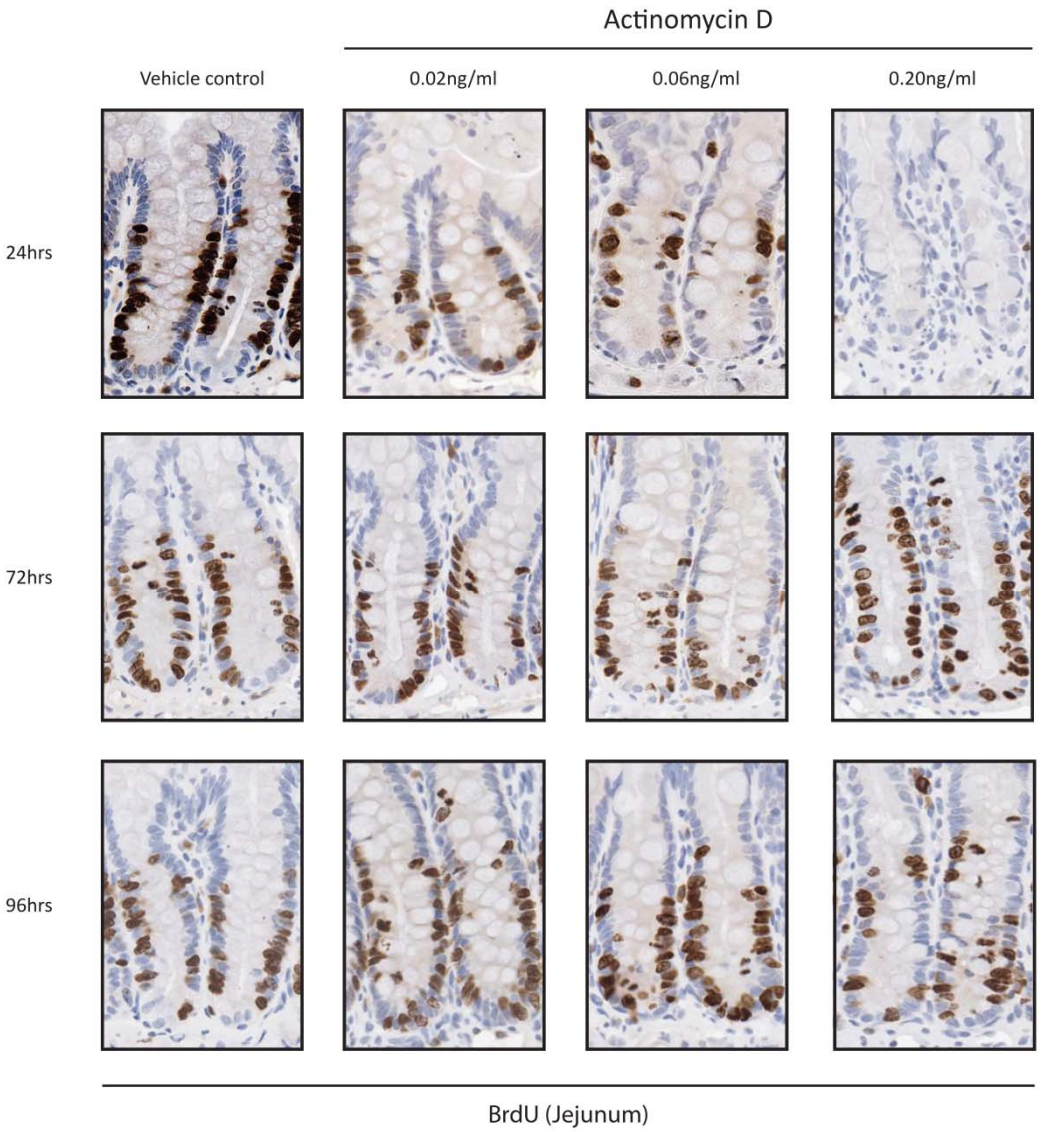

Figure 5: Actinomycin D does not induce cell cycle arrest of gastrointestinal cells

Animals were injected (*i.p*) with either vehicle control or actinomycin D (0.02, 0.06 or 0.20mg/kg) and euthanized 24, 72 and 96 hours after injection. Cellular proliferation was determined via immunohistochemical staining for BrdU. Representative images of jejunum sections shown at 400x magnification.

