From the DEPARTMENT OF MICROBIOLOGY, TUMOUR AND CELL BIOLOGY
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P53 TRANSCRIPTIONAL ACTIVITY AS A TOOL TO UNCOVER NOVEL AND DIVERSE DRUGGABLE TARGETS IN CANCER

Marcus James Graeme Watson Ladds

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p53 Transcriptional Activity as a Tool to Uncover Novel and Diverse Druggable Targets in Cancer

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By

Marcus James Graeme Watson Ladds

Principal Supervisor:
Prof. Sonia Laín
Karolinska Institutet
Department of Microbiology, Tumor and Cell Biology

Co-supervisor(s):
Prof. David Lane
Karolinska Institutet / A*Star Singapore
Department of Microbiology, Tumor and Cell Biology / SciLifeLab / p53 Lab

Dr. Ingeborg van Leeuwen
Karolinska Institutet
Department of Microbiology, Tumor and Cell Biology

Opponent:
Prof. Antony Braithwaite
University of Otago
Department of Pathology

Examination Board:
Prof. Nico Dantuma
Karolinska Institutet
Department of Cell and Molecular Biology

Prof. Aristidis Moustakas
Uppsala University
Department of Medical Biochemistry and Microbiology

Docent Kaisa Lehti
Karolinska Institutet
Department of Microbiology, Tumor and Cell Biology

To my Wife, for her understanding, caring, love, and support

To my Mother, for giving me life, love and support for all my years

To my Sister, for believing in me

To those who may benefit from our work

"Orthodoxy means not thinking – not needing to think.

Orthodoxy is unconsciousness"

– George Orwell, 1984

ABSTRACT

The transcription factor p53 is one of the most studied tumour suppressors with over 90 000 publications in PubMed referring to the protein. It is also the most frequently mutated gene across all cancer types with around 50% of cancers presenting as mutant p53, and when it is not mutated, it is frequently inactivated to circumvent its tumour suppressor function. Therapeutic targeting of both mutant and wild-type p53 has been a key focus ever since its first discovery as "the guardian of the genome". For our drug development programme, we have focused on visualising the induction of p53 transcriptional activity as a readout for a desirable phenotype. This screen used two stably transfected reporter cell lines, the T22 murine fibroblasts, and the ARN8 human melanoma cell line. Using this forward chemical genetic approach, we have entered into our drug development programme in a target-blind manner.

For **Paper I** we screened 30 000 compounds in both T22 and ARN8 cells and selected those that were capable of increasing p53 transcriptional activity in the ARN8 tumour cells, but not in the T22 murine fibroblasts. We selected a compound from the hits that had a drug-like structure as well as possessing a chiral centre and christened it HZ00. HZ00 was found to induce p53 protein in a dose-dependent manner, selectively kill tumour cells whilst inducing a reversible G1 arrest in normal human dermal fibroblasts (HNDFs), and increase p53 synthesis at early timepoints without stabilising the protein or increasing levels of p53 mRNA. HZ00 also synergised with the inhibitor of p53 degradation, nutlin 3, both *in vitro* and *in vivo* in a tumour xenograft model. Following target deconvolution using a knowledge-based approach we identified DHODH, a key enzyme in the *de novo* pyrimidine nucleotide synthesis pathway, as the target of HZ00. At this point we re-screened 30 000 compounds in ARN8 cells that were previously screened in the T22 cell line for another study. We found that those that were able to activate p53 in ARN8 cells also largely inhibited DHODH. This yielded 12 other chemotypes capable of inhibiting DHODH. At this point we tested HZ00 analogues and identified a much more potent compound we named HZ05. HZ05 phenocopied HZ00 and demonstrated enantiomer-selective inhibition of DHODH with (*R*)-HZ05 inhibiting DHODH with an IC_{50} of 11 nM. We obtained a crystal structure of (*R*)-HZ05 in complex with DHODH and found that it occupied the same quinone tunnel as the known inhibitors brequinar and teriflunomide (A77 1726). HZ05 caused a number of tumour cells to accumulate in S-phase. We found that a slower cycling cell line, U2OS, required pre-treatment with HZ05 to accumulate cells in S-phase prior to treatment with nutlin 3a to achieve tumour cell kill, as co-treatment resulted in G1 arrest. We therefore theorised that accumulating cells in S-phase with high levels of p53 predisposed them to cell kill upon application of a blocker of p53 degradation.

The first sets of compounds found back in 2008 by the Laín laboratory were the tenovins. Tenovin 1 was the first compound identified from the screen, which used the T22 murine fibroblasts to establish its ability to activate p53 transcriptional activity in the reporter assay. Tenovin 1 was, however, not particularly soluble and therefore a more soluble analogue

called tenovin 6 was synthesised. Tenovin 6 elicited many of the same cellular phenotypes as tenovin 1, and therefore target identification was conducted using tenovin 6. Tenovin 6 was subsequently identified as an inhibitor of SirT1 and SirT2 in a yeast genetic screen, biochemical assays and further target validation in mammalian cells. Tenovin 1 and 6 displayed a very similar profile – they both induced p53 transcriptional activity and both increased acetylation of both p53 and tubulin. This is where the similarity ends, however, as it was discovered, through extensive structure-activity relationship studies, that the targeting profiles of both molecules was markedly different.

In **Paper II** we built upon previous studies that identified tenovin 6 as a compound capable of inhibiting autophagy. In this paper we conducted structure-activity relationships using tenovin analogues to understand the mechanism by which tenovins affect autophagy. We confirmed that tenovins capable of perturbing autophagy do so by inhibition of autophagic flux, in a similar manner to chloroquine, by raising the pH of lysosomes. We also isolated the portion of the molecule, a tertiary amine at the end of an aliphatic chain, as the reason for blockage of autophagic flux. Finally, we found that blockage of autophagic flux by tenovins is required to eliminate tumour cells in culture and that this blockage of autophagy is capable of killing mutant B-Raf tumour cells arrested in G1 by vemurafenib treatment.

In **Paper III** we further explored the targeting profile of the tenovins and tested whether tenovins were capable of inhibiting DHODH. We found that tenovins 1 and 6 were capable of inhibiting DHODH at 113 nM and 500 nM respectively. We also conducted a thermal shift assay and identified tenovins 1, 6 and 39OH as being capable of interacting with DHODH *in vitro*. We then obtained a crystal structure of tenovin 6 occupying the same quinone tunnel as HZ05, brequinar and teriflunomide. Phenotypically, tenovin 1 and 33 had their ability to induce p53 transcriptional activity ablated upon addition of either uridine or orotate, but not dihydroorotate, whilst tenovin 6 had its ability to induce p53 transcriptional activity partially prevented by addition of uridine or orotate. Tenovin 39 and 39OH displayed no difference upon supplementation. Tenovin 1 and 33 also had their growth inhibitory effect markedly reduced upon orotate or uridine supplementation, but no other tenovin, including 6, showed any effect of supplementation. We also discovered another target of the tenovins – the ability to inhibit nucleoside uptake. We discovered that uridine uptake was blocked by tenovin 6, 33, 39, 39OH and 50. This paper, therefore, highlights the shifting targeting profile of the tenovins due to small molecular changes and that a phenotypic readout may remain static even as the targeting profile changes, as well as highlighting both the benefits and cautions of targeting multiple disparate targets in cells.

Unlike our other projects, **Paper IV** focused on understanding the structure and function of DHODH. We studied a purified DHODH lacking the transmembrane domain using native protein nano-electrospray mass spectrometry (nESI-MS). Firstly, we identified MS conditions that allowed for the DHODH to spray and isolated a high m/z range that corresponded to the molecular weight of the enzyme plus the bound FMN cofactor. Ion mass spectrometry was conducted to differentiate between the holo- and apo- DHODH, with the

holo-DHODH corresponding to a compact formation suggesting that folded DHODH with FMN present can be preserved in the gas phase. We next incubated lipids that constitute the human mitochondrial membrane with DHODH and analysed the interaction in the gas phase. Complexes with both PE and CDL were evident, but complexes with PC were not easily detected. The next finding was that an intact protein-cofactor complex was required for the DHODH inhibitor, brequinar, to bind thus confirming that brequinar binding to DHODH is not random, but requires properly structured DHODH. Finally, MD simulations were conducted using both full length and truncated protein associated with a model PE bilayer. These models established that DHODH sits on the surface of the lipid bilayer loosely and is anchored in place by the transmembrane helix and this anchorage holds DHODH in the correct orientation to allow insertion of coenzyme-Q10 into the quinone tunnel of DHODH.

LAY SUMMARY

Cancer is a highly devastating disease that affects people worldwide with around 14 million cases each year and around 8 million deaths around the world every year. Currently we have a lot of medicines for cancer, but unfortunately many of those therapies are very toxic with horrific side effects that make it difficult for patients to keep up with the treatment. It is for this reason that we want to develop new therapies that can activate and take advantage of particular proteins in our cells that are known as tumour suppressors. One such tumour suppressor we are interested in is called p53, and has been referred to as the "guardian of the genome" as it helps to protect our cells from all sorts of cancer-causing events.

In **Paper I**, we describe the development of a new family of compounds called the HZ series. We found the compound HZ00 by using a screening method where we examine the activity of the anti-tumour protein p53. HZ00 was very good at killing melanoma cells in culture and did not appear to harm non-tumour cells. We also found that HZ00 was able to cause the cell to make new p53 after only a few hours of treatment. Through our knowledge-based approach to finding the target of HZ00, we discovered that it binds to an enzyme called dihydroorotate dehydrogenase. This enzyme is part of a chain of enzymes that produce building blocks of our RNA, which is used to make our proteins, and our deoxynucleotides that make our DNA. Having low levels of deoxynucleotides while your cell is dividing and making new DNA causes cells to die, but most healthy cells can sense this low level and stop before making new DNA. Many cancer cells cannot respond to this, and therefore they try to divide without enough deoxynucleotides and die. After identifying the target, we tested chemicals that had a similar structure to HZ00 and found HZ05 – a much more potent compound that could kill tumour cells in culture at a concentration more than 10 times less than that of HZ00. We tested HZ05 in combination with a compound that stops the cell from breaking down p53 called nutlin 3a. We thought that HZ05 increasing the amount of new p53 made, and nutlin 3a preventing p53 from being broken down, would lead to a greater level of tumour cell killing. Excitingly, we found that nutlin 3a killed more cancer cells with HZ05 than either compound did on their own.

Paper II explores a new idea using a series of compounds the Laín research group discovered back in 2008 called the tenovins. These compounds inhibit two proteins called SirT1 and SirT2. These proteins work together to remove molecules from the p53 protein, decreasing its stability so it is more likely to be broken down by cells. We conducted a series of studies with new tenovins with chemical structures closely related to the original compounds. Other research groups had seen that tenovin 6 was able to block a process in the cell called autophagy. Autophagy is a word that comes from ancient Greek that means to "self eat". This process in cells recycles old molecules and parts of the cell and breaks them down to the building blocks used by the cell to make new molecules that it needs to carry out its normal function. Cancer cells often rely on autophagy to survive as they need to consume a lot of nutrients as they grow faster and have faster metabolism than normal cells. Therefore blocking autophagy is a potentially good way to kill tumour cells. In this paper we found the

part of the tenovin molecule that is responsible for blocking autophagy and found that the tenovins with this structural feature can travel into small organelles in the cell, called lysosomes, and stop their function, which causes a cessation of autophagy. We found that tenovin molecules capable of blocking autophagy were able to eliminate all tumour cells in culture. We even found that a tenovin that blocks autophagy was able to kill cells that did not respond to one of the current therapies against melanoma called vemurafenib.

Paper III uses the tenovins once again. This time, however, we discover two new targets of these molecules. We found that four tenovins (tenovin 1, tenovin 33, tenovin 6 and tenovin 39OH) were capable of blocking DHODH, the same protein that HZ00 inhibits. We also found that tenovin 6, tenovin 33, tenovin 39, tenovin 39OH and tenovin 50 were capable of blocking a protein on the cell surface that transports nucleosides, such as uridine, from entering the cell. Despite the fact that these compounds are so closely related with only a small change in one or two atoms between them, they demonstrate that a small change in their structure can change the protein they target in the cell.

We have, so far, discovered two series of compounds that inhibit the protein DHODH, but we don't know enough about how the protein works inside the cell. **Paper IV** is a study using a technique known as mass spectrometry, which in simple terms is using a machine to "weigh" molecules, that is determine their charge and their mass ratio (m/z). In this case, we wanted to examine an intact protein, in particular, DHODH. Not only did we want to be able to examine DHODH, we wanted to be able to see if anything could bind to DHODH and be seen in the mass spectrometer at the same time. One important point about DHODH is that it is located inside the mitochondria, a small organelle in the cell that is responsible for energy production, otherwise known as the powerhouse of the cell. Because DHODH sits inside the mitochondria on the inner mitochondrial membrane, it binds to lipids that make up the membrane. We used mass spectrometry to determine the following three things: 1, that we could "see" DHODH in the mass spectrometer; 2, that we could determine if lipids were attached to DHODH; 3, that we could determine if an inhibitor of DHODH was bound to the protein. As part of these studies we also conducted computer modelling of DHODH and the membrane to see what direction the protein faced towards the membrane. We found that DHODH was attached to the membrane loosely by a small part of the protein, called the transmembrane domain, which inserted inside the membrane and keeps DHODH anchored in place.

We hope that with **Paper I** we have started to develop a potential new anti-cancer therapy that we can build on into the future. With **Paper II** we show how we can target autophagy and that autophagy blockage may be useful for cancer therapy. **Paper III** shows that a small change in a molecule can lead to a new target. Finally, with **Paper IV** we hope we have opened up new understanding of how DHODH acts inside the cell.

