Soft Tissue Sarcoma: Biology and Therapeutic Correlates.

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by published work[1]

incorporating publications arising from research carried out in the department of Surgical Oncology at the University of Texas MD Anderson Cancer Centre, Houston, Texas, USA.

Summary

Soft tissue sarcomas (STS) comprise a heterogenenous group of greater than 50 malignancies of putative mesenchymal cell origin and as such they may arise in diverse tissue types in various anatomical locations throughout the whole body. Collectively they account for approximately 1% of all human malignancies yet have a spectrum of aggressive behaviours amongst their subtypes. They thus pose a particular challenge to manage and remain an under investigated group of cancers with no generally applicable new therapies in the past 40 years and an overall 5-year survival rate that remains stagnant at around 50%.

From September 2000 to July 2006 I undertook a full time post-doctoral level research fellowship at the MD Anderson Cancer Center, Houston, Texas, USA in the department of Surgical Oncology to investigate the biology of soft tissue sarcoma and test novel anti-sarcoma adenovirus-based therapy in the preclinical nude rat model of isolated limb perfusion against human sarcoma xenografts. This work, in collaboration with colleagues as indicated herein, led to a number of publications in the scientific literature furthering our understanding of the malignant phenotype of sarcoma and reported preclinical studies with wild-type p53, in a replication deficient adenovirus vector, and oncolytic adenoviruses administered by isolated limb perfusion. Additional collaborative and pioneering preclinical studies reported the molecular imaging of sarcoma response to systemically delivered therapeutic phage RGD-4c AAVP.

Doxorubicin chemotherapy is the single most active broadly applicable anti-sarcoma chemotherapeutic yet only has an approximate 30% overall response rate with additional breakthrough tumour progression and recurrence after initial chemo-responsiveness further problematic features in STS management. Doxorubicin is a substrate for the multi-drug resistance (*mdr*) gene product p-glycoprotein drug efflux pump and exerts its main

mode of action by induction of DNA double-strand breaks during the S-phase of the cell cycle. Two papers in my thesis characterise different aspects of chemoresistance in sarcoma. The first shows that wild-type p53 suppresses Protein Kinase Calpha (PKCα) phosphorylation (and activation) of p-glycoprotein by transcriptional repression of PKCα through a Sp-1 transcription factor binding site in its -244/-234 promoter region. The second paper demonstrates that Rad51 (a central mediator of homologous recombination repair of double strand breaks) has elevated levels in sarcoma and particularly in the S-G2 phase of the cell cycle. Suppression of Rad51 with small interfering RNA in sarcoma cell culture led to doxorubicin chemosensitisation. Reintroduction of wild-type p53 into STS cell lines resulted in decreased Rad51 protein and mRNA expression via transcriptional repression of the Rad51 promoter through increased AP-2 binding.

In light of poor response rates to chemotherapy, escape from local control portends a poor prognosis for patients with sarcoma. Two papers in my thesis characterise aspects of sarcoma angiogenesis, invasion and metastasis. Human sarcoma samples were found to have high levels of matrix metalloproteinase-9 (MMP-9) with expression levels that correlated with p53 mutational status. MMP-9 is known to degrade extracellular collagen, contribute to the control of the angiogenic switch necessary in primary tumour progression and facilitate invasion and metastasis. Reconstitution of wild-type p53 function led to decreased levels of MMP-9 protein and mRNA as well as zymography-assessed MMP-9 proteolytic activity and decreased tumour cell invasiveness. Reintroduction of wild-type p53 into human sarcoma xenografts *in-vivo* decreased tumour growth and MMP-9 protein expression. Wild-type p53 was found to suppress *mmp-9* transcription via decreased binding of NF-κB to its -607/-595 *mmp-9* promoter element. Studies on the role of the $VEGF_{165}$ in sarcoma found that sarcoma cells stably transfected with $VEGF_{165}$ formed more aggressive xenografted tumours with increased vascularity, growth rate, metastasis, and resistance to chemotherapy. Use of the anti-VEGFR2 antibody DC101 enhanced doxorubicin sensitivity at sub-conventional dosing, inhibited tumour growth, decreased

development of metastases, and reduced tumour micro-vessel density while increasing the vessel maturation index. These effects were explained primarily through effects on endothelial cells (e.c.s), rather than the tumour cells *per se,* where DC101 induced e.c. sensitivity to doxorubicin and suppressed e.c. production of MMPs.