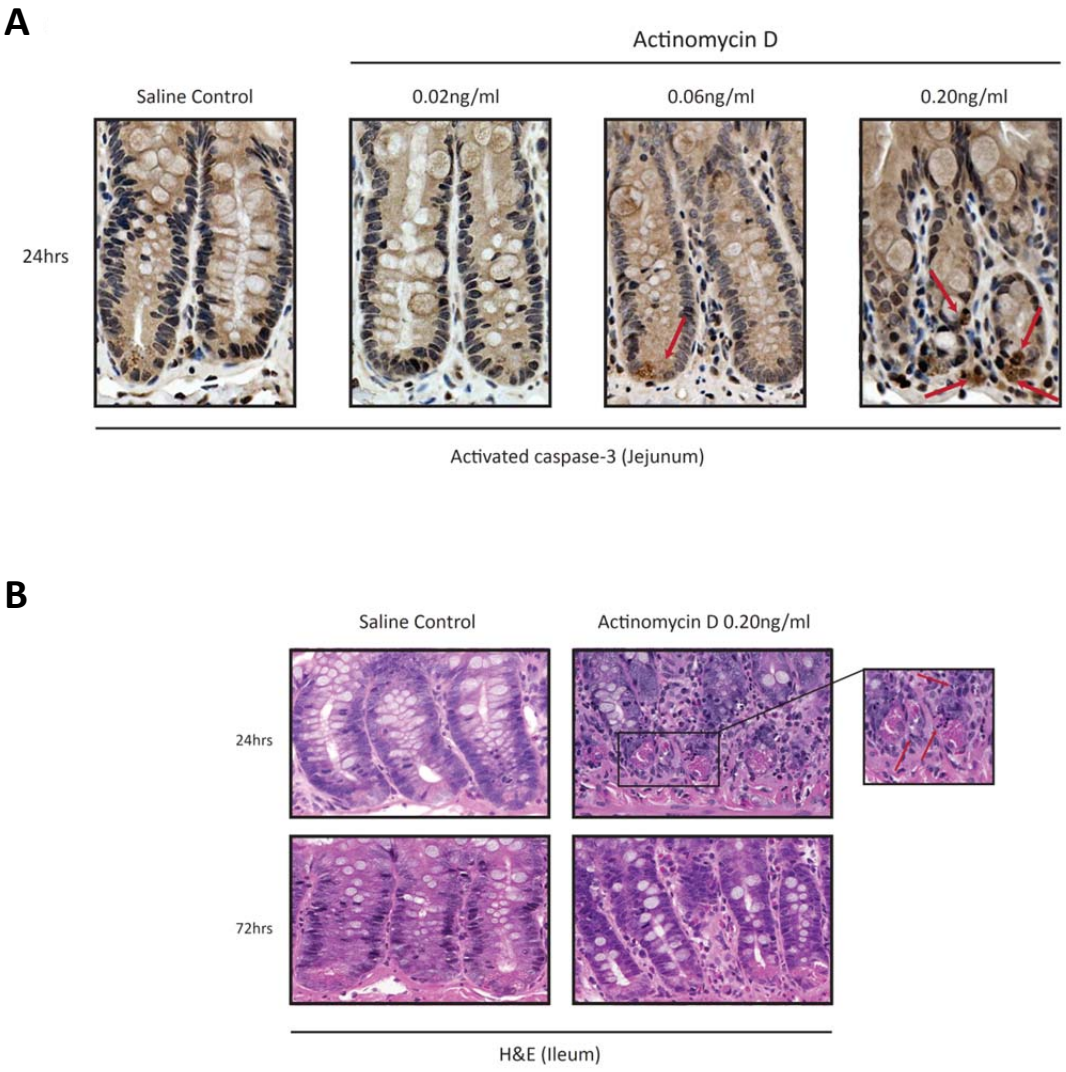

Figure 6: Actinomycin D treatment induces apoptosis.