LIST OF SCIENTIFIC PAPERS

I. **Marcus J.G.W. Ladds**, Ingeborg M.M. van Leeuwen, Catherine J. Drummond, Su Chu, Alan R. Healy, Gergana Popova, Andrés Pastor-Fernández, Tanzina Mollick, Suhas Darekar, Saikiran K. Sedimbi, Marta Nekulova, Marijke C.C. Sachweh, Johanna Campbell, Maureen Higgins, Chloe Tuck, Mihaela Popa, Mireia Mayoral Safont, Pascal Gelebart, Zinayida Fandalyuk, Alastair M. Thompson, Richard Svensson, Anna-Lena Gustavsson, Lars Johansson, Katarina Färnegårdh, Ulrika Yngve, Aljona Saleh, Martin Haraldsson, Agathe C.A. D'Hollander, Marcela Franco, Yan Zhao, Maria Håkansson, Björn Walse, Karin Larsson, Emma M. Peat, Vicent Pelechano, John Lunec, Borivoj Vojtesek, Mar Carmena, William C. Earnshaw, Anna R. McCarthy, Nicholas J. Westwood, Marie Arsenian Henriksson, David P. Lane, Ravi Bhatia, Emmet McCormack and Sonia Laín. A DHODH inhibitor increases p53 synthesis and enhances tumor cell killing by p53 degradation blockage. *Nature Communications* **9**, 1107 (2018).

II. **Marcus J.G.W Ladds**, Andrés Pastor-Fernández, Gergana Popova, Ingeborg M M van Leeuwen, Kai Er Eng, Catherine J Drummond, Lars Johansson, Richard Svensson, Nicholas J Westwood, Anna R McCarthy, Fredrik Tholander, Mihaela Popa, David P Lane, Emmet McCormack, Gerald M McInerney, Ravi Bhatia and Sonia Laín. Autophagic flux blockage by accumulation of weakly basic tenovins leads to elimination of B-Raf mutant tumour cells that survive vemurafenib. *PLOS ONE* **13**(4): e0195956 (2018).

III. **Marcus J.G.W. Ladds**, Andrés Pastor-Fernandéz, Gergana Popova, Ingeborg M M van Leeuwen, Maria Håkansson, Björn Walse, Fredrik Tholander, Srinivasaraghavan Kannan, Chandra Verma, Ravi Bhatia, David Lane, and Sonia Laín. Polypharmacology and multiple routes of p53 activation within a family of small molecules. *Manuscript to be submitted.*

IV. Joana Costeira-Paulo, Joseph Gault, Gergana Popova, **Marcus J.G.W. Ladds**, Ingeborg M M van Leeuwen, Médoune Sarr, Anders Olsson, David P Lane, Sonia Laín, Erik G Marklund and Michael Landreh. Lipids Shape the Electron Acceptor-Binding Site of the Peripheral Membrane Protein Dihydroorotate Dehydrogenase. *Cell Chemical Biology* **25,** p1-9 (2018).

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CONTENTS

LIST OF ABBREVIATIONS

8-oxo-dG	8-oxo-2'-deoxyguanosine
O^6-meG	6-*O*-methylguanine
ABL	Abelson murine leukaemia viral oncogene homologue 1
ActD	Actinomycin D
AML	Acute myeloid leukaemia
AMPK	5' AMP-activated protein kinase
APC	Adenomatous polyposis coli
ATM	Ataxia telangiectasia mutant
ATP	Adenosine tri-phosphate
ATR	Ataxia telangiectasia and rad3-related protein
BafA1	Bafilomycin A1
BAX	Bcl-2-associated X protein
BCE	Before common era
Bcl-2	B-cell lymphoma 2
Bcl-X_L	B-cell lymphoma-extra large
BCR-ABL	Breakpoint cluster region protein – Abelson murine leukaemia viral oncogene homologue 1
BrdU	Bromodeoxyuridine
BaP diol epoxide	(+)benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide
c-myc	Avian myelocytomatosis viral oncogene homologue
CAR-T	Chimeric antigen receptors
CBP	CREB-binding protein
CD8	Cluster of differentiation 8
CD19	Cluster of differentiation 19
CD20	Cluster of differentiation 20
CD28	Cluster of differentiation 28
CD80	Cluster of differentiation 80
CD86	Cluster of differentiation 86
Cdc6	Cell division cycle 6
CDKN1A	Cyclin-dependent kinase inhibitor 1
CDL	Cardiolipin
CETSA	Cellular thermal shift assay
CML	Chronic myelogenous leukaemia
CNS	Central nervous system

CNT	Concentrative nucleoside transporters
CoQ10	Coenzyme Q10
COX2	Prostaglandin-endoperoxidase synthase 2
CPRG	Chlorophenol red-β-D-galactopyranoside
CQ	Chloroquine
CRISPR	Clustered regularly interspaced short palindromic repeats
CTD	C-terminal domain
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CTP	Cytidine triphosphate
CXCL1	Chemokine (C-X-C motif) ligand 1
CYP450	Cytochrome p450
DBD	DNA binding domain
DHODH	Dihydroorotate dehydrogenase
DNA	Deoxyribonucleic acid
DRAM	Damage-regulated modulator of autophagy
DSG	Muscular dystrophy–associated dystrophin glycoprotein complex
EGFR	Epidermal growth factor receptor
ENT	Equilibrative nucleoside transporters
ESCRT	Endosomal sorting complex required for transport
FBXO11	F-box only protein 11
FDA	Food and drug administration
FMN	Flavin mononucleotide
Fox-O1	Forkhead box protein O1
G-CSF	Granulocyte-colony stimulating factor
GABARAP	Gamma-aminobutyric acid receptor-associated protein
Gadd45	Growth arrest and DNA damage
Gate-16	Golgi-associated ATPase Enhancer of 16 kDa
GTP	Guanosine-5'-triphosphate
Hdm2	See mdm2 (Mouse double minute 2 homologue)
HER2	Human epidermal growth factor receptor 2 / Receptor tyrosine-protein kinase erbB-2
hERG	Human ether-á-go-go-related gene
HIF-1α	Hypoxia-inducible factor 1-alpha
hMOF	Human males-absent-on-the-first
HNDF	Human normal dermal fibroblast
HSC70/HSPA8	Heat shock 70 kDa protein 8

IC_{50}	Half maximal inhibitory concentration 50
IKKβ	IκB kinase
IL-4	Interleukin 4
IL-6	Interleukin 6
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-13	Interleukin 13
iNOS	Inducible isoform nitric oxide synthase
iPSC	Induced pluripotent stem cells
IRES	Internal ribosomal entry site
iTRAQ	Isobaric tag for relative and absolute quantitation
IUPAC	International Union of Pure and Applied Chemistry
IκB	Inhibitor of kappa B
LC3	Microtubule-associated proteins 1A/1B light chain 3A
LD	Low dose
LeptoB	Leptomycin B
LogP	Partition coefficient
MAP	Microtubule-associated protein
MD	Molecular Dynamic
Mdm2	Mouse double minute 2 homologue
MET	Tyrosine-protein kinase Met
MGMT	O^6-alkylguanine DNA alkyltransferase
miR-34a	Micro ribonucleic acid 34a
MOPP	Mustargen Oncovin Procarbazine Prednisone
MQ	Methylene quinulidnone
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MSC	Multiple sclerosis
mTOR	Mammalian target of rapamycin
MVB	Multivesicular body
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NBR1	Neighbour of BRCA1 gene 1 protein
NCI	National Cancer Institute
NEDD8	Neural precursor cell expressed, developmentally down-regulated 8

nESI-MS	Nano electrospray-mass spectrometry
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
nM	Nanomolar
NMR	Nuclear magnetic resonance
NOD	Non-obese diabetic mice
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
NSAID	Non-steroidal anti-inflammatory drug
OD	Oligomerisation domain
OECD	Organisation for Economic Co-operation and Development
OGG1	8-oxoguanine glycosylase
OGG2	8-oxoguanine glycosylase
O-Glc-NAc	*O*–*N*–acetylglucosamine
$p14^{ARF}$	ARF tumour suppressor
p21/Waf1/Cip1	Cyclin-dependent kinase inhibitor / CDK-interacting protein 1
p300	P300-CBP coactivator family
p53	Tumour protein p53 / cellular tumour antigen p53
PAIN	Pan-assay interference compounds
PAS	Phagophore assembly site
PBMC	Peripheral-blood mononuclear cells
PC	Phosphatidyl choline
PCNA	Proliferating cell nuclear antigen
PD-1	Programmed cell death protein 1
PDGF	Platelet-derived growth factor
PE	Phosphatidyl ethanolamine
PI3K	Phosphatidylinositol 3-kinase
pO_2	Partial pressure of oxygen
POMP	Proteasome maturation protein
PRMT	Protein arginine N-methyltransferase 1
PRR	Proline-rich region
PUMA	P53 upregulated modulator of apoptosis
RB	Retinoblastoma
REDD1	Regulated in development and DNA damage response 1
RNA	Ribonucleotide
RNAi	Ribonucleotide interference

ROS	Reactive oxygen species
SCID	Severe combined immunodeficiency
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SERCA	Sarco/endoplasmic reticulum $Ca2^{+}$-ATPase
SILAC	Stable-isotope labelling by amino acids in cell culture
SirT1	NAD-dependent deacetylase sirtuin-1
SirT2	NAD-dependent deacetylase sirtuin-2
SM	Small molecule
Smyd2	Histone methyltransferase
SRB	Sulforhodamine B
Src	Proto-oncogene tyrosine-protein kinase Src
STAT3	Signal transducer and activator of transcription 3
SUMO	Small ubiquitin-like modifier
SV40	Simian vacuolating virus 40
TAD	Transactivation domain
TAM	Tumour-associated macrophage
TIGAR	TP53-inducible glycolysis and apoptosis regulator
TIP60	Tat-interactive protein of 60 kDa
TMT	Tandem mass tag
TNF	Tumour necrosis factor
UDP	Uridine diphosphate
UGT	Uridine 5'-diphospho-glucuronosyltransferase
ULK1	Unc-51 like autophagy activating kinase 1
UMP	Uridine monophosphate
UTP	Uridine triphosphate
UV	Ultraviolet
V-ATPase	Vacuolar-type H^{+}-ATPase
VCX1	Variable charge X-linked protein 1
VDAC	Voltage-dependent anion channel
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

1 PREFACE

One overwhelming thought that stuck in my mind during the writing of this thesis is the sheer volume of work that has occurred, the great personal sacrifices of every individual involved, and the passion of all the researchers and other supporting staff in the pursuit of uncovering new paradigms, treatments and questions with regards to cancer. When one engages in one's research projects, it is often incredibly easy to lose sight of the wider context of our work, and the personal stories behind every paper, every finding and every advance. Adding a small drop in the ocean of research can often seem insignificant, yet every drop makes a splash, one that ripples out from the point of impact, and thus one's work should never be viewed in a vacuum.

Reading broad appeal books such as "The Emperor of All Maladies" [1] or "The Immortal Life of Henrietta Lacks" [2] helps to bring me out of my comparatively narrow field and take a wider look at the world around me. It also makes me realise that the so-called "war on cancer" is a relatively recent one, and though it has been one peppered with both success and failure, it evokes a feeling of how far we have come in our relative understanding of cancer. Our therapeutic arsenal has expanded from the nitrogen mustards, adapted from their wartime use of poisoning our fellow humans to being anti-cancer agents, all the way to targeted therapies, immunotherapy, and the future technologies of gene editing with CRISPR in tumour cells. We have refined our approach from the bone saw to the surgical knife; or in the context of chemotherapy, a genotoxic and systemic approach to a localised and highly specific approach. All of these advances have come about thanks to a fundamental understanding of cancer and its unique pathogenesis. Hanahan and Weinburg have captured this paradigm shift eloquently in their seminal paper "The Hallmarks of Cancer" back in the year 2000 [3], which as our understanding has grown has expanded to include new hallmarks and was revisited again in 2011 [4].

It is to this end that I add another drop in the ocean. I hope that the phenomenal pace of research continues into the future, and that the next "hallmarks" update includes a section under each hallmark with a treatment that safely and effectively targets each aspect of cancer as we edge ever closer to the elusive cure we all chase.

2 INTRODUCTION

2.1 CANCER

2.1.1 What is cancer – a short history of disease

"The longer you can look back, the farther you can look forward"

– W. Churchill, 1944

2.1.1.1 Ancient times

The disease we now refer to as cancer has been documented throughout human history. One of the oldest references and treatments for cancer comes from the ancient Egyptians, with tumours being detailed in the Ebers papyrus, Smith papyrus and the Petrie papyrus, which date from 1600 BCE [5]. It is also here that the first treatments for cancer were referenced from incisions and poultice application, to cauterisation with an instrument called the "fire drill" all the way through to a more effective contribution to cancer treatment with caustic arsenic pastes applied to superficial skin lesions [5].

It was then that the ancient Greeks picked up where the Egyptians left off. Platonic and Aristotelian theories helped shape the ideas behind the perception of the world, with Aristotle expanding the Platonic theories to include form and matter [5]. This was a further explanation of physiological processes using the "basic elements" as described by Empedocles as well as incorporating the idea of humors, spirits and faculties [5]. It was through this lens of understanding that Hippocrates devoted study to the treatment of cancer. It was he who denoted various kinds of aberrant growths such as cancer, carcinoma, ulcer, indurated, hidden, superficial or deep-seated cancer and the associated terminology [6]. In the writings of Hippocrates, his clinical observations of cancer also included considerations for treatment including the age, gender, type of tumours and the organs being treated – a very enlightened approach at the time [6]. It was Galen, however, that took the classification of tumours to a new level by distinguishing between names of diseases that refer to symptoms or due to their similarities to exogenous objects [6]. Indeed, it is Galen we should thank for naming the disease "cancer" from the greek "*karkinos*" meaning crab to describe a lesion he found on a patient [7]. Less enlightened though was the humoral theory that underpinned the prevailing view as to development of disease. The body was believed to contain four "humors" – blood, phlegm, yellow bile and black bile [6,8]. Galen, adopted the humoral theory to explain the pathogenesis of cancer believing that tumours result from the bile from the liver (black bile). Black bile at the site of an organ in particular was thought to be the cause of cancer and this theory persisted for almost 16 centuries before being displaced [8].