The p53 tumour suppressor pathway is the most frequently mutated pathway in sarcoma. Recapitulation of wild-type p53 function in sarcoma exerts a number of anti-cancer outcomes such as growth arrest, resensitisation to chemotherapy, suppression of invasion, and attenuation of angiogenesis. Using a modified nude rat-human sarcoma xenograft model for isolated limb perfusion (ILP) delivery of wild-type *p53* in a replication deficient adenovirus vector I showed that functionally competent wild-type p53 could be delivered to and detected in human leiomyosarcoma xenografts confirming preclinical feasibility - although not efficacious due to low transgene expression. Viral fibre modification to express the RGD tripeptide motif led to greater viral uptake by sarcoma cells *in vitro* (transductional targeting) and changing the transgene promoter to a response element active in cells with active telomerase expression restricted the transgene expression to the tumour intracellular environment (transcriptional targeting). Delivery of the fibre-modified, selectively replication proficient oncolytic adenovirus Ad.hTC.GFP/ E1a.RGD by ILP demonstrated a more robust, and tumour-restricted, transgene expression with evidence of anti-sarcoma effect confirmed microscopically. Collaborative studies using the fibre modified phage RGD-4C AAVP confirmed that systemic delivery specifically, efficiently, and repeatedly targets human sarcoma xenografts, binds to αv integrins in tumours, and demonstrates a durable, though heterogeneous, transgene expression of 1-4 weeks. Incorporation of the Herpes Simplex Virus thymidine kinase (HSVtk) transgene into RGD-4C AAVP permitted CT-PET spatial and temporal molecular imaging *in vivo* of transgene expression and allowed quantification of tumour metabolic activity both before and after interval administration of a systemic cytotoxic with predictable and measurable response to treatment before becoming apparent clinically.

These papers further the medical and scientific community's understanding of the biology of soft tissue sarcoma and report preclinical studies with novel and promising anti-sarcoma therapeutics.

Contents

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Dr Nora Janjan (former Professor of Radiation Oncology and Symptom Research at The University of Texas M.D. Anderson Cancer Center) and Prof. David George (former Professor of Surgery, University of Glasgow) for encouragement to pursue a period of research apart from clinical commitments to properly learn the discipline of laboratory based research and make a meaningful contribution to cancer research.

Dr Raphael E Pollock (former Professor of Surgical Oncology, Chairman of the Department of Surgical Oncology, and Chief of the Division of Surgery, University of Texas M.D. Anderson Cancer Center) for appointing me as his research fellow, funding, and patientience in guiding & constraining my involvement in the projects that I undertook.

Dr Dihua Yu (Professor of Molecular and Cellular Oncology, University of Texas M.D. Anderson Cancer Center) for clear and experienced scientific advice.

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The many members of the Pollock, Yu, and Lev labs for daily helpful advice, encouragement and long lasting friendship. Particular thanks are due to Drs Maocheng Zhan, Juehui Liu, and Lianglin Zhang for close collaborative and mutual help as well as their ready agreement to include their papers that I contributed to in this thesis.

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Finally, special thanks to the Leonardo family, USA for their love, warmth, and generosity in providing me more than a home-from-home while I undertook my fellowship in the USA.

Declaration

I hereby declare that, where indicated and itemised in the accompanying explanatory essay, I personally conceived and undertook the research projects, performed the experimentation, interpreted the results, drafted, edited, and submitted the accompanying peer-reviewed published papers arising from my Surgical Oncology post-doctoral research fellowship at the University of Texas M.D. Anderson Cancer Center, Houston Texas, USA during the period of September 2000 through June 2006.

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Jonathan AF Hannay.

Abbreviations

AAVP	adeno-associated virus phage
AP-2	activator protein 2
CAR	cocsackie-adenovirus receptor
DNA	deoxyribonucleic acid
e.c.	endothelial cells
HSVtk	Herpes Simplex Virus thymidine kinase
GIST	gastro-intestinal stromal tumour
ILP	isolated limb perfusion
mdr-1	multi-drug resistance gene 1
MMP-2	matrix metallo-proteinase 2
MMP-9	matrix metallo-proteinase 9
mRNA	messenger ribonucleic acid
NER	nucleotide excision repair
NF-κB	nuclear factor kappa B
NSAIDs	non-steroidal anti-inflammatory drugs
p53	tumour suppressor p53 gene
p53	tumour suppressor p53 protein
PDGFRα	platelet derived growth factor receptor alpha
PDGFRβ	platelet derived growth factor receptor beta
PKCα	protein kinase C alpha
rad51	rad51 gene
Rad51	Rad51 protein
RGD	arginine-glycine-aspartic acid tripeptide
siRNA	short inhibitory ribonucleic acid
SP-1	specificity protein 1
STS	soft tissue sarcoma
VEGF	vascular endothelial growth factor
VEGFR1	vascular endothelial growth factor receptor 1
VEGFR2	vascular endothelial growth factor receptor 2
VEGFR3	vascular endothelial growth factor receptor 3
WHO	World Health Organisation