A. Animals were injected (*i.p*) with either vehicle control or actinomycin D (0.02, 0.06 or 0.20mg/kg) and euthanized 24, 72 and 96 hours after injection. Transverse intestinal sections (duodenum, ileum, jejunum and colon) were stained for activated caspase-3 (marker of apoptosis). Representative immunohistochemistry staining from 24 hour jejunum sections shown at 40x magnification. Red arrows indicate apoptotic cells.

B. Representative H&E staining of ileum sections from animals treated as in (A) 24 and 72 hours post injection. Images shown at 40x magnification. Red arrows indicated apoptotic cells.

In summary, the study described here attempted to define the dose of actinomycin D required to induce prolonged growth arrest of rat intestinal cells. Our results suggest that pre-treatment with actinomycin D will not be a feasible modality in alleviating chemotherapy induced mucositis, as administration of actinomycin did not induce cell cycle arrest at any of the concentrations tested in this particular rodent model. At the highest actinomycin D dose tested (0.20mg/kg) significant transient apoptosis was induced. Although our goal was not achieved, further studies investigating the potential role of implementing p53 activating agents such as Nutlin-3a to shield normal cells from chemotherapy induced mucositis should be considered in alternative *in vivo* models.

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Conclusion

CONCLUSION

Sarcomas are a complex heterogeneous group of rare solid bone and soft tissue malignancies that disproportionally affect the young. Currently, treatment is multimodal with surgical removal and radiotherapy used as cornerstones for local control, along with chemotherapy for systemic disease (chemo-sensitive subtypes only). Despite these aggressive treatment strategies, 50% of patients succumb to disease within 5 years of primary diagnosis, primarily due to metastatic disease. As such there is an imperative need for the development and implementation of new targeted therapeutics to improve outcomes for sarcoma patients.

The p53 tumour suppressor, often referred to as the 'guardian of the genome' orchestrates several complex cell cycle and apoptotic signal transduction pathways in response to cellular insult. Due to its central role in maintaining genomic integrity, p53 is frequently inactivated in cancer primarily through mutation or deletion. In contrast, aberrations in p53 are rarely observed in most sarcoma subtypes, making them an ideal model for the testing of p53 targeted therapies (discussed in Chapter 1). As such, the primary aim of work presented in thesis has been to investigate whether pharmacological activation of p53 represents a new therapeutic means for the treatment sarcomas.

Targeted therapy encompassing p53 activating agents has been an area of active investigation, with early-phase clinical trials underway. At the forefront of p53 targeted therapy is Nutlin-3a (RG7112), a cis-imidazoline agent which sterically interferes with the binding of MDM2 and p53. Indeed our results conclusively demonstrate that treatment of Ewing sarcoma cell lines with Nutlin-3a results in rapid stabilisation and biological activation

of p53 leading to robust apoptosis *in vitro* (Chapter 2). Furthermore, Nutlin-3a synergized with current Ewing sarcoma chemotherapy protocols (Vincristine, Actinomycin, Doxorubicin and Etoposide), further supporting p53 activation as a novel systemic therapeutic approach for this disease.

In the age of personalised medicine, the use of genetic markers to predict patient response and resistance, will be critical for the optimal clinical implementation of Nutlin-3a in sarcoma treatment protocols. Using a novel *ex vivo* methodology we have explored the role of p53 pathway alterations and downstream targets in their ability to confer sensitivity to Nutlin-3a (Chapter 3). This is the first study to use primary sarcoma tumour tissue to validate that Nutlin-3a elicits robust apoptotic and cytostatic cellular responses in a subset of sarcomas. Our findings refute the accepted opinion that wild-type p53 status and *MDM2* amplification are critical determinants for Nutlin-3a sensitivity and we have identified new p53 target genes specifically unregulated during Nutlin-3a induced apoptosis. Lastly, we show that epigenetic silencing of these targets may be a mechanism through which many sarcomas possess intrinsic resistance to Nutlin-3a.