2.1.1.2 The middle ages

Whilst progress virtually stagnated for hundreds of years through the middle ages, there were some glimmers of understanding from scholars such as Paul of Aegina (625–690 CE) [6,7]. It has been recorded that he opined that "some say that cancer is so called because it adheres with such obstinacy to the part it seizes that, like a crab, it cannot be separated from it without difficulty" [7]. Doctrine from the church and the prohibition of autopsies made the study of the finer points of medicine difficult for even the most enthusiastic of practitioners. Thus progress proceeded the pace of molasses up until the first real challenge to the Galenic theories came from three French physicians and surgeons, Lanfranc (1252–1315), Henri de Mondeville (1260–1320), and Guy de Chauliac (1300–1368) [9]. Another noteworthy challenge to the theories of Galen came from a man of poisons and founder of modern toxicology, Paracelsus, or to be precise, Philippus Aureolus Theophrastus Bombastus von Hohenheim (1493-1541) [8]. The same man that uttered the immortal phrase "Alle Dinge sind Gift, und nichts ist ohne Gift; allein die Dosis machts, dass ein Ding kein Gift sei", publically burned books on Galen and Avicenna. Though he failed to sway his colleagues through this rather rash and obstreperous action, he did prove his actions and words were as bombastic as his real name (rather than his more oft used nom de plume) [10-12]. Following Paracelsus came the landmark discovery by William Harvey (1578–1657) who finally described that blood circulated in vessels in 1628. Lymphatics were next on the discovery list thanks to Thomas Bartholin (1616–1680) who, in 1656, introduced a concept of coagulation and fermentation of the blood and lymph as a cause of cancer. He also made the observation of organ deterioration in systems harbouring cancer. Thomas Sydenham (1642–1689) who took a Hippocrates-like approach to collation of information and recording patient information served to advance the theories of the disease process through his writings.

2.1.1.3 Cancer during the renaissance and the enlightenment

Following this relative torpor came a frenetic increase in understanding during The Enlightenment, starting with theories of a renowned Dutch physician named Herman Boerhaave (1668–1738) and Jean Astruc (1684–1766). Boerhaave and Astruc believed that cancer formed due to accumulated bad humors thanks to stagnation, coagulation and blockage of body secretions [9]. They believed that this chronic inflammation played a crucial role in the pathogenesis of cancer. As a point of interest, Astruc was also well-known for other rather more entertaining experiments including cooking a piece of breast cancer next to a piece of meat, eating them both and proclaiming that he couldn't taste the difference [9]! The role of inflammation and cancer was further cemented by London surgeon, John Hill (1716–1775), who wrote a book on the use of snuff [7]. He found that the use of large quantities of snuff could lead to ulcerated cancer after many years of use [9]. Another environmental hazard was pivotal for our understanding of inflammation and cancer – the case of the London chimney sweeps [8]. It was Percivall Pott (1714–1788) that noted that the chimney sweeps' scrotal cancer was caused by exposure to soot, which contained liberal quantities of arsenic, sulphur and ammonia [8,9]. One of the most enlightened approaches of the age came from a French physician, Bernard Peyrilhe (1735–1804). He was the first

person to write an entire monograph on cancer, though he did espouse the view that it was a cancer virus (of unknown origin) that caused the disease [8]. Peyrilhe's misstep in his assigning cancer to a viral origin meant that he justified the isolation of cancer patients to cancer wards as he felt that the cancerous virus was transmitted from parents to other children through the air, saliva and bodily secretions [8].

2.1.1.4 The great leap forward during the 19th century

To usher in a new era of discovery, four English surgeons published their book on the signs and symptoms of cancer. James Hill (1703–1776), Benjamin Bell (1749–1806), John Pearson (1758–1826) and Joseph Adams (1756–1818) all contributed in unique ways to the treatment and advancement of the use of surgery to remove tumours. They surmised that generous excision was required to remove the tumour to prevent relapse [13]. Metthew Baillie (1761–1823) published some of the most important pathological analyses of the era, noting pathological changes of organ systems as well as illustrating common and rare tumours. A fellow delver into pathology, Xavier Bichat (1771–1802), theorised that cancer, as well as other diseases, developed from pathological change within tissues. This idea was revolutionary, as was his idea regarding the composition of tumours whereby he distinguished the stroma and parenchyma [5,7,13]. Another Frenchman, whose embarrassment during examining a women's heartbeat led to his development of the stethoscope, named René Laennec (1781–1826), played an important role in distinguishing pulmonary tuberculosis from lung cancer. He also played an important role in coining the term melanoma as well as colloid cancer [13]. In a home run for the French physicians, it was another French physician who popularised the term metastasis. Joseph Recamier (1774–1852) found by watching cancer growing and spreading he was able to identify which blood vessels were invaded, and thus he proposed compression treatment to restrict blood supply to lessen the spread [8,13].

The next leap forward occurred just a short jump across the channel back in London where Thomas Hodgkin (1798–1866) noted the appearance of enlarged spleen and lymph nodes in patients whilst he was a pathologist at Guy's Hospital. Hodgkin made a very detailed study of his discovery accompanied by illustrations of the disease he named lymphogranuloma malignum [13]. It took 33 years for his observation to be named after him by noted pathologist Samuel Wilks (1824–1911). The discovery of a lymphoma leads nicely into the next major breakthrough in the history of cancer – that we humans are composed of cells. It was Theodor Schwann (1810–1882) who made this discovery in 1838 [13]. In that very same year, Johannes Muller (1801–1858), who happened to be Schwann's supervisor, published a treatise that described cancers as abnormal structures composed of cells and stroma and that cancer came from the formation of new cells that were destructive and able to spread to other parts of the body by invading blood vessels [13]. He also distinguished epithelial and connective tissue tumours and developed further subdivisions for other types of tumour as well [13]. It is at this point we take a different tack and switch to the development of one of the first biochemical assays for cancer. Henry Bence Jones (1814– 1873) discovered that a

substance precipitated out of the urine of an ill patient upon the addition of nitric acid. Upon the death of this patient, haemorrhagic cavities were discovered in their bones, and thus the link of this precipitate in urine was linked to myeloma [13].

Rudolph Virchow (1821–1902), another student of Johannes Muller, was one of the next pioneers in the cancer theory. He finally put the humoral theory to death along with the blastema theory by introducing the concept that all cells derive from other cells [14]. Virchow noted the presence of leucocytes in the tumours he examined, thus concluding that inflammation influenced tumour progression [13,14]. He also divided all neoplasias into two types: homologous growths, which were made up of an increase in size and number of cells of a type that were present in normal tissues, and heterologous tumours, which are mostly malignant and made up of new types of cells that are not present in normal tissues [13,14]. It was also Virchow that gifted us the term leukaemia from his observation of two different kinds of leukaemia – myeloid and lymphocytic during his studies [13,14]. Whilst his legacy overall was of amazing strides forward, we would be remiss in not acknowledging that even the greatest of pioneers make errors along the way. Virchow firmly believed, right up until his death, that epithelial tumours originated from connective tissues, and not from the surface epithelium. This held progress back for a number of years before the theory was overturned [13].

2.1.1.5 Towards modern medicine in the early 20th century

We now enter into the most productive period in terms of advancement of our understanding of cancer. We start with a Berlin pathologist with the name of Julius Cohnheim (1839–1884), the first person who proposed that tumours develop either from collections of cells that are misplaced during embryonal development, or cells that had retained embryonal characteristics [15]. It was Cohnheim that first proposed that changes in vascularity through angiogenesis may cause these cells to develop into tumours [15]. Moritz Wilhelm Hugo Ribbert (1855–1920) took this theory one step further by adding that mechanical irritation predisposed these cells to developing into cancer [15]. Development in other areas of biology such as the discovery of nucleotin and nucleoprotein (now referred to as DNA) in every cell helped further understanding of cancer [15]. It was during the 19th and early 20th centuries in particular that surgery for therapy became widely used, especially spurred on by the more universal use of anesthesia in surgery [16].

Though overshadowed by the surgical achievements of Halsted *et al*. there were advances in chemistry and bacteriology that helped to contribute to the field [15]. Both fields attempted to find cancer-causing chemicals in water, soil and other locales but to no avail at this point. It was only upon observation of the occupational risk of lung cancer in miners that mine dust may be responsible for the pathogenesis of the disease. Eventually links were formed between cancer and arsenic, bismuth, cobalt, nickel as well as industrial tar, coal tar and paraffin [15,17,18]. These were some of the first carcinogens identified after the study of the chimney sweeps in Britain many years prior. In the field of microbiology, Reginald Harris (1838–1908) also noted that there was an elevated incidence of cancer in Egyptians infected

with a parasite called *Schistosoma haematobium* [15,19]. This provided evidence that organisms infecting or colonising the body could also increase ones predisposition to developing cancer.

There was also a paradigm shift in studying tumours in experimental novels. A Russian veterinarian named Mstislav Novinsky (1841–1914) managed to transplant a malignant venereal tumour from an affected dog into unaffected dogs [15,20]. Following this success, spontaneous rat vulvar carcinomas were transplanted into the testes of male rats, and there was a successful transplantation of a murine carcinoma through 17 generations of mice [15]. The next breakthrough occurred when Wilhelm Rontgen (1845–1923), discovered a form of electromagnetic radiation that he termed x-rays [21]. This led to a new era of diagnostic medicine with radiology units opening across Europe and the United States. It was found that these x-rays were both tumour destroying as well as tumour causing [15]. Two other groups of scientists, namely Antoine Becquerel (1852–1908) along with Marie Curie (1867–1934) and her husband Pierre (1859–1906), discovered the concept of radioactivity whilst working with uranium and radium respectively [22,23]. The use of radioactivity to treat tumours is covered in chapter 2.1.3. Further to the use of x-rays for diagnostic purposes, nontraumatic biopsy using an aspiration syringe was implemented as a means to obtain samples from tumours for microscopic analysis [24]. The diagnosis of leukaemia also took a leap forward thanks to the pathologist, James Ewing (1866–1943). By studying blood smears, Ewing was able to distinguish two forms of leukaemia – the myeloid and lymphoid, and noted that chronic leukaemias progressed slowly, whereas acute leukaemia progressed quickly [25].

It is at this point we enter into the modern age with the American Association for Cancer Research being founded in 1907 and American Society for the Control of Cancer coming into being in 1913 [15]. It was with one final set of discoveries that we enter the world of molecular biology and begin to drill down to the molecular hallmarks of cancer. The seminal paper by Peyton Rous (1879–1970), published in 1910, showed how a sarcoma in hens could be transmitted to normal hens by an injection of cell-free filtrates taken from the original sarcoma [26]. The agent in the filtrate was identified later to be an RNA virus, and was named after its discoverer as the Rous sarcoma virus. At a similar time, Theodor Boveri (1862–1915) found that the development of cancer could be initiated by chromosomal mutation [27,28]. It was at this point that two scientists, Alexis Carrel (1873–1944) and Montrose Burrows (1884–1947) started to culture tumour cells *in vitro* from the Rous chicken sarcoma [29]. It was not long before a culture of osteosarcoma cells was established in the same laboratory [30] and that differences between malignant and normal cells could be seen *in vitro* [31]. Further to the earlier experiments where tumours were transferred between animals, it was also now possible to induce tumours in rats using x-rays thanks to the previous work of Becquerel [32].

Thus we see the gradual progression, from the naming and identification of the disease all the way up to the 20th century where tumour cells were finally beginning to be cultured outside the body and *in vitro* experimentation took off. We can now consider the molecular basis of

cancer and, rather than the crude descriptions of the disease detailed above, it is time to dissect the various factors that allow us to see how cancer is initiated, and how it succeeds against all the biological controls we possess to prevent its success.

2.1.2 The biology and pathogenesis of cancer

As we have seen, cancer is an old disease that has been documented throughout human history. The first and most important fact to establish is that cancer is a disease of dysregulation whereby normal cells become abnormal through a progressive selective pressure due to exposure to cellular insults, acquisition of genomic instability, mutation at either the somatic or germline level and a plethora of other initiating events [33-35]. These events lead to a situation where the cells become self-sufficient and possess an apparent endless replicative ability, eventually gaining the ability to spread and colonise new areas of the body.

First and foremost it is important to start to define the key characteristics of a cancer cell. The best place to start is by looking at the seminal papers by Hanahan and Weinburg [3,4] that summarise in an elegant and concise manner the means by which cancer develops, persists, and succeeds in replicating when other cells would die or undergo senescence. To be successful, a cancer cell has to resist cell death, acquire genome instability and mutation, induce angiogenesis, activate invasion and metastasis, enable replicative immortality, evade the immune system, evade growth suppressors, sustain proliferating signalling, deregulate cellular energetics and exist in a pro-inflammatory environment as summarised in fig 1 [4].

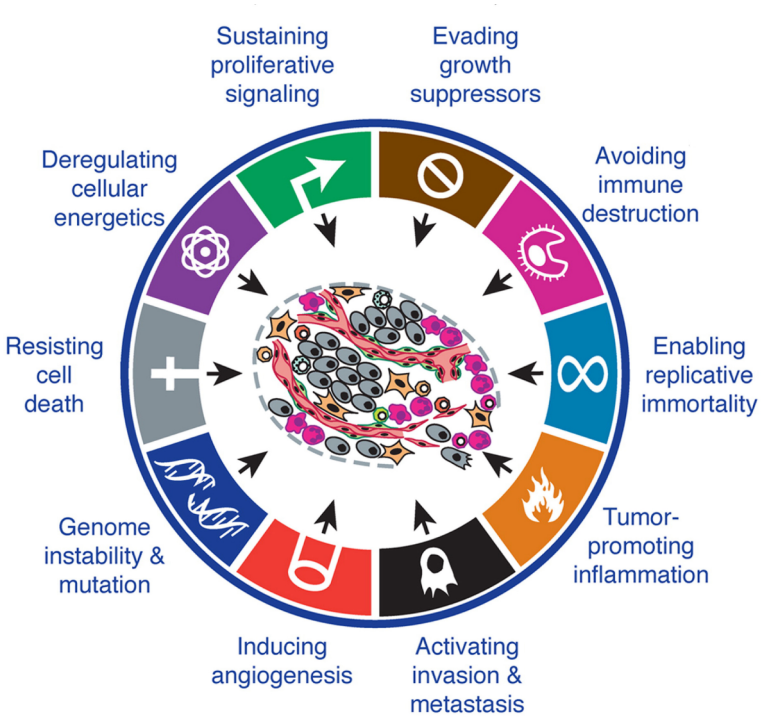


Fig 1. The hallmarks of cancer in their most recent iteration. Diagram adapted from Hanahan and Weinberg [4]. Reproduced with permission from Elsevier all copyright retained (2011).