Explanatory Essay

Introduction

Soft tissue sarcomas (STS) comprise a heterogenenous group of greater than 50 malignancies of putative mesenchymal cell origin. As such they may arise in diverse tissue types in various anatomical locations throughout the whole body including muscle, fat, peripheral nerve components, and fibrous tissue types, etc. Considering that mesenchymally derived tissue accounts for over 75% of the mass of an adult human it is surprising that STS is not encountered more frequently than the 1% of all malignancies it, as a group, accounts for. None-the-less, in 2012 it was anticipated that there would be over 11,000 new cases of STS in the USA[1] and over 2,300 in England and Wales[2, 3]. The World Health Organization (WHO) recognizes that diagnosis and management of these cancers pose a particular challenge for clinicians due to the following compounding layers of complexity[4]:

1. The relative rarity of STS: the incidence of STS as a group is approximately 30/ million accounting, as mentioned above, for around 1% of adult human malignancies.
2. The heterogeneity of subtypes: over 50 different sub-types of STS are categorized in the World Health Organization Classification of Tumours: Pathology and Genetics of Tumours of Soft Tissue and Bone.
3. The diverse lineages or host / parental tissue types that STS arises in and the resultant diverse histopathologic characteristics.
4. The wide range of behaviours from fairly indolent to highly invasive and aggressive.

5. The age range of affected patients: STS accounts for 15% of paediatric malignancies and the overall incidence rises with each decade of life to a peak around 65years of age.
6. The incidence of benign counterparts is around 100-to-1.

Although these tumours are by nature challenging, a thorough appreciation of STS biology and their management is still necessary to avoid compromised outcome if the initial management of patients with these tumours is sub-optimal[5]. Tragically, though, in spite of multimodal treatment in 'centres of excellence' and despite the relative successes in some STS subtypes - such as imatinib for the treatment of gastro-intestinal stromal tumours (GISTs) - the 5-year survival rate for patients with STS remains stagnant at around 50%.

Perhaps because of their relative rarity and complexity, STS remains an under-investigated tumour group in recent decades in contrast to the milestones of generally applicable biologic insights gained previously from basic science research of sarcomas - dating from Peyton Rous' seminal work on the transmission of avian spindle cell sarcomas in Plymouth Rock hens, published in 1911[6]. No doubt related to this paucity of investigation, there have been no broadly applicable new therapies for STS in the past 40 years beyond the use of doxorubicin and ifosphamide based chemotherapy regimens.

This baneful state compels investigation into soft tissue sarcoma biology with the hopeful view of rational development of therapeutic correlates - the purpose of this thesis by published work.

Fellowship and research background

In September 2000 I undertook a full-time, laboratory-based, Surgical Oncology Research Fellowship at the University of Texas' MD Anderson Cancer Center, Houston, Texas, USA under the direction of Dr Raphael Pollock, Chief of Surgery (now at the University of Ohio). Dr Pollock's laboratory was, and is, one of the few laboratories in the world focusing on the research and treatment of soft tissue sarcoma and was run with co-directorship from Dr Dihua Yu (Professor of Molecular & Cellular Oncology, University of Texas) during my first 4 years and Dr Dina Lev (Assistant Professor of Cancer Biology, University of Texas) during my later 2 years in the lab. My recruitment to this post-doctoral research fellowship drew from my previous experience in molecular biology research and surgical training allowing me to contribute to active research projects in the lab while refining and utilising the group's animal model for delivery of novel anti-sarcoma therapeutics.

At the commencement of my research fellowship the main strands of research within the group were the role of the tumour suppressor p53 in the development of the malignant hallmarks of sarcoma, wild-type p53 reconstitution as a potential therapeutic following adenoviral delivery, and angiogenesis induction by sarcoma. Collaborative work within our group, and within the institution, led to the publication of a number of co- and first-author papers of which 6 have been selected for submission in this thesis by published work entitled "Soft Tissue Sarcoma: Biology and Therapeutic Correlates".

Publications and contribution.

The publications submitted in this thesis are:

1. “Isolated limb perfusion: a novel delivery system for wild-type p53 and fiber-modified oncolytic adenoviruses to extremity sarcoma.”

   Hannay J, Davis JJ, Yu D, Liu J, Fang B, Pollock RE, Lev D.

   Gene Ther. 2007 Apr;14(8):671-81.

   PMID: 17287860

My contribution to this paper was:

- hypothesis generation and testing,
- experiment design: all figures,
- experiment execution
  - Figure 1: all panels,
  - Figure 2: all panels,
  - Figure 3: all panels,
  - Figure 5,
- data interpretation,
- manuscript writing, editing, submission, and reply to reviewer’s comments.

2. “Rad51 overexpression contributes to chemoresistance in human soft tissue sarcoma cells: a role for p53/activator protein 2 transcriptional regulation.”

   Hannay JA, Liu J, Zhu QS, Bolshakov SV, Li L, Pisters PW, Lazar AJ, Yu D, Pollock RE, Lev D.