Tumourigenesis is a multifaceted process that requires dysregulation of several pathways that are essential for cellular growth and survival; hence monotherapuetic strategies will inevitably lead to acquired resistance. As such we explored whether targeted activation of both the intrinsic and extrinsic pathway of apoptosis through the combination of Nutlin-3a and Drozitumab (agonistic monoclonal antibody directed against TRAIL receptor DR5) (Chapter 4) will be an effective therapeutics means for sarcomas. Although p53 knockdown experiments revealed that p53 status alone has no influence on the ability of Drozitumab to

impart its cytotoxic effect; pre-activation of the p53 pathway through Nutlin-3a did sensitise sarcomas to Drozitumab induced apoptosis both *in vitro* and *ex vivo.*

Another approach to non-specifically activate the p53 pathway in sarcomas involves inhibiting the downstream ubiquitin-proteasome system. The 26S proteasome has emerged over the past decade as an attractive therapeutic target in the treatment of cancers. Recently Bortezomib (a potent proteasome inhibitor) has been approved as a frontline treatment for multiple myeloma. In spite of its success, Bortezomib possesses numerous undesirable characteristics including a very narrow therapeutic range, lack of specificity for cancer cells, severe side effects and almost uniformly development of resistance. As such, this thesis has reported new tripeptide aldehydes that are highly specific for the chymotrypsin-like catalytic activity of the proteasome (Chapter 5). These new proteasome inhibitors demonstrated potent p53 dependent apoptotic activity specifically in sarcoma cell lines whilst having no effect on non-malignant primary cells. Collectively our results suggest that activation of the p53 pathway through the inhibition of the proteasome using these new tripeptide aldehydes represents a new treatment avenue for sarcomas and possibly a replacement for Bortezomib.

Although Nutlin-3a shows great promise for solid malignancies, its clinical application has yet to be approved. Fortunately, high throughput screening of natural product libraries by Sir David Lane and colleagues identified actinomycin D as a compound that mimics Nutlin-3a in the highly specific activation of p53 dependent transcription when administered at low doses. As actinomycin D is the only p53 activator with current FDA approval, this provides a unique opportunity to expedite the implementation of p53 activating agents into existing sarcoma treatment protocols. As such we conducted a study to define the

p53 dependent concentration of actinomycin D required to induce Ewing sarcoma regression *in vivo* (Chapter 6). Although our results confirm that Ewing sarcoma cell lines are sensitive to the p53 dependent anti-tumour activity of low-dose actinomycin D both *in vitro* and *in vivo*, doses required to elicit sarcoma regression *in vivo* (mouse model) led to severe cutaneous ulceration. Due to the unfavourable pharmacokinetic properties of actinomycin D, further investigations into the delivery of this agent are required as a constant low dose cannot be achieved through bolus intravenous administration.

p53-based cyclotherapy has emerged as a new paradigm in cancer treatment that aims to specifically protect normal tissues from the cytotoxic effects of chemotherapy, whilst maintaining the genotoxicity of chemotherapy to tumour cells. Despite its efficacy the use of chemotherapy is frequently curtailed by the acute toxicity of these agents, thereby compromising the efficacy of clinical outcomes. Therefore this thesis addressed whether the p53 activating agent actinomycin D (low dose) could protect normal proliferating cells such as those found in the intestinal epithelium from the non-specific cytotoxic effects of chemotherapeutic agents by inducing transient cell cycle arrest (Chapter 7). Our results suggest that pre-treatment with actinomycin D will not be a feasible modality in alleviating chemotherapy induced mucositis (damage of the gastrointestinal epithelium), as administration of actinomycin D did not induce cell cycle arrest at any of the concentrations tested in our particular rodent model. Although our goal was not achieved, further studies investigating the potential role of implementing p53 activating agents to shield normal cells from chemotherapy induced mucositis should be considered in alternative *in vivo* models. Indeed, recently Sir David Lane and colleagues have defined the dose of actinomycin D

required to induce transient cell cycle arrest of mouse gastrointestinal cells (*personnel communication*).

In summary, this thesis has validated that pharmacological activation of p53 through agents such as Nutlin-3a and low dose actinomycin D represents an exciting new means for the targeted treatment of sarcomas. It is hoped that findings presented will make significant inroads towards alleviating the burden of sarcoma in our community.