When viewed individually, it is clear that certain hallmarks are markedly more complex than others and may have multiple routes that allow tumours to circumvent the mechanisms put in place to ensure normal cellular function. As each category could span an entire book, for the sake of brevity I will elucidate a few factors that each contribute to the accumulation of adaptations that permit the tumours to circumvent regular controls.

2.1.2.1 Of oncogenes and tumour suppressors

Firstly, let us consider the genetic components of cancer. It was first the renowned scientist Theodor Boveri that postulated in 1914 that there may be a factor that became amplified to allow for tumourigenesis [27]. This theory lay dormant for a number of years before being taken up again by George Todaro and Robert Huebner in the late 1960's [36,37]. In fact, this research built on the original studies by Rous 60 years previously in chickens, whereby injection of a cell-free filtrate from a chicken sarcoma caused development of a sarcoma in a healthy chicken [26]. Huebner and Todaro used the term "oncogene" to describe a particular genetic code that predisposed a cell to transform from a normal cell into a tumour cell [36,37]. It wasn't until a few years later that the first oncogene was isolated and named *src* [38,39]. It was found that mutation of the proto-oncogene, or gene that had the ability to become an oncogene, was required to cause either expression of a mutated form of the protein product and/or overexpression of the oncoprotein as *src* was found to be present in normal cells as well [40]. Shortly after, the nucleotide sequence of *v-src* was painstakingly identified and the proposed amino acid sequence of the first oncoprotein suggested by Czernilofsky *et al.* [41]. These initial experiments paved the way for further research into oncogenes with dozens being identified further down the track including *c-myc* and *ras* oncogenes, two highly important pathways in cancer [42,43]. The main function of most of these oncogenes is to overcome the normal replicative limits, circumvent controls designed to keep cell proliferation in check, or to override signals that would otherwise cause cells to die or undergo senescence [44].

Every villain needs its counterpart hero, and in the case of the oncogene, we have tumour suppressor genes. There was somewhat of a lag between the discovery of oncogenes and the discovery of our protagonists, but in 1971 there were hints of there being a specific repressor of the development of cancer, and it was Alfred Knudson that formulated the two-hit hypothesis based on the observation of retinoblastoma incidence in families [45,46]. It was also observed that this stemmed from the mutation of specific genes [47]. At a similar time in 1979, there were hints of there being a protein that was detected upon transformation of murine cells. This protein was not detected in normal cells, but only in transformed cells [48,49]. One year later it was discovered that this mysterious protein was associated with the large T protein that was produced upon the oncogenic SV40 infection of cells. This protein became known as p53 – so named after its approximate migration on a SDS-PAGE gel [48-51]. *TP53* was originally isolated by three groups independently from the mouse [52-54], with the human form following shortly after [55]. The protein was also found frequently in several human tumour cell lines [56]. It was also found to respond to DNA damage in cells

upon irradiation [57]. Subsequently, it was theorised that *TP53* was an oncogene thanks to the cloning of a mutated p53 for the cDNA library, but in actuality it turned out to be a gene that was frequently mutated during tumourigenesis [58,59]. Thus, the villain became the hero with the designation of p53 as the "guardian of the genome" in 1992 [60]. It was at this time that the tumour suppressor or "anti-oncogene" field exploded and by 1993, seven tumour suppressor genes had been cloned including *TP53*, *RB1* and *APC* [61]. These tumour suppressors largely serve to control the cell cycle, maintain genomic integrity, modulate cellular responses to stress and, when necessary, promote apoptosis in cells that have suffered damage that could lead to aberrant behaviour [44].

2.1.2.2 The microenvironment and immune system

We come back to a key figure in the history of cancer – Rudolph Virchow. It was he who first speculated on the role of inflammation in the pathogenesis of cancer upon his observation of leucocytes infiltrating tumours [14]. This theory came back to the forefront with the groundbreaking paper by Dvorak in 1986 where he referred to tumours as "wounds that do not heal" [62]. Yet again, we go back in history to the ancient Greeks who first identified the cardinal signs of inflammation – dolor, rubor, calor and tumor [63]. Dvorak argued that all of these cardinal features existed both in cancer and in wound healing, as did many of the same signalling factors [62]. The idea of crosstalk between the stromal compartment of tumours and the tumour itself was a pivotal change in thinking as no longer was cancer an isolated abnormal growth of cells, but it was also an entity that could remodel its surroundings to better serve its survival and growth as well as derive factors from its surroundings to allow it to proliferate and survive. A quote that stands out to me is the following taken from Balkwill and Mantovani in 2001 [64]:

> *"If genetic damage is the match that lights the fire of cancer, some types of inflammation may provide the fuel that feeds the flames"*

The inflammatory environment of tumours is characterised by host immune cell infiltration. One of the first cell types to explore are the tumour associated macrophages (TAM). These cells are widely present in most if not all solid tumours [65]. The TAMs not only secrete factors that promote inflammation but respond to tumours secreting factors such as prostaglandin E_2, which causes COX2 expression and increased levels of PD-L1 expression, leading to downregulation of $CD8^+$ T-cells and thus the suppression of the immune response to the tumour [66]. Two pathways, the M1 or M2 pathway, can activate macrophages. Those associated with tumours largely progress down the M2 pathway leading to high expression of IL-10, IL-4 and IL-13 [67]. There have been a number of studies showing that the TAMs can also secrete factors such as CXCL1, IL-6 and G-CSF whilst simultaneously inhibiting the expression of anti-tumour factors such as TNF-α and IL-12 [68]. When it comes to lymphocytes, natural killer cells are not often found in the tumour, instead the dominant types found are memory cells [69]. These tumour-infiltrating T cells often express high levels of PD-1, a factor that causes downregulation of the $CD8^+$ T-cells, and thus have a similar

immunosuppressive effect as the TAMs [70]. A further key factor that the inflammatory environment suppresses is p53. A factor, known as migration inhibitory factor, has been shown to actually downregulate p53 activity, pointing to another important selective pressure a pro-inflammatory environment applies [71,72].

This infiltration accompanied with a rich cytokine network that overexpresses inflammatory cytokines, growth factors, and chemokines, but without expression of cytokines responsible for a sustained immune response, assists the tumour in its replicative ability, and to survive the cytotoxic portion of the immune system that would otherwise clear the aberrant cells [73,74]. Immune cells not only assist the tumour, but the tumour also signals to the immune cells, and one way it does this is through the factor NF-κB [75,76]. This factor in particular is incredibly important in regulating cell survival through BCL-X_L expression, inflammation through COX2, iNOS, TNF and IL-6 expression and also further stimulation of the tumour associated immune cells [75]. The immune cells as a feedback also signal using the cytokine inhibitor of kappa B kinase (IKKβ), which causes degradation of the inhibitor of kappa B (IκB), the negative regulator of NF-κB [75,77].

The second consideration of the microenvironment that is highly important for the propagation of a tumour is the ability to induce angiogenesis to allow for nutrient transport, oxygen transport and for a route of metastasis [78]. As a tumour reaches a certain size, poor blood supply due to leaky and blind-ending vasculature and hypoxic regions are characteristic of solid tumours [79]. There are a number of important factors involved in signalling for angiogenesis, but one we covered earlier is also a key driver of this phenomenon – NF-κB [77]. One of the downstream effectors of NF-κB is the family of factors known as vascular endothelial growth factor (VEGF) [80]. VEGF is a member of the platelet-derived growth factor (PDGF) family, a family of four homodimeric glycoproteins (VEGF-A, VEGF-B, VEGF-C and VEGF-D) [80]. There are three main VEGF receptors numbered 1–3. The primary driver of angiogenesis, VEGF-A binds to both VEGFR-1 and VEGFR-2, which promotes angiogenesis, vascular permeability, cell migration and gene expression [81,82]. Equally, just as inflammation drives this process, hypoxia from the poor blood supply also leads to activation of the VEGF pathway through HIF-1α signalling, the primary pathway responsible for the cellular response to low oxygen levels [83]. These signalling pathways add up to produce an environment that is immunosuppressive, pro-growth, anti-apoptotic and able to stimulate the division of endothelial cells to form new tumour blood vessels.

2.1.2.3 Carcinogenesis and cellular insults

Whilst analysing factors that contribute to the success and continued proliferation and survival of tumours is highly important, it is also of paramount importance to understand the mechanisms by which selective pressure can be applied to a normal cell that leads to tumourigenesis.

Prior to understanding genetic determinates of carcinogenesis, scientists have noticed that exposure to certain environmental conditions has the effect of increasing one's propensity to

develop cancer. One of the pivotal observations eluded to in section 2.1.1.3 was that London chimney sweeps were predisposed to a rare form of occupational cancer – cancer of the scrotum [84]. In fact, even in more modern times, chimney sweeps were exposed to environmental toxins that predisposed them to a number of tumours (amongst other maladies) [85]. Clearly there was a component present within the soot that was highly carcinogenic. In fact, as far back as the 1930s there was a clear link between certain compounds and their ability to induce tumours in animals [86]. Whilst the mechanism was not understood at the time, now we have a good idea as to how these compounds can cause cancer. For the sake of simplicity, we'll take a look at one particular carcinogen that has been well studied for over 100 years – benzo[*a*]pyrene. This compound is particularly interesting, as it requires metabolism in the liver for it to be a carcinogen. As can be seen in fig 2, benzo[*a*]pyrene can undergo metabolism by endogenous cytochrome p450 enzymes (CYP450) and epoxide hydrolase through to (+)benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide (BaP diol epoxide) [87-90]. This electrophilic compound is able to attack and bind to the nucleophilic guanine bases in DNA [91]. The base excision repair system that repairs adducts like those created by benzo[*a*]pyrene can sometimes misrepair and cause GC ➔ TA transversions, altering the code and, if within a coding region of the DNA, altering the amino acid sequence of the gene product [91-94].

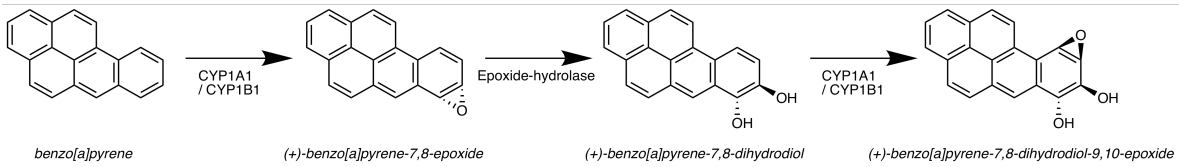


Fig 2. Bioactivation of benzo[*a*]pyrene to a compound capable of intercalating DNA. Diagram produced from information within [87-89].

There are a vast array of carcinogens like benzo[*a*]pyrene known these days and many of them are either direct or indirect DNA damaging agents [95,96]. The International Union of Pure and Applied Chemistry (IUPAC) publish monographs on the subject after considering a plethora of data both *in vitro*, *in vivo* and epidemiological in nature [97]. Carcinogenicity testing has become a routine analysis since moving from testing the ability to induce tumours in animals. In fact, mutagenicity testing can now be carried out *in vitro* thanks to the advent of the Ames test [98]. The Ames test relied upon a very simple premise, the use of a *Salmonella typhimurium* strain that carries mutations in their genes that are involved in histidine synthesis, meaning they require histidine but cannot produce it themselves [99]. Therefore, upon plating on an agar plate with limited histidine, they are unable to proliferate without a genetic reversion to allow them to synthesise their own histidine again [98,100]. A number of different mutant strains can be used, but the most commonly used are TA-1537 and TA-1538 that carry frameshift mutations, or TA-1531 that carries a point mutation in the genes required to synthesise histidine [100]. Thus the test is able to detect two different kinds of genetic mutation. As pointed out with the case study of benzo[*a*]pyrene, some compounds require metabolic activation for their carcinogenic potential to be unlocked, and therefore

incubation with and without a liver homogenate or fraction (such as S9) is recommended during the testing of each compound [99]. In further improvements to the testing system, the bacteria often carry a mutation in their lipopolysaccharide synthesis pathway resulting in a leaky cell wall, as well as in the excision repair system to make the test more sensitive [100,101]. The readout of the test is incredibly simple – assess the number of colonies formed upon treatment with a potential mutagen relative to natural reverts (background) [99,100]. Whilst the test is indicative, it is not perfect and there is still the possibility of false positives or even false negatives. That said, the technique is still in use in a modified form to this day to detect potential mutagens. These days, the test has been refined to yield the "fluctuation method", which relies upon exactly the same premise, except involves the mixture of a pH indicator into the medium, allowing a visual change to be noted upon proliferation of the bacteria as they release metabolic products causing acidification of the well contents [102]. The greatest advantage of this test is that it can be conducted on 384-well plates. The Ames test is just one of a number of *in vitro* and *in vivo* methods for determining mutagenicity, but it is certainly one of the most simple to conduct along with the micronucleus assay [103] and comet assays [104].