   Mol Cancer Ther. 2007 May;6(5):1650-60.

   PMID: 17513613

My contribution to this paper was:

- hypothesis generation and testing,
- experiment design: all figures,
- experiment execution
    - Figure 1: all panels,
    - Figure 2: all panels,
    - Figure 3: all panels,
    - Figure 4: all panels,
    - Figure 5: D - preparation of viruses and virally infected cells,
- data interpretation,
- manuscript writing, editing, proof reading, and submission.

3. “Vascular endothelial growth factor overexpression by soft tissue sarcoma cells: implications for tumor growth, metastasis, and chemoresistance.”

    Zhang L[1], Hannay JA[1], Liu J, Das P, Zhan M, Nguyen T, Hicklin DJ, Yu D, Pollock RE, Lev D.

    Cancer Res. 2006 Sep 1;66(17):8770-8.

    PMID: 16951193

    ([1] co-first authorship)

My contribution to this paper was:

- experiment design: figures 2 and 6,
- experiment execution (shared): figures 2 and 5: all panels,
- data interpretation,
- manuscript preparation, editing, submission, reply to reviewer’s comments, rewriting, and resubmission.

4. “A preclinical model for predicting drug response in soft-tissue sarcoma with targeted AAVP molecular imaging.”

   Hajitou A, Lev DC, Hannay JA, Korchin B, Staquicini FI, Soghomonyan S, Alauddin MM, Benjamin RS, Pollock RE, Gelovani JG, Pasqualini R, Arap W.

   Proc Natl Acad Sci U S A. 2008 Mar 18;105(11):4471-6.

   PMID: 18337507

My contribution to this paper was:

- hypothesis generation and testing,
- experiment execution (shared)
  - Figure 2: all panels,
  - Figure 3: all panels,
  - Figure 4: all panels,
- data interpretation,
- manuscript proof-reading and editing.

5. “Transcriptional repression of protein kinase Calpha via Sp1 by wild type p53 is involved in inhibition of multidrug resistance 1 P-glycoprotein phosphorylation.”

   Zhan M, Yu D, Liu J, Glazer RI, Hannay J, Pollock RE.

   J Biol Chem. 2005 Feb 11;280(6):4825-33.

   PMID: 15563462

My contribution to this paper was:

- independently repeat and confirm reproducibility of the western blot analyses (figure 1),

- amplify, purify, and titrate the viruses used in figure 4: A-C, and provide experimental assistance,
- data interpretation,
- manuscript proof-reading and editing.

6. “Wild-type p53 inhibits nuclear factor-kappaB-induced matrix metalloproteinase-9 promoter activation: implications for soft tissue sarcoma growth and metastasis.”

   Liu J, Zhan M, Hannay JA, Das P, Bolshakov SV, Kotilingam D, Yu D, Lazar AF, Pollock RE, Lev D.

   Mol Cancer Res. 2006 Nov;4(11):803-10.

   PMID: 17077165

My contribution to this paper was:

- assist in sarcoma tissue sample selection, processing, and data retrieval - figure 1 and table 1,
- amplify, purify, and titrate the viruses used in figure 5 A, and provide experimental assistance,
- data interpretation,
- manuscript preparation, editing, and proof-reading.

Rather than submit the papers in a strictly chronological sequence according to their appearance in the literature, or according to degree of contribution, I present the papers as follows in thematic pairs.

Paper 1: “Transcriptional repression of protein kinase C-alpha via Sp1 by wild type p53 is involved in inhibition of multidrug resistance 1 P-glycoprotein phosphorylation.” and paper

2: “Rad51 overexpression contributes to chemoresistance in human soft tissue sarcoma cells: a role for p53/activator protein 2 transcriptional regulation.” both relate to the problem of soft tissue sarcoma’s resistance to chemotherapy. These papers describe the characterisation of two separate molecular mechanisms of chemoresistance exhibited by soft tissue sarcoma in the context of p53 mutation status - the tumour suppressor most frequently abrogated in human cancer and of particular importance in soft tissue sarcoma. One of the principal mechanisms of chemoresistance development by cancer cells is the up-regulation of chemotherapy drug efflux pumps. The *mdr-1* gene product p-glycoprotein drug efflux pump is up-regulated in sarcoma cells and extrudes doxorubicin - the mainstay anti-sarcoma chemotherapeutic - as well as many other drugs. Wild-type p53 activation was already known to transcriptionally repress *mdr-1* gene transcription however, paper 1 demonstrated another new layer of control of the doxorubicin efflux pump in sarcoma in that wild-type p53 activation transcriptionally represses the activating kinase of p-glycoprotein (PKCa) through inhibition of the SP-1 transcription factor binding on the PKCa promoter. In sarcoma, therefore, loss of wild-type p53 may lead to chemoresistance by increased drug extrusion from tumour cells through two levels of loss of control: at the transcriptional level of the *mdr-1* gene, and at the functional level of the p-glycoprotein pump.