Another key mutagen that we are all exposed to, and one we all find quite hard to avoid unless we spend our lives underground, is UV radiation from the sun. It is in fact UVB radiation with a wavelength of between 280-315 nm that is the most energetic and damaging component of solar radiation [105]. This form of radiation can in fact cause direct damage of DNA bases due to the absorption of UVB photons primarily by the pyrimidine bases, though the purines can also be targeted [106]. UVB can cause direct adducts between two pyrimidines forming cyclobutane dimers and (6-4) photoproducts [107]. Additional reactions, like the splitting of water to form hydroxyl radicals and subsequent reactions with nucleophilic guanine to form 8-oxo-dG are also common [107]. In fact, from an interesting evolutionary standpoint, 8-oxo-dG lesions are so common that there are dedicated enzymes responsible for removing these DNA adducts such as OGG1 and OGG2 [108,109].

One can see, therefore, that we are surrounded by chemical and environmental factors that can cause somatic mutations within our cells. Thankfully we have DNA repair mechanisms such as MGMT to remove O^6-meG, nucleotide excision repair, transcription domain-associated repair, base excision repair, single strand break repair, mismatch repair, and double strand break repair which includes homologous recombination and non-homologous end joining [110,111]. Each of these methods, whilst generally high fidelity, can fail and lead to misrepair of the DNA [112]. The best analogy I can think of is the Russian roulette challenge – the more times one pulls the trigger, the more likely one is to get shot, and thus it is the same for DNA repair. The more cellular insults your cells endure, the greater the chance for the repair mechanisms to go awry, despite the evolutionary brilliance of their design, and for a particular mutation to fall within a highly important region of a tumour suppressor or proto-oncogene that could lead to repression or activation respectively.

In summary, it is clear that cancer is a highly complex and multifaceted disease with numerous factors that determine its pathogenesis and its ability to survive. This chapter provides a brief overview of but a few potential causes of cancer and a limited number of factors that serve to explain what cancer is and why its eradication has eluded us thus far.

2.1.3 Treatment of cancer through the ages

2.1.3.1 Early cancer therapy

Once again the treatment of cancer harks back to the Ebers papyrus as a source for the first possible cancer therapeutic as well as the first surgical techniques [7]. It was in these texts that the ancient Egyptians used numerous techniques to attempt to stymie cancer, often without success. They used cauterisation as well as trying an early version of chemotherapy with salts. But it was their use of an arsenic paste that was the most striking of their discoveries, and one that came closest to the therapy that started the chemotherapy revolution spearheaded by Paul Ehrlich in 1910 [113]. Many of the early civilisations such as the Sumerians, Chinese, Indians, Persians and Hebrews resorted to remedies such as tea, fruit juices, figs and boiled cabbage, but in persistent cases did turn to rather extreme measures with pastes of copper, iron, sulphur and mercury [114]. The Greeks were next of the ancient civilisations to make a mark on therapy and they, like the Egyptians, used poultices made from numerous different ingredients depending on the symptoms displayed [6]. Ultimately, though, they realised that it was only surgery that could cure, and even then only at an early stage [6,114]. We now jump a good hundred years to Paracelsus once again, who unlike the previous physicians who rather haphazardly applied herbs and compounds of various ingredients, took a rather more systematic approach to therapy [10]. He introduced mercury, lead, sulphur, iron, zinc, copper, arsenic, iodine and potassium as therapies to be introduced internally, though he added a cautionary note that many of these substances were toxic if the dose was too high [115,116].

Surgery of tumours was still commonplace and many surgeons attempted to grapple with the difficulty of operating on cancer patients. It was Ambroise Pare (1510–1590) that first proposed a wide excision around the tumour. He treated breast cancer by placing a lead sheet covered in mercury on the tumour [9]. A German surgeon named Johannes Scultetus (1595–1645) published a detailed and illustrated guide to surgical treatment of tumours by excision and amputation [9,117]. Another German, Guilielmus Hildanus (1560–1634) proposed a modified radical mastectomy by removing the ancillary lymph nodes to the tumour [118]. In a hat trick for German surgeons, the man who has been referred to as the Father of German surgery, Lorenz Heister (1683–1758), postulated that for breast cancer, the removal of the breast, ancillary lymph nodes, and the pectoralis major muscle was the best way to achieve cure [119]. This technique became known as a radical mastectomy. Heister's brilliance did not just extent to the radical, but also to the conservative as he also detailed a method by which to remove just the tumour in what was referred to as a lumpectomy [9,119].

2.1.3.2 Modern surgery and radiotherapy

Whilst knowledge of cancer biology advanced rapidly, the treatment of cancer rather languished, though surgical precision and knowledge did improve throughout the 18th and early 19th centuries. It wasn't until the end of the 19th century and into the 20th century that therapy began to advance rapidly. The first advance, building on the earlier radical mastectomies performed by Heister, was from Halsted in 1891 [120,121]. This treatment had an incredible 42% 3-year disease-free survival, demonstrating the first convincing curative method for dealing with a particular type of cancer [121]. The second advance followed the discovery of x-rays as covered in chapter 2.1.1.5. It was found that not only did x-rays cause tumours as was found in a technician who worked with them, but they were also able to eliminate tumour cells [15]. In 1897, x-ray radiation was used to treat a woman with advanced, inoperable breast cancer. The discoveries of Marie and Pierre Curie paved the way for the use of radium as a means to treat cancer [23]. The first treatment occured on patients in London where two cases of basel cell carcinoma were treated [122]. These uses of x-rays and ionising radiation launched the discipline of radiotherapy, which is still widely used today to treat cancer [123]. The third advance at the end of the 19th century came from Paul Ehrlich whom we mentioned previously. It was not only his use of arsenic compounds in 1910 that earned him fame, but also his investigations into the use of other purified compounds for the treatment of cancer. Ehrlich published his findings in the first book on chemotherapy [124].

2.1.3.3 The chemotherapy era

It was at this point the First World War broke out. It has often been said that the horrors of war spur innovation out of necessity, but it was actually the end of the war that provoked one of the most startling discoveries in the field of chemotherapeutics. The use of a chemical weapon, in particular a nitrogen mustard, during the First World War led to leucopoenia in those exposed. This observation led to the testing of a particular nitrogen mustard to treat lymphomas and leukaemias [125]. Later, the focus shifted to a more mechanistic understanding of how a nitrogen mustard leads to the elimination of tumour cells. They found that it was the formation of an ethylene immonium ring, an alkylating intermediate, reacts with electron-donors on nucleic acids and proteins [126,127]. The next pivotal finding came from Sidney Farber in which he found that treatment with antifolates led to remission in acute leukaemia [128]. It was the synthesis of folate analogues and the subsequent inhibition of dihydrofolate reductase that led to these drugs being the first compounds that gained widespread use in numerous cancers including breast, bladder, and head and neck cancers [116,126,129]. The most incredible finding with the antifolates was that it could be used on solid tumours, a feat thought of as impossible until this discovery [130]. The finding of novel therapeutic strategies snowballed at this point, and the first microtubule poison, a vinca alkaloid, was discovered [131,132].

It was in the late 1960s the first two combination regimens was devised consisting of either a nitrogen mustard, vincristine, procarbazine and prednisone (MOPP) [133-135] or

methotrexate, vincristine, a purine analogue and prednisone (POMP) [136]. The next therapies developed in the 1960s were the taxanes and camptothecins. The taxanes were another anti-microtubule agent, but this time acted by stabilising the tubules preventing their depolymerisation rather than disrupting their polymerisation, providing yet another novel mechanism for an anti-tumour agent [137-139]. Camptothecin was another curious compound that had a novel mechanism of action. It targeted topoisomerase I, preventing the re-ligation of DNA, causing the induction of DNA damage and, ultimately, apoptosis [140]. Unfortunately, the use of camptothecin proved to be problematic *in vivo* as it was not stable at neutral pH, and it was not until 1996 that irinotecan, a stable camptothecin analogue, could be used in therapy [126,141,142]. Cisplatin and other platinum drugs were next to be discovered and yet again, possessed a novel mechanism of action, this time preferentially forming adducts with guanine in the DNA [143-145]. As guanine adducts are frequently dealt with by nucleotide excision repair, this is actually one of the primary mechanisms by which tumour cells overcome susceptibility to cisplatin [146]. All of these drugs are still in the clinic to this day and have proven to be highly efficacious in a number of different tumours [126]. The unfortunate side effect of nearly all of these therapies is, unfortunately, DNA damage, which may prove to be mutagenic down the line and therefore a new strategy to treat cancer was needed [147-151].

2.1.3.4 Targeted therapies and personalised therapy

In the late 1980s, the understanding of genetics and the forays into molecular biology opened up new avenues of research for cell biologists. This opened up new avenues for targeting specific molecules within the cell, and designing a therapy that would best achieve tumour cell kill without adversely affecting the function of normal cells. Chronic myeloid leukaemia presented the low hanging fruit thanks to the tumour being driven by a reciprocal chromosomal translocation between chromosomes 9 and 22, causing the expression of the constitutively active fusion protein, BCR-ABL [152]. ABL is a tyrosine-kinase, a protein that is capable of transferring a phosphate group from ATP to a protein in the cell, thus allowing signal transduction and activation of various pathways in the cell [153]. The BCR-ABL fusion causes an abnormal, constitutively active ABL, which permits cytokine independent proliferation and prevents apoptosis in response to DNA damage or cytokine depravation [154,155]. As the ATP binding domain of protein kinases had been solved, a number of small molecules were rationally designed and synthesised as part of a drug discovery project [156]. Imatinib was demonstrated to be the most potent inhibitor of ABL kinase, was able to ablate growth of cells in culture and in xenograft studies, and was relatively non-toxic to normal cells in culture [156]. Imatinib was approved for use for CML in 2001 and was the first rationally designed and targeted therapeutic on the market [157,158]. The approval of imatinib was followed by a slew of small molecule tyrosine kinase inhibitors, starting with gefitinib, an inhibitor of the epidermal growth factor receptor (EGFR) amongst others [159-162].

Since the discovery of antibodies, they have been utilised in research, for example for detecting proteins separated by SDS-PAGE and western blotting [163]. Monoclonal antibodies are highly specific and are capable of binding to one or very few targets. The first humanised antibodies that were developed for therapies were chimeric, consisting of murine variable regions that are capable of antigen recognition [164]. The first antibody therapy approved for cancer treatment was rituximab, which targets CD20 and is used in recurrent or refractory B-cell lymphoma [165]. The next step was to develop a fully humanised antibody for therapy to overcome some of the possible immunogenicity that one may elicit with chimeric antibodies [166]. The first of these came in the form of trastuzumab, an anti-HER2 antibody [164,167]. Following development of a number of therapies, new antibody-drug conjugates attempted to take advantage of not only the anti-tumour activity of the antibody binding to its target, but also to deliver a small molecule to the tumour [168]. The first therapy approved as brentuximab vedotin in 2011 with the antibody, brentuximab, directed against CD30 and the vedotin being a potent inhibitor of microtubule polymerization [169].

Whilst the addition of exogenous agents to cause cell death in tumours by targeting particular pathways or by exploiting the increased proliferative rate of tumour cells has been the workhorse for chemotherapy thus far, the thought of utilising the immune system against the tumour is an old idea and was accidentally exploited in the past. A young surgeon named William Coley (1862–1936), in an attempt to rid patients of persistent tumours, introduced a bacterial toxin to cause inflammation [170]. This treatment was unfortunately ended due to extreme toxicity, but there were positive responses from a few tumours, proving the principle that activation of the immune system may cause cancer regression [170]. The challenge with immunotherapy is threefold; firstly, there must be specific tumour antigens that are accessible; secondly, there must be an effector T-cell response; thirdly, the immune system must overcome the immunosuppressive environment either from the tumour or from the other tumour associated immune cells such as the TAMs [171]. The first problem of antigen presentation was addressed by a treatment regimen known as sipuleucel-T [172]. This treatment is a personalised treatment that involves the removal and reinsertion of peripheral-blood mononuclear cells (PBMCs) that have been activated with a recombinant fusion protein called PA2024, a prostatic acid phosphatase that is fused to granulocyte-macrophage colony-stimulating factor [173,174]. This treatment was intended for metastatic castrate-resistant prostate cancer and has demonstrated an overall survival improvement in a number of phase III clinical trials [175,176]. A second therapy known by the rather long and onerous name, tisagenlecleucel, uses what are known as CAR-T cells for the treatment of pediatric and young adults with relapsed B-cell precursor acute lymphoblastic leukaemia [177]. This therapy involves the removal and reprogramming of the patient's own T-cells to express a modified T-cell-receptor construct targeting CD19 before being reintroduced to the patient [178]. This therapy was only approved in 2017 by the FDA, making it an incredibly recent addition to the anti-cancer arsenal [178].

One final aspect of immunotherapy addresses the third consideration of mobilising the immune system against the tumour – the ability to overcome the immunosuppressive signals

tumours possess [171]. The first therapy to target this pathway was an anti-CTLA-4 therapy called ipilimumab [179]. Cytotoxic T-lymphocyte antigen-4 (CTLA-4) is a factor that binds CD80 and CD86 – cell surface markers on antigen presenting cells [180]. This binding to CD80 or CD86 interferes with the binding to CD28, thus preventing the co-stimulation mediated by CD28 [180]. Therefore, the theory came that blockade of CTLA-4 would lead to increased immune system activation and an immune response towards tumours [181]. Clinical trials were encouraging and the FDA approved ipilimumab in 2011 for use against melanoma [179,181-183]. The second checkpoint therapy came in the form of an anti-PD1 therapy called nivolumab [184]. Programmed cell death 1 (PD1) is a receptor generally expressed on T cells, natural killer (NK) cells, B cells and some myeloid cells [185]. PD1 functions as a regulator of immune response and is highly important for tolerance to self-antigens [186,187]. It turns out that the ligand of PD1, PDL1, is often expressed by tumour cells to dampen the immune response, and therefore the theory came about that either targeting the ligand or the receptor would lead to an increased immune response [188]. Nivolumab went through clinical trials and demonstrated clinically meaningful responses against advanced melanoma [189,190]. The FDA therefore approved nivolumab and another therapy with the same target, pembrolizumab, for treatment of a number of cancers [191-193]. Now, both anti-CTLA-4 and anti-PD1 therapies are being tested in concert to provide two different mechanisms to overcome immunosuppression from tumours [194,195].