Paper 2 characterises a mechanism of chemoresistance in sarcoma that relates to the functional mechanism of the mainstay anti-sarcoma agents: doxorubicin chemotherapy and ionising radiation. Both of these agents have as their principle mode of action the induction of double-strand DNA breaks. Cells are critically sensitive to this form of DNA damage and, in cycling cells, repair such breaks via homologous recombination. The central mediator of homologous recombination is the protein Rad51. Paper 2 demonstrates that Rad51 is over-expressed in soft tissue sarcoma, particularly in cells harbouring *p53* mutation, and accumulates in the G2 phase of the cell cycle where doxorubicin causes cycling sarcoma cells to arrest. Suppression of Rad51 by siRNA

rendered sarcoma cells with elevated Rad51 levels to become more chemosensitive at clinically meaningful concentrations of chemotherapeutic. Additionally, reconstitution of wild-type p53 function led to suppression of Rad51 levels via transcriptional repression mediated through AP2 binding to the *rad51* promoter. This paper was the first in the literature to investigate and characterise the role of Rad51 in sarcoma chemoresistance yielding, what should be, a therapeutically relevant insight.

Paper 3: "Wild-type p53 inhibits nuclear factor-kappaB-induced matrix metalloproteinase-9 promoter activation: implications for soft tissue sarcoma growth and metastasis." and paper 4: "Vascular endothelial growth factor overexpression by soft tissue sarcoma cells: implications for tumor growth, metastasis, and chemoresistance." both relate to soft tissue sarcoma growth, invasion, and metastasis. Paper 3 was the first report in the literature examining MMP-9 in sarcoma and shows that MMP-9 is over-expressed in sarcoma and that expression levels correlate with both metastasis and p53 mutational status. Reconstitution of wild-type p53 function led to down regulation of functional MMP-9 and suppression of *mmp-9* transcription via altered NF-kB binding in the *mmp-9* promoter.

Paper 4 demonstrates that over-expression of the VEGF isoform 165 in sarcoma leads to a more aggressive phenotype for growth, invasion, and metastasis and additionally confers an enhanced chemoresistant phenotype *in vivo*. Combining the anti-VEGFR2 antibody DC101 with low-dose doxorubicin as a biochemotherapy strategy led to attenuation of the aggressive phenotype more than with conventional schedule and dosing of doxorubicin. Paper 5 provides evidence that this mechanism of suppression is mediated through the biochemotherapy effect on tumour endothelial cells.

Paper 5: "Isolated limb perfusion: a novel delivery system for wild-type p53 and fiber-modified oncolytic adenoviruses to extremity sarcoma." and paper 6: "A preclinical model for predicting drug response in soft-tissue sarcoma with targeted AAVP molecular

imaging." relate to testing rationally designed biologic agents in the preclinical setting. Following on from the findings presented in the earlier papers that wild-type p53 reconstitution suppresses different pathways and mechanisms pertinent to the malignant phenotype in sarcoma, paper 5 demonstrates that functional wild-type p53, in an adenoviral vector, can be successfully delivered to human soft tissue sarcoma by the established surgical procedure of isolated limb perfusion (ILP). Efficacy, however, is poor and strategies to improve anti-sarcoma treatment with therapeutic adenoviruses led to the demonstration that fibre-modified, selectively replication-proficient oncolytic adenoviruses have greater potential as clinical therapeutics against sarcoma. The finding, reported in paper 5, that fibre-modified viruses containing the arginine-glycine-aspartic acid (RGD) tripeptide motif have greater tropism for human soft tissue sarcoma led to the collaborative study, published in paper 6, examining the effect of systemically delivered RGD-fibre modified bacteriophage as a vector for the herpes simplex virus thymidine kinase (HSVtk) gene. Uptake and expression of the exogenous HSVtk gene allows whole body imaging with CT-PET and the radio nucleotide (^{18}F)-FEAU to identify regions of expression (and regions of metabolic activity with (^{18}F)-FDG) prior to administration of the non-toxic prodrug gangcyclovir that is then converted by HSV-TK to it's toxic metabolite. Subsequent CT-PET imaging may then be used to ascertain sarcoma response to therapy at the cellular, metabolic, and molecular levels before any change occurs (if at all) in tumour volume.

As can be seen, these papers have contributed to the medical and scientific community's understanding of the biological processes that are at play and perturbed in soft tissue sarcoma and furthermore, show-case exploratory studies with rationally designed preclinical anti-sarcoma therapeutics.

These papers collectively form my thesis by published work of "Soft Tissue Sarcoma: Biology and Therapeutic Correlates".