Thus we see that treatment of tumours has evolved through the ages from the crude, systemic application of therapy or indiscriminate surgery to the highly specific targeting of particular aspects that drive tumour growth and survival.

2.2 P53 – "THE NEXUS OF TUMOUR BIOLOGY"

2.2.1 Signalling, sensing, and acting

Previously in chapter 2.1.2.1 we touched upon the idea of tumour suppressor proteins and delved into the story of how p53 became known as "the guardian of the genome" [60]. But what is it in particular about p53 that makes it unique compared to other regulatory proteins within the cell?

2.2.1.1 Background to p53 and its family

The p53 family is highly evolutionarily conserved and its origins can be traced back almost 1–2 billion years ago to the dawn of multicellular organisms [196]. The strong evolutionary history of this gene points to its importance in allowing life to the success of organisms. As shown in fig 3, most vertebrates share very common features of the p53 pathway including the presence of two closely related proteins – p63 and p73 as well as the same negative regulators of p53 protein function, MDM2 and MDMX [197]. The two p53 family members, p63 and p73, share significant structural homology to p53, but do not necessarily act

primarily as tumour suppressors in the same way as p53 does [198,199]. They do, however, appear to play a significant role in development and differentiation [200,201].

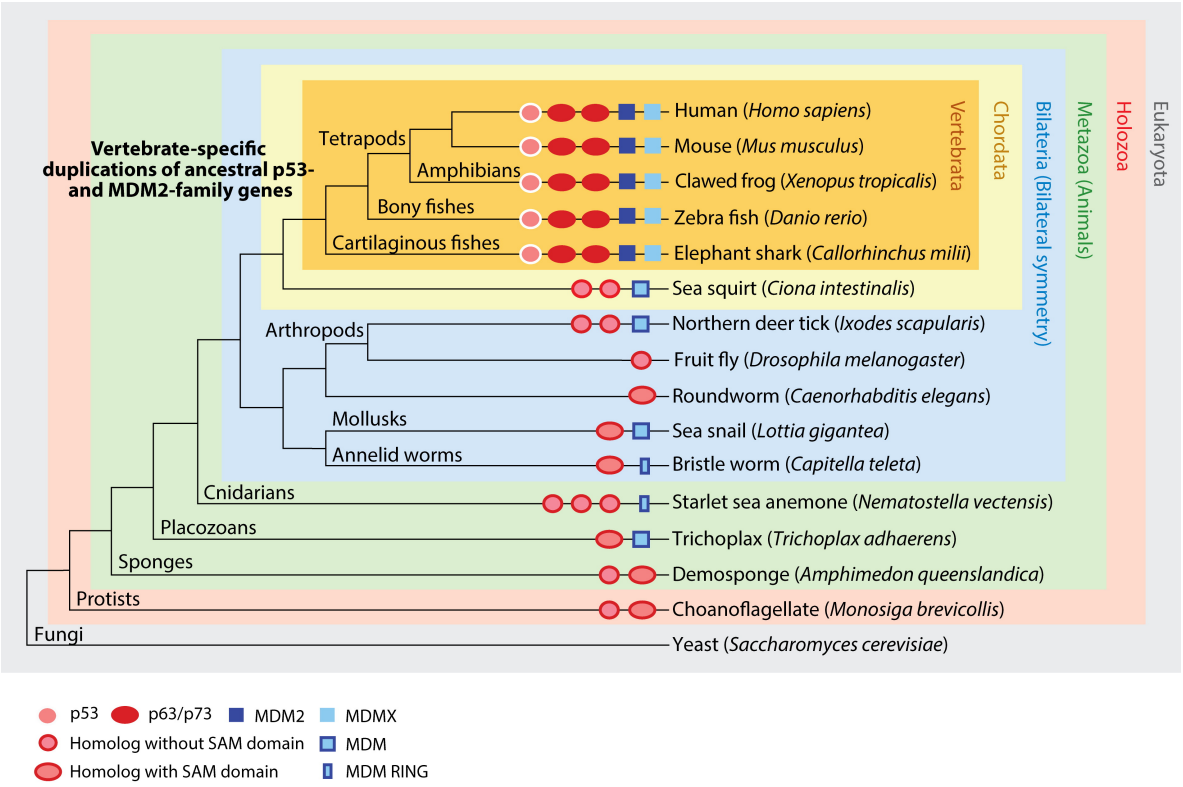
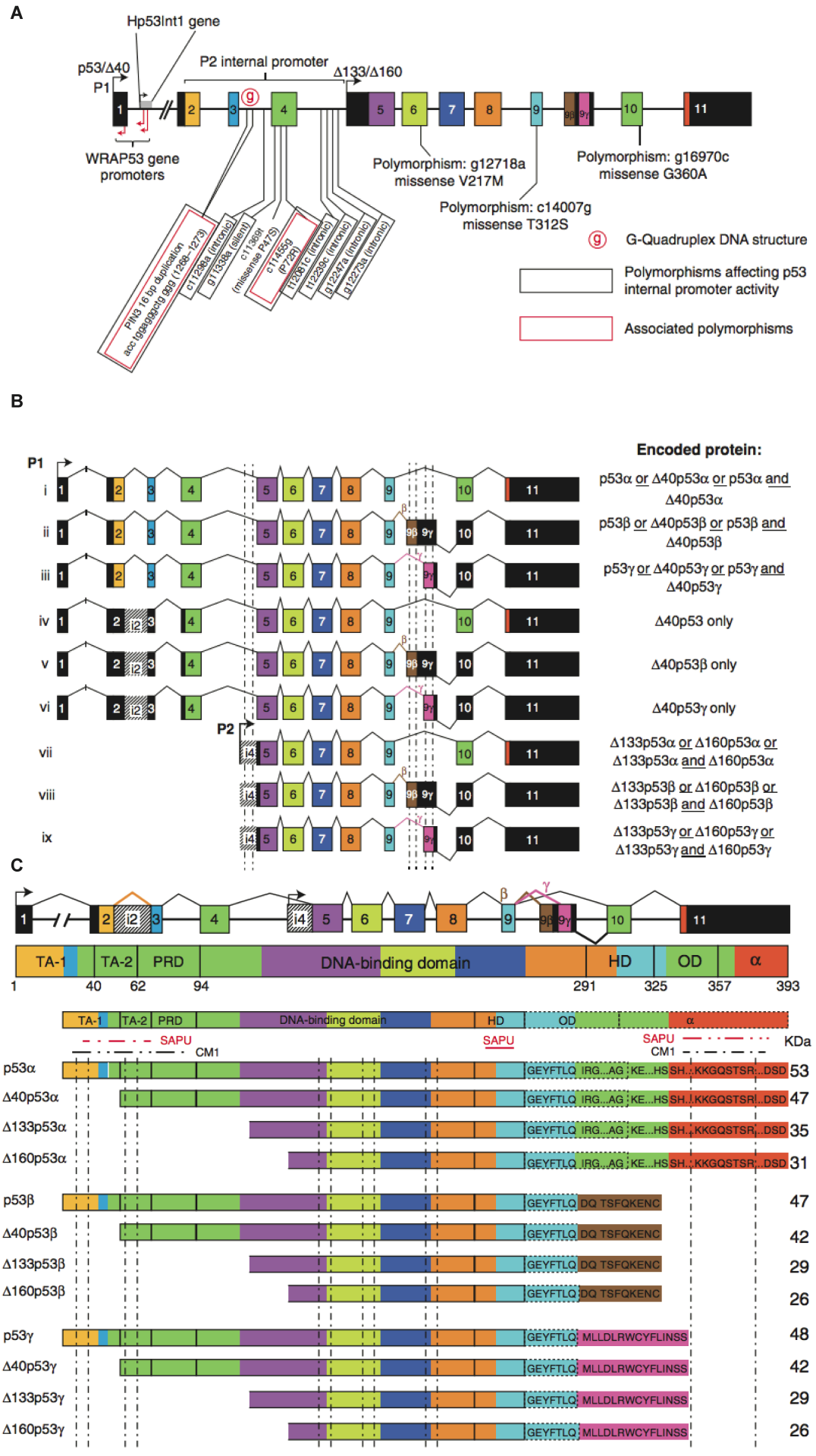


Fig 3. p53 and MDM2-family evolution constructed from genome-wide data obtained from [202]. Diagram taken from Joerger & Fersht [203]. Reproduced with permission from Annual Reviews. All copyright is reserved by the authors and publishers (2016).

2.2.1.2 The TP53 gene

The p53 gene is located on the human chromosome 17p13 [204] and consists of 13 exons (11 exons and two cryptic exons), one of which is non-coding [205,206]. As with many genes, the *TP53* gene encodes a number of different isoforms through alternative splicing as shown in fig 4 [206]. Whilst the canonical p53 protein is the most abundantly expressed isoform encoded by *TP53*, different isoforms are expressed at different levels depending on the cell-type and tissue localisation [207]. It was in the mid-1980s that the first splice variants were noted [208], and currently there are approximately nine p53 mRNAs in normal tissue encoding 12 different p53 isoforms [209]. Two promoters, P1 and P2 have been identified with the promoter P1 situated in front of exon 1 and P2 after intron 4 [210]. The fully spliced p53 transcript encodes the canonical p53 (p53α) as well as the Δ40p53α thanks to the presence of an internal ribosomal entry site (IRES) [211-213]. Both exon-9β and exon-9γ contain stop codons so exon-10 and -11 are non-coding in β and γ transcripts of p53 [206]. The mRNAs transcribed from the P2 promoter can start at either codons 133 or 160 yielding the Δ133 or Δ160 p53 protein isoforms, all of which can exist as α, β, or γ forms [206].


A
Hp53Int1 gene
p53/Δ40
P1
P2 internal promoter
Δ133/Δ160
WRAP53 gene promoters
Polymorphism: g12718a missense V217M
Polymorphism: g16970c missense G360A
Polymorphism: c14007g missense T312S
G-Quadruplex DNA structure
Polymorphisms affecting p53 internal promoter activity
Associated polymorphisms
B
Encoded protein:
p53α or Δ40p53α or p53α and Δ40p53α
p53β or Δ40p53β or p53β and Δ40p53β
p53γ or Δ40p53γ or p53γ and Δ40p53γ
Δ40p53 only
Δ40p53β only
Δ40p53γ only
Δ133p53α or Δ160p53α or Δ133p53α and Δ160p53α
Δ133p53β or Δ160p53β or Δ133p53β and Δ160p53β
Δ133p53γ or Δ160p53γ or Δ133p53γ and Δ160p53γ
C
TA-1
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PRD
DNA-binding domain
HD
OD
α
SAPU
CM1
KDa
p53α
Δ40p53α
Δ133p53α
Δ160p53α
p53β
Δ40p53β
Δ133p53β
Δ160p53β
p53γ
Δ40p53γ
Δ133p53γ
Δ160p53γ

Fig 4. The *TP53* locus and p53 mRNAs that derive from that. Black represents non-coding sequences with coding sequences coloured. (A) The *TP53* gene locus structure with the 11 standard exons and two cryptic exons (9β and 9γ). (B) p53 mRNAs showing the nine possible protein products of each of them. (C) The 12 human p53 protein isoforms showing each domain of the protein. Diagram taken from Joruiz and Bourdon [206]. Reproduced with permission. Copyright is retained by Cold Spring Harbor Laboratory Press (2016).

2.2.1.3 p53 protein structure

The p53 protein is active as a homotetramer, and has a length of 393 amino acid residues [214]. The N-terminal portion of the protein possesses an intrinsically disordered transactivation domain (TAD) and a proline-rich region [215]. This transactivation domain is involved in protein-protein interactions with proteins that regulate p53 function, such as mdm2, which interacts as residues 19–26 of p53 [215,216]. The N-terminal region is, in its native state, unfolded, though it does demonstrate some residual secondary structure due to functionally important hydrophobic regions [217]. This region of disordered protein is actually often seen in the TAD of transcription factors, and the p53 TAD has a short molecular recognition feature of 20 residues that allows the transition from a disordered to an ordered state upon binding [218,219].

The core domain of p53 was first solved in 1994 [220] followed by its DNA-free form later on [221,222]. This core domain has an immunoglobulin-like β-sandwich to establish its basic structure for DNA to bind [214]. The loops that form the DNA binding surface (L2 and L3) are stabilised by a zinc ion, which is coordinated to a histidine and three cysteine side chains (Cys-176, His-179, Cys-238 and Cys-242). The loss of this zinc causes an increased tendency to aggregate and the loss of DNA binding specificity [223,224]. The regulation of gene transcription by p53 hinges on recognition of a decameric, palindromic sequence of the form 5′-RRRCWWGYYY-3′ where R can be A or G, W can be A or T, and Y can be C or T [225]. There are key residues within the p53 core domain that make contact with DNA – Lys-120, Arg-248, Arg-273, Ala-276, Cys-277 and Arg-280 [220,226]. There is also evidence that the acidic domain of mdm2 is capable of binding to the DNA binding domain in the core domain of p53. This binding occurs after interaction between the mdm2 and the p53 N-termini due to a conformational change in the mdm2 acidic domain and it is this binding that promotes the E3 ligase activity of mdm2 [227].

Within the p53 structure is a tetramerisation domain towards the C-terminal region of the protein in residues 325-356 [228]. The p53 tetramer structure has been visualised by both X-ray crystallography and by NMR [229-231]. The way in which p53 interacts to form a tetramer has been described as a dimer of two primary dimers due to the difference in interactions that cause the quaternary structure to form [214]. The primary dimer is formed by two monomers forming an antiparallel intermolecular β-sheet and antiparallel helix with the Leu-330, Ile-332 and Phe-341 forming the hydrophobic core of this dimer [214]. The tetramer forms from two of these primary dimers, which associate through their α-helices and

stabilise due to hydrophobic interactions between Leu-344 and Leu-348 to provide the tetramer structure [230].