Field Developments & Future Directions

During my research fellowship the strands of research that I was involved with opened a number of avenues for development of novel therapeutics or revealed biological processes in soft tissue sarcoma that therapeutics in development, at the time, may have had applicability. Concomitant and subsequent to my fellowship a number of other overlapping developments occurred in the field of sarcoma biology and related therapeutics which resonate with and relate to my work in this thesis.

Advances in the Use of Hyperthermia

Hyperthermia (induction of tissue temperatures of over 39ºC) is one of the oldest treatments for cancer and an established component of hyperthermic isolated limb perfusion therapy. Hyperthermia is recognised to act as a radio- and chemosensitiser and a recent review[7] of over 100 clinical trials incorporating some form of hyperthermia identified over 20 trials where addition of hyperthermia to standard chemo- and radiotherapy regimens led to significant improvement in clinical outcomes and notably in soft tissue sarcoma[8]. Subgroup analysis of a recently reported phase 3 trial comparing neo-adjuvant chemotherapy alone or with regional hyperthermia for localised high-risk soft-tissue sarcoma showed that for abdominal and retroperitoneal sarcoma (where surgical resection is often on vital structures), addition of hyperthermia to chemotherapy enhanced local control and disease-free survival for patients undergoing R0 and R1 macroscopic disease clearance but not those with macroscopically incomplete R2 resections[9]. While it is encouraging that evolution of technology now allows application of hyperthermic treatment to regions other than limbs with improved local control, overall survival was no different between the two groups underscoring the need for new systemic agents.

Of interest, treatment of sarcoma cell lines and primary cell cultures from patients with transgene-expressing vaccinia virus still demonstrated T-cell activating transgene expression in the face of exposure to hyperthermia and ifosfamide[10] - albeit in vitro. High pyrexia would be expected to both attenuate viral activity inside human cells and, with enhanced accumulation of intracellular cytotoxic agent at hyperthermic temperatures, greatly interfere with transcription & translation cellular machinery. The persistence of an immune-stimulatory virus-based therapy in a field that has been treated with current 'maximal anti-sarcoma therapy' opens an intriguing avenue of research.

Developments in Adenovirus-based Therapy

Pre-clinical and early clinical studies with replication deficient adenovirus delivering wild-type p53 showed promise in the treatment of aero-and-upper-digestive tract malignancies leading to government licensing in 2003 for treatment of head and neck cancers in patients in the USA & China. This was followed two years later with approval of replication-selective virus for the treatment of nasopharyngeal cancer. However, although use of viruses as anticancer agents remains popular in China and other parts of Asia, results with adenovirus based mono-therapy or systemically delivered viruses have been roundly disappointing.

A number of factors, not all purely clinical, have lead to waning enthusiasm for the use of recombinant viruses in anti-cancer strategies. First, without the ringing endorsement of clear clinical need that can't be recapitulated with another modality then pharmaceutical companies are unlikely to invest in the development of new manufacturing technologies for large-scale production of therapeutic viruses. Manufacturing plants that produce biologically active agents are different from those involved with industrial chemistry producing chemicals and small molecules. Second, and allied to the previous disincentive for pharmaceutical companies, are the disparate and entrenched intellectual property

rights associated with viral and recombinant technologies that would need to be engaged with and brokered before the agents could be 'brought to market'. Third, marketability of virus based therapy would need to overcome the current western cultural mindset of deep suspicion of recombinant-DNA being put into humans and particularly if the thought is prevalent that 'Big Pharma was doing it for a profit'. Many people if offered the choice between swallowing a pill or being injected with a 'manipulated virus' will preferentially opt for swallowing the pill.

Since completing my research fellowship a number of findings have emerged characterising host-adenovirus factors that impinge upon adenoviral therapy in the clinic (for recent reviews see[11] and[12]). Studies from the 1990s established adenoviral up-take occurring through a two step process involving viral engagement of its fibre first with the cell-surface Cocsackie-Adenovirus Receptor (CAR) and then with the penton base interacting with cellular integrins. Modification of peptides within the virus fibre allowed altered tropism of the virus for cells expressing receptors other than CAR. However, systemic delivery of adenoviruses still yielded very low tumour or target tissue transduction. Elucidation of the host factors leading to clearance, principally by the liver, of systemically delivered adenovirus made clear that systemic delivery of adenoviruses is unlikely to be a viable therapeutic approach.

First, adenoviruses are ubiquitous in the environment and by the time most people reach adult hood they have developed neutralising antibodies to the common adenoviruses - notably serotype 5 and -2 which were the 'workhorse' viruses for adenoviral gene therapy.

Second, the innate immune system was found to have several redundant and powerful mechanisms of neutralising systemically delivered adenoviruses in vivo. Adenoviruses in the blood stream bind IgM, complement factors, and blood clotting factors leading to clearance in the liver by both hepatocytes and Küpffer cells. The coagulation factor X, for

example, binds the adenovirus' major capsid protein hexon and bridges binding to hepatocyte surface heparin sulphate molecules[13]. Although this seemingly main mechanism of clearance has been of interest in gene delivery to hepatocytes, decoration of adenoviruses with factor X also leads to rapid triggering of NFkB early response genes following detection of internalised factor X in macrophages and monocytes[14]. In experiments where factor X binding was abrogated, IgM and complement factors (mainly C1q and C4) had increased binding to adenovirus particles and increased extra-hepatic clearance through the reticuloendothelial system again activating a pro inflammatory state. In rats the systemic intravenous injection of adenovirus vectors brought about shock that was due to the release of platelet activating factor from the reticuloendothelial system[15] which may be an anticipated consequence of systemic delivery to humans should liver clearance mechanisms be overcome. Internalisation of virus particles bearing complement C3 has also recently been found to activate a 'mitochondrial antiviral signaling' cascade that induces pro-inflammatory cytokine secretion as well as directly targeting C3 tagged viruses for immediate proteosomal degradation[16].

While these findings above currently nullify adenoviruses for systemic delivery a role may yet remain for their restricted regional delivery in hyperthermic isolated limb perfusion of locally advanced irresectable limb sarcoma. The finding that adenoviruses have inherent mechanisms to circumvent the effects of a deleterious intracellular milieu was capitalised on in the generation of early oncolytic adenoviruses[17]. Subsequent studies of the biological effects of the adenoviral E1b gene product found additional roles to its p53-binding function. During pyrexia the nucleopore transport mechanism is closed and nascent mRNAs (such as heat shock protein mRNAs) are exported via a nucleopore independent pathway that E1b is also able to utilise to guide viral RNAs out of the nucleus. Dysregulation of this export mechanism in cancer cells at 37ºC was found to underpin the mechanism of E1b-deleted oncolytic adenoviruses[18, 19]. This mechanism goes part way to explain the finding that adenovirus-delivered transgenes often have

robust expression in situations of hyperthermia and would further encourage consideration of use of adenovirus based therapy in the hyperthermic isolated limb perfusion context.

New Systemic Therapies.

Doxorubicin still remains the benchmark anti-sarcoma therapy for systemic treatment of non-GIST tumours that escape local control. Intensified doxorubicin plus ifosfamide in combination demonstrated no superiority to doxorubicin alone in a recently published trial[20]. In this report overall survival was was 12-14 months, progression-free survival 4-7months, and overall response to treatment 14% in the doxorubicin alone group compared to 26% in the combination group. There clearly still remains a desperate need for new systemic therapies.

Trabectedin

Trabectedin (ET-743, Yondelis®) is a synthetic anticancer agent derived from the Caribbean marine tunicate, Ecteinascidia turbinata and now approved for clinical use in sarcoma in over 70 countries having received first approval for use in sarcoma in the European Union in 2007. Trabectaedin has a number of modes of action. First, it's principle activity is as a guanine alkylating agent binding the minor groove of the DNA double helix leading to a conformational bend in DNA and inhibiting local transcription factor binding as well as inducing DNA double strand breaks through interference in transcription-coupled nucleotide excision repair (NER). Second, trabectedin consequently leads to apoptosis and sensitisation to Fas-mediated cancer cell death. Third, trabectedin has the unusual property of significantly altering the cancer cell environment through selective killing of tissue monocytes and tumour macrophages while sparing neutrophil and lymphocyte populations[21]. This effect both suppresses angiogenesis[22, 23] and

alters the tumour environment associated inflammatory state[24]. Of interest, a recent retrospective study assessing DNA repair gene expression patterns in advanced sarcoma samples from 245 patients treated with trabectedin found that active NER genes were associated with trabectedin sensitivity but active homologous repair pathway genes were negatively associated leading to the proposal of a DNA repair 'signature' that may predict response of patients with advanced sarcoma to trabectedin[25]. Neither Rad51 nor p53 were assessed in the study.

Trabectedin has been tested in phase I, II, and phase III trials both in combination with established chemotherapeutics and as mono-therapy in patients who have failed on standard chemotherapeutics (for a recent review see[26]). Generally response rates are less than 12% with tendency to improved progression-free survival but rarely improved overall survival. Response appears to be greatest when trabectedin is administered as a 24h infusion, combined with doxorubicin, or if the sarcoma subtypes are leiomyosarcoma, round cell liposarcoma, or sarcomas with translocation gene products where trabectedin has been investigated as mono-therapy[27-29]. Currently, trabectedin has established itself as a new systemic therapy that appears to be at least as good as doxorubicin and ifosfamide in soft tissue sarcoma.

There as yet no published studies assessing the combination effect of trabectedin with adenoviral gene therapy. Since trabectedin diminishes local macrophage / monocyte populations and consequently still possesses an anticancer effect even in the face of trabectedin resistant tumour cells[21], it is intriguing to consider that this indirect effect of trabectedin may enhance regional anti-sarcoma adenoviral therapy and merits investigation.