The final domain to consider of the p53 protein is the C-terminal domain, which is normally intrinsically disordered, however, it can become stabilised upon binding to other proteins or nonspecific DNA [232-234]. These residues at the end of p53 undergo extensive posttranslational modification including acetylation, ubiquitination, phosphorylation, sumoylation, methylation and neddylation that allow for regulation of p53 levels and function within cells [235-237].

2.2.1.4 p53 posttranslational modification

As described in section 2.2.1.3, the C-terminal domain in particular is a site for multiple posttranslational modifications. Additional to the C-terminus, the N-terminus also undergoes significant phosphorylation, but not other types of posttranslational modification, see fig 5. Each of the modifications plays a role in the regulation of p53, whether it be to stabilise, increase transcriptional activity, or to cause its proteasomal degradation [238].

Phosphorylation is one of the most common modifications in response to cellular stress [238,239]. One of the most commonly examined phosphorylations is at Ser15, a site that undergoes phosphorylation predominantly by two responders to DNA damage – ATM and ATR [240,241]. This modification actually mediates the further phosphorylation of other residues within p53 [242,243]. In fact, it is phosphorylation at Ser15 or Ser20 that reduces the affinity of p53 to its negative regulator, hdm2 (the human form of mdm2), and increases its recruitment of transcriptional coactivators [235]. Phosphorylations at Thr-55, Ser-376 and Ser-378 appear to be present constitutively in unstressed cells [244,245].

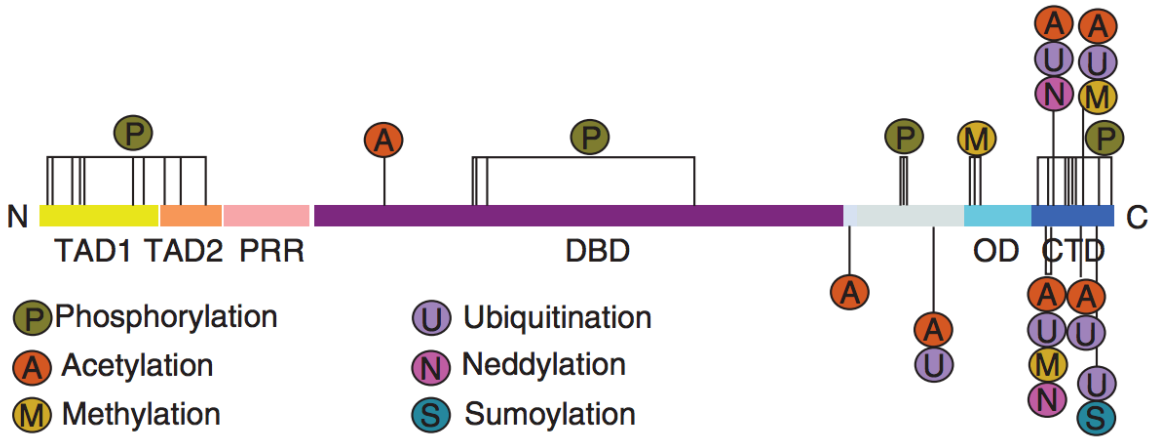

Fig 5. Sites of the most frequent posttranslational modifications of p53. Each of the domains is as follows: transactivation domain (TAD), proline-rich region (PRR), DNA-binding domain (DBD), oligomerisation domain (OD) and C-terminal domain (CTD). Diagram taken from Chillemi *et al* [246]. Reproduced with permission. Copyright retained by Cold Spring Harbor Laboratory Press (2017).

Acetylation of p53 primarily occurs at the C-terminus and plays a major role in activating its function [239]. Lysines in the C-terminal domain that undergo ubiquitination can also undergo acetylation, and as these are mutually exclusive events, it is correct to say that acetylation blocks the ubiquitination and subsequent direction of p53 for proteolytic degradation [238]. The histone acetyltransferase p300 and CREB-binding protein (CBP) are capable of acetylating p53 at Lys-164, -305, -370, -372, -373, -381, -382 and -386 [247-250]. Human males-absent-on-the-first (hMOF) and tat-interactive protein of 60 kDa (TIP60) are also able to acetylate p53 at Lys-120, a lysine residue that has been implicated as being essential for the induction of apoptosis by p53 [251,252]. In fact, it has been shown that acetylation of eight lysine residues (Lys-120, -164, -370, -372, -373, -381, -382 and -386) is required for the disruption of the physical interaction between p53 and MDM2 at target gene promoters [253]. Certain deacetylases are able to remove these acetyl groups from p53, and one such example is SirT1, which is capable of deacetylating p53 at Lys-320 and Lys-382 [254,255].

Ubiquitination, unlike the phosphorylation and acetylation of p53, is primarily associated with its inactivation and degradation [256]. There are three main requirements for the ubiquitination of a protein; activation, conjugation and ligation of the ubiquitin to the target protein [257]. The enzymes that are responsible for this are known as E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme, and E3 ubiquitin-ligating enzyme with the E3 ubiquitin ligase acting as a "docking protein" to bind to substrate proteins and E2 [257]. In the case of p53, its ubiquitin ligase is mdm2, or in the human case, hdm2 [258]. Ubiquitination of p53 by mdm2 occurs at Lys-370, -372, -373, -381, -382 and -386, and this polyubiquitination results in the nuclear export of p53 and for it to be targeted for proteasomal degradation [259]. The ubiquitination of p53 does not appear to involve the conjugation of a ubiquitin tree to one residue, instead, it appears that mdm2 monoubiquitinates multiple residues [260]. Interestingly it appears that mdm2 preferentially targets p53 dimers, rather than tetramers, for ubiquitination leading to increased nuclear export and degradation [261]. There are a plethora deubiquitinating enzymes that can, directly or indirectly, remove ubiquitin from a target protein [262]. One such deubiquitinase found recently is USP3, which antagonises the mdm2-mediated ubiquitination of p53 and increases p53 stability [263].

The protein neural precursor cell expressed developmentally downregulated protein 8 (NEDD8) functions like ubiquitin in that, just like ubiquitin, it can be conjugated to p53. Additionally, just like with ubiquitin, mdm2 is capable of neddylating p53 at K370, K372 and K373 [264] and in fact, phosphorylation of mdm2 at Tyr-281 and Tyr-302 by *src* switches mdm2 from a ubiquitylating E3 ligase to a neddylating ubiquitin ligase [265,266]. Neddylation does not appear to induce changes in p53 localisation or stability, but neddylation by FBXO11 appears to inhibit p53 transcriptional activity [267]. There remains one final mechanism of control of p53 in the form of conjugation of small ubiquitin-like modifier (SUMO). Much like NEDD8 and ubiquitin, SUMO is conjugated to lysine residues and currently one main site at Lys-386 has been described for p53 [268]. The actual function

of sumoylation is rather confused with some studies pointing to promotion of p53 transcriptional activity, whilst other studies demonstrate increased cytoplasmic localisation of p53 upon sumoylation [269,270].

The final form of modification that p53 undergoes is methylation. One of the key regulations of p53 occurs due to arginine methylation at Arg-333, -335 and -337 [271]. Interestingly, Arg-335 and -337 were dimethylated, whereas there was only one methyl group transferred to Arg-333 and this is mediated by N-methyl transferases (PRMT) [271]. Type I and type II PRMTs can transfer methyl groups, and both types of PRMT can transfer either one or two methyl groups, but type I PRMTs add an asymmetric methyl group, whilst type II PRMTs add a symmetrical one [272]. Methylation at these residues is actually quite complex, as it is possible that the same methyltransferases capable of methylating p53 can actually lead to the downregulation of p53 [273,274]. This, however, looks to be very context-dependent, as methylation also appears to occur at these sites as a response to UV-induced DNA damage [271]. It is not just at the arginines that p53 undergoes methylation, once again the lysines are highly prone to modification, and in particular smyd2 at Lys-370, Set7/9 at Lys-372, and set 8 at Lys-382 are capable of methylating p53 [275-278]. Methylation at Lys-370 and Lys-382 lead to repression of p53 activity, whereas conversely methylation at Lys-372 assists the acetylation of p53 by Tip60 and prevents methylation of Lys-370 by smyd2 [275-279].

2.2.2 The function and regulation of p53

We have now a good idea of the genetics, the structure, and the posttranslational modifications of the p53 protein, yet we have little explored how p53 exerts its effect on the cell.

2.2.2.1 p53 as a transcription factor

Firstly, p53 is primarily a transcription factor that responds to a vast number of cellular stresses and can attempt to repair the damage, arrest the cell to stop it from dividing, or if the damage is too severe, induce apoptosis [280]. These responses are in response to oncogene activation, nutrient deprivation, DNA damage, ribonucleotide depletion, hypoxia, oxidative stress, and telomere shortening as detailed in fig 6. p53 has a number of downstream effectors and a few of the key ones will be summarised here.

One of the most well studied downstream targets upregulated by p53 is CDKN1A, otherwise known as p21/Waf1/Cip1. It was identified back in 1993 as a direct target of p53 [281]. This protein has many roles, with the most notable being its effect on the cell cycle and its inhibition of cyclin-dependent kinase complex [282]. p21 bind to cyclins directly through specific domains, termed DK and Cy motifs, present in the N-terminal domain of the protein [283]. It is also possible for p21 to regulate the cell cycle in a manner separate from its ability to inhibit cyclins by binding to proliferating cell nuclear antigen (PCNA) [284]. PCNA is a protein that plays a role in DNA repair and replication, and therefore the binding of p21 to

PCNA may interfere with its role in DNA synthesis [285]. p21 also plays a role in transcriptional regulation both in a positive and negative manner. By preventing phosphorylation of Rb-family proteins, p21 inactivates E2F-dependent transcription [286]. p21 can also function as a transcriptional cofactor that regulates proteins such as NF-κB, Myc, E2F, p300, STAT3 as well as oestrogen receptor family proteins [286-290]. Thus, one can see that p21 is a major effector of p53 through its myriad of functions.

p53 also plays a major role in maintaining genomic stability and repairing DNA. One such protein downstream of p53 is known as growth arrest and DNA damage 45 (gadd45) and it plays a role in global genomic repair [291]. Gadd45 works by recruiting nucleotide and/or base excision repair factors to gene specific loci as well as mediating DNA demethylation [292]. The maintenance of genomic stability derives from the fact that p53 is capable of regulating centrosome duplication [293].

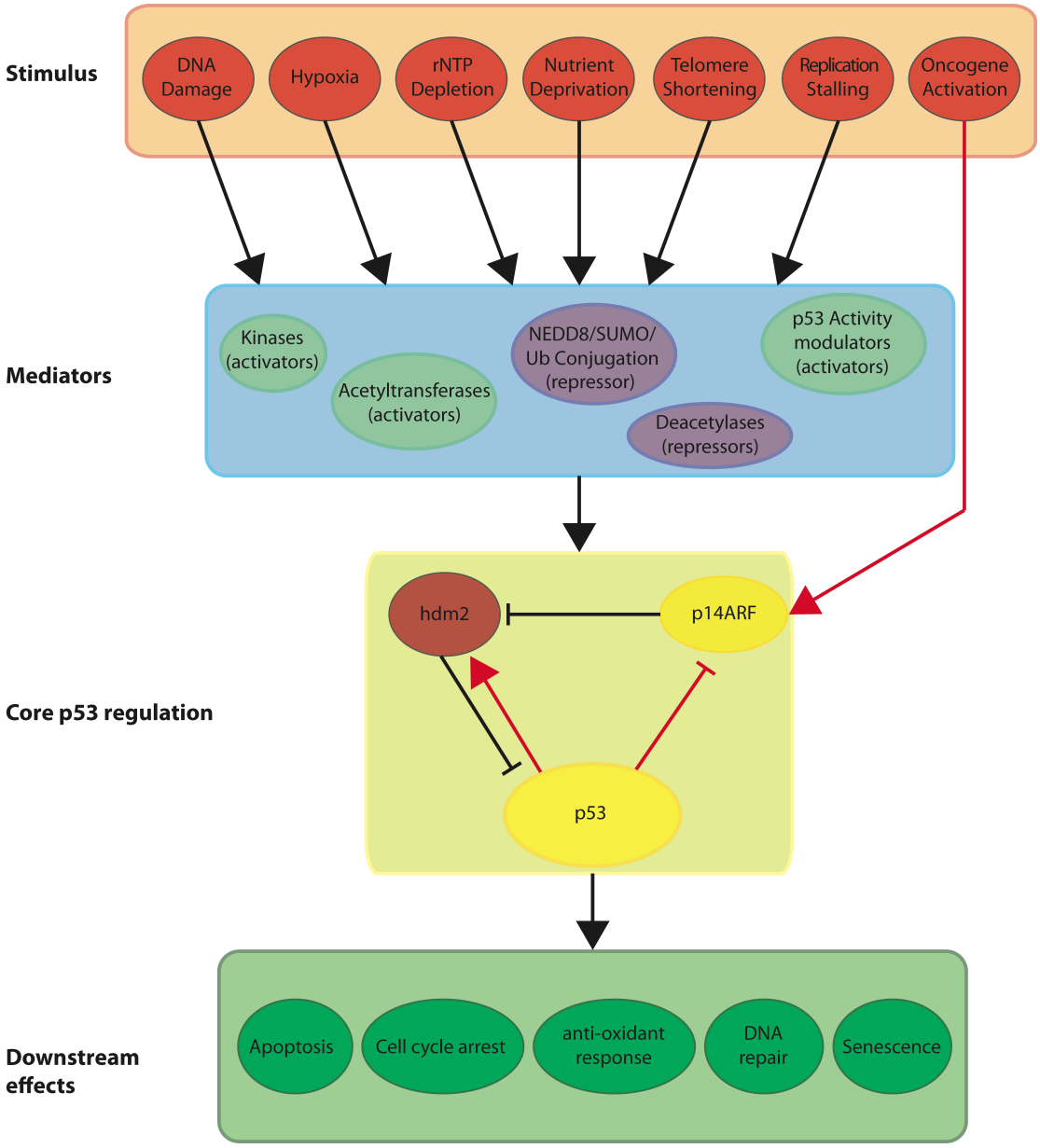

Fig 6. Stimuli, mediators, regulation and downstream effects of p53.