Pazopanib

Pazopanib (GW786034, Votrient®) is a small molecule inhibitor of tyrosine kinases that was first developed against VEGFR2 but was found to have activity against related tyrosine kinases VEGFR1, VEGFR3, PDGFRα, PDGFRβ and c-kit. Pazopanib was first approved for use against metastatic renal cell carcinoma in the USA in 2009 but has also been found to have statistically significant activity against metastatic soft tissue sarcoma while preserving a low side effect profile (reviewed in [30] and [31]). Although a number of anti-angiogenic agents such as monoclonal antibodies and small molecule inhibitors have entered the general anticancer armamentarium, and not as first-line mono-therapy, only pazopnib has been regarded as an agent worth considering as standard of care in non-adipocytic sarcoma following on from results of phase II and phase III trials. Recently published analysis of pooled data from phase II and phase III studies of pazopanib in the European Union found that out of 344 patients with STS treated with pazopanib median progression free survival was 4.4. months and median overall survival 11.7 months. However, in approximately a third of treated patients PFS was over 6 months and OS over 18 months with one-in-thirty patients surviving over 2 years on therapy[32]. Pazopanib's superiority in sarcoma over related tyrosine kinase inhibitors may be due to it's principle target being VEGFR2. Recent data indicates that amongst the pro-angiogenic receptor tyrosine kinases, VEGFR2 over expression in human STS samples correlates most tightly with poorer survival[33].

Innate and acquired resistance mechanisms against pazopanib have yet to be studied in soft tissue sarcoma. Common mechanisms of resistance to antiangiogenesis therapy in other cancer types that would be of particular relevance for sarcoma include acquisition of a phenotype for survival in hypoxic environments, up regulation of redundant proangiogenic signalling pathways, recruitment of tumour associated fibroblasts & circulating marrow-derived cells for denser pericyte coverage of tumour neovasculature, up regulation of *mdr* genes, and p53 mutation[34].

mdr-1 inhibitors

Since the discovery of *mdr*-1 and its gene product p-glycoprotein there has been interest in identifying inhibitors of this drug efflux pump that is a potent effector of chemoresistance in tumour cells. Many agents identified as effective inhibitors in vitro to restore chememosensitivity have had the drawback of being too toxic in vivo either due to too broad inhibitory action on other necessary efflux pumps (such as verapamil) or requiring infeasibly high tissue concentrations without generating toxicity (such as cyclosporin A). Since the completion of my fellowship further studies of *mdr*-1 in sarcoma have identified an effect of non-steroidal anti-inflammatory drugs (NSAIDs) in reversing multi drug resistance in uterine sarcoma cell lines[35] and also that delivery of anti-*mdr*-1 siRNA on nanoparticles can suppress chemoresistance in preclinical studies of osteosarcoma[36]. More recently, however, newer small molecules of promise have been identified via high throughput screening assays that are able to reverse the chemoresistant phenotype at micro molar tissue concentrations[37, 38].

MDM2-p53 interfering drugs

Mutation hot-spots in the p53 gene occur in regions altering DNA binding, folding, and additionally shorten the half-life of the fully folded protein at 37^{o}C. The discovery that p53 C-terminal modification, including by binding with antibiodies, can lead to reactivation of DNA binding by mutant p53 led to a search for p53 stabilising agents. Identification of agents that stabilise p53 and 'reactivate' mutated p53 are attractive therapeutics since they would circumvent hurdles associated with gene-therapy reintroduction of functional wild-type p53 (reviewed in [39] and [40]). Strategies to reactivate native p53 broadly fall

into two categories: those agents that bind p53 directly or those that inhibit p53's negative regulator MDM2. The most investigated of these compounds are the MDM-2 antagonists and in particular the nutlins and their derivatives which bind the p53 binding pocket of MDM2 with greater affinity than p53. These agents have been shown to enhance chemosensitivity and radiosensitivity, change MDM2 folding, reverse p-glycoprotein mediated chemoresistance, suppress tumour associated angiogenesis and lead to sarcoma regression in preclinical and clinical studies (reviewed in [41]).

Summary

Although a number of years have passed since the completion of my fellowship and the incorporation of this thesis arising from findings during my fellowship, soft tissue sarcoma remains an under investigated tumour type. The promise of viral gene therapy from the late 1990s has paled and further developments with small molecule inhibitors of characterised biologic pathways hold out future promise for rationally designed and personalised treatment of patients with sarcoma. The surgical oncologist still retains a central role in the holistic care of the patient with sarcoma and additionally should be an integrated figure in bridging the development of new therapies between the laboratory and bedside patient care: a role I still seek to develop.

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Paper 3

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([1] co-first authorship)

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Paper 6

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