If DNA repair fails, one of the options left is to force the cell into apoptosis and p53 can mediate this through the expression of pro-apoptotic target genes. One such gene under direct control is *BAX*, whose product is an inhibitor of the pro-survival bcl-2 protein [294]. The binding of BAX to bcl-2 results in the release of cytochrome c from the mitochondria through the mitochondrial voltage-dependent anion channel (VDAC) [295]. Another pro-apoptotic gene under direct control of p53 is p53 upregulated modulator of apoptosis (*PUMA*). Like BAX, PUMA binds directly to bcl-2, localises to the mitochondria and induces the release of cytochrome c [296]. On an interesting aside, it is PUMA that is the downstream effector of p53 that impairs the formation of induced pluripotent stem cells iPSC [297]. One more interesting pro-apoptotic factor that is under direct control of p53 is the microRNA miR-34a [298]. miR-34a is thought to work by direct inhibition of bcl-2, SirT1, c-MYC and MET amongst others causing apoptosis or G1-arrest [299].

The antioxidant response is also one of the key pathways that p53 is capable of modulating. Rather than just activating a compensatory mechanism, p53 is capable of promoting an antioxidant response under normal conditions, but actually switches to a pro-oxidant response under severe stress to eliminate damaged cells [300]. One way in which p53 can reduce oxidative stress is by induction of TIGAR, which causes a shift in metabolism to the pentose phosphate pathway resulting in an increase in NADPH [301,302]. One of the key antioxidant factors, Nrf2 is also induced by p53 [303]. Nrf2 has a large number of downstream effects including upregulation of glutathione S-transferase, a key enzyme in the glutathione antioxidant pathway [304].

It is often said that too much of a good thing is deleterious, and indeed, p53 is its own worst enemy in this regard. The main negative regulator of p53, mdm2 is a direct transcriptional target of p53 [305,306]. Another regulator of p53, mdmx, also known as mdm4 or hdmx in humans, is a negative regulator by forming hetero-oligomers with mdm2 [307,308]. Mdmx is, however, not normally transcribed by p53, and even then it is only at its alternative promoter, resulting in an mdmx with a long N-terminus [309,310]. p53 also acts as a negative regulator of a protein called $p14^{ARF}$ [311]. $p14^{ARF}$ is able to complex with mdm2 and p53 complexes and stabilise them [311]. What happens after binding to mdm2 depends on the cellular background as in HeLa cells, $p14^{ARF}$ promotes mdm2 destabilisation due to expression of viral proteins [312], however, in non-virally infected cells, $p14^{ARF}$ leads to an increase in mdm2 levels [313].

We can see clearly that p53 plays a key role in a number of cellular responses to stresses that may, in fact, be deleterious to cells and allow for the development of cancer. So the key question now is, how does the “guardian of the genome” behave in cancer, and is there a way to exploit p53 therapeutically?

2.2.3 p53 and cancer

2.2.3.1 Inactivation and mutation of p53 in cancer

By some measures, p53 is the most mutated gene in human cancer [314] with approximately 50% of cancers possessing a p53 mutation [315], and particularly of note, inactivating mutations are highly common [316]. Unfortunately, p53 mutation has also been linked to poor prognosis for most cancers as well, making understanding its biology more important [317]. When p53 is not mutated in cancer, its activity is attenuated or ablated by upregulation of compensatory mechanisms including the upregulation of negative regulators such as mdm2 due to gene amplification or similar mechanisms [318-320], or the deletion or mutation of an upstream positive regulator such as $p14^{ARF}$ [321].

The p53 protein has a number of sites that are frequently mutated, known as "hotspot mutations". Nearly all of the most common mutations occur within the p53 DNA binding domain as shown in fig 7. The most frequent mutations are R175H, R248Q, R273H, R248W, R273C, R282W, G245S and R249S [322]. These eight mutations account for around 28% of all missense mutations in p53 [322]. Broadly, these mutations are divided into two categories, structural mutants that lose their intrinsic structure required for function, and contact mutants that are unable to bind to DNA [323]. Some of these mutants, such as the R175H mutant are completely defective for transactivation due to their unfolding under physiological pH, whereas R273H has normal protein conformation, but little capacity for transactivation [324,325].

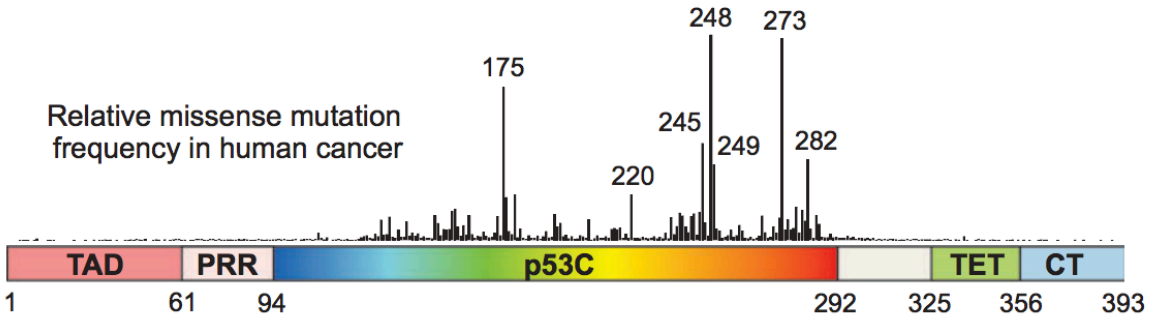

Fig 7. Sites of mutation across the p53 protein and their relative frequency. Diagram taken from Joerger and Fersht [214]. Reproduced with permission from Annual Reviews. All copyright (2008) retained by author and publisher.

Not all mutations of p53 are complete loss of function mutations, but one can also see gain of function mutations as was theorised back in 1993 [326]. Now, gain of function mutations have been encountered that lead to a number of highly deleterious effects such as increased invasion, altered cell migration, increased proliferation, drug resistance, avoidance of cell death, overcoming anoikis, increased colony formation, and increased genomic instability [323].

p53 mutation can also occur at the germline level. In the "classic" cases of Li Fraumeni syndrome, there are detectable p53 mutations in between 60–80% of the patients [327-329]. Li Fraumeni patients suffer early onset tumours with 82% of women developing a tumour before the age of 45, whilst men are less likely to develop cancer, but still demonstrate a highly increased risk relative to the general population with 27% of all carrier males developing a tumour before the age of 45 [330].

2.2.3.2 p53 therapy

There are two primary focuses for p53 therapies targeting cancer: restore wild-type p53 activity by overcoming the compensatory mechanisms forcing repression of its activities in tumours, or reactivate mutant p53 in tumours. As p53 is such a prolific responder to numerous cellular stresses as well as being modulated and regulated by so many factors there are a plethora of possible targets one could explore for p53 therapy. For the sake of brevity a few will be elucidated here.

One of the first strategies conceived was to modulate the binding of the negative regulator of p53 in tumours containing wild-type p53. The first reagent published that achieved this was a synthetic mdm2-binding mini protein made by a 12 amino acid peptide from the mdm2-binding domain of p53 into the *E.coli* thioredoxin gene [331]. This synthetic mini protein was known as SuperTIP. In an mdm2 binding assay, SuperTIP had an IC_{50} of 300 nM, which compared favourably to full-length p53, which had an IC_{50} of 400 nM in the same assay [331]. The idea of adding a "staple" using a hydrocarbon bridge across the helical domain that binds to mdm2 generated a lot of interest as this overcame the difficulty of using such a short peptide sequence that may not form a stable secondary structure [332]. These staples stabilised the α-helix of the p53 transactivation domain sequence responsible for binding to the hydrophobic cleft at the N-terminus of mdm2 [333]. The peptide sequences have been further refined to yield better binding to mdm2, and at the same time have demonstrated efficacy *in vivo* in tumour models and have reached phase I clinical trials [334-336].

In between the generation of SuperTIP and the advent of peptide stapling came a series of small-molecule antagonists of mdm2, named the nutlins of which nutlin 3 was the most potent inhibitor [337]. Nutlin 3 was interesting as it possessed a chiral centre and demonstrated enantiomer-specific effects, much like our DHODH inhibitor featured in **paper I** [337,338]. Nutlin 3 enantiomers are referred to as nutlin 3a (active) and nutlin 3b (less active). There have been a number of nutlin analogues synthesised that demonstrate improved activity as well as improved pharmacokinetics [339]. Roche has engaged in a phase III clinical trial of the nutlin analogue, idasanutlin for AML [340].

A further approach to reactivating wild-type p53 that has been explored is to increase the stability of the p53 protein by inhibiting proteins responsible for removing beneficial posttranslational modifications. One such strategy involves the inhibition of SirT1 and SirT2 [255,341]. A series of compounds targeting the sirtuins, named the tenovins, were used in **paper II** and **paper III** [342]. These compounds have demonstrated success in a number of

preclinical models, and most strikingly, were capable of eliminating CML and AML cancer stem cells upon combination regimens [343,344]. New inhibitors of SirT1 are being developed currently in an attempt to find a safe and efficacious compound to take to the clinic [345-347].

Two older therapies that deserve a brief mention for their novel mechanisms of action are actinomycin D (ActD) and leptomycin B (LeptoB). Actinomycin D inhibits RNA synthesis by binding to guanine residues in the DNA and preventing RNA polymerase I from catalysing transcription [348,349]. This causes double strand breaks. Low dose ActD (LDActD) overcame the DNA damage by inhibiting RNA polymerase II, which appears to induce the accumulation of p53, possibly due to inhibition of mdm2 caused by binding of free ribosomal proteins, such as L11, as these have been shown to be potent binders of mdm2 [350,351]. LeptoB works in a completely different way to ActD by inhibiting nuclear export of p53 [352]. LeptoB binds to the nuclear export mediator CRM1, which is responsible for the export of proteins carrying a leucine-rich nuclear export signal [353]. Mdm2 is thought to cause nuclear export of p53 through its ubiquitin ligase activity, therefore, inhibition of export of ubiquitinated p53 may be the mechanism by which leptomycin increases p53 transcriptional activity [354]. Both compounds have been tested together to attempt to synergistically increase p53 activity [355].

Mutant p53, unlike wild-type p53, is a much more difficult prospect to target. As covered previously in chapter 2.2.3.1, there are numerous p53 mutations. As also discussed, there are roughly two groups of mutations of p53 – structural or contact mutants and each lose transcriptional activity for markedly different reasons [323]. One such compound discovered to restore mutant p53 function is the small molecule PRIMA-1 [356]. PRIMA-1 is believed to reactivate mutant p53 by covalent modification of thiol groups in the mutant p53 DNA binding domain [357]. A methylated form of PRIMA-1, called APR-246, has now been developed and has made it to a phase I/IIa clinical trial [358]. APR-246 is converted to methylene quinuclidinone (MQ), which is a Michael acceptor capable of reacting with cysteines in the p53 core in the same way as PRIMA-1. Modification of Cys-124 and Cys-277 are required for the thermostabilisation of both R175H and R273H p53 mutants [359].

Another compound that was found to specifically target the R175H p53 mutant was NSC319726, a compound that became known as ZMC1 [360]. ZMC1 is believed to reactivate the R175H mutant by chelating zinc as this compound class are well-known iron, copper and zinc chelators [361]. It has been previously shown that altering zinc coordination can change the structure and function of p53 [223], and indeed in certain p53 mutants there is a defect in zinc binding, leading to a change in the conformation, and thus an altered transcriptional activity of the protein. By delivering zinc to the p53-R175H mutant, ZMC1 allows the mutant p53 to fold properly [362-364].

Taken together, it is clear there are a plethora of pathways one can utilise to drug the p53 pathway, and indeed in **paper III** we explore the idea that one can activate p53 through

different mechanisms and yet maintain the same phenotypic readout in terms of p53 transcriptional activity.

2.3 DRUG DESIGN AND DISCOVERY

2.3.1 What is a drug?

Throughout history humans have used exogenous compounds in an attempt to improve their condition. Some of the first recorded therapeutics date back to 2600 BCE in Mesopotamia, with a few therapies such as cedar, cypress, liquorice, myrrh and poppy juice still being in use today [365]. There were mentions of over 700 therapies in the Ebers papyrus from the ancient Egyptians, which was written in 1600 BCE demonstrating the widespread use of trial and error in finding therapeutics [366]. The Greeks also produced compendiums of medicine as well, one of which is still referenced to this day – *De Materia medica* by Dioscorides [367]. One of the oldest, most complete, and well-studied compendiums of medicine came from Chinese medicine, which forms a complimentary medical system in contemporary China alongside western medicine [368]. This form of medicine relies upon empirical evidence and holistic treatment through trials either on the practitioner of the art, or on a cohort of patients, and believed in healing based upon the five elements of Chinese medicine [368,369]. We should also hark back to the father of modern toxicology, Paracelsus, who theorised about the "doctrine of signature" – the idea that there was an active ingredient in every plant or herb to cure every disease [365,370]. It took a while more before a more rational and targeted approach came from Emil Fischer and his theory on the "lock and key" fit of exogenous therapeutics into a specific target within the body [371,372]. It is at this point we reach a modern definition of a therapeutic drug – a purified substance that interacts with one or few targets to elicit a therapeutically beneficial effect.

2.3.2 (Ir)rational drug design

Modern drug design is a highly multi-disciplinary field involving biologists, chemists, pharmacologists, and even legal professionals at the later stages. It also tends to be an incredibly labour intensive and expensive endeavour due to the various regulatory challenges in introducing a new therapeutic to the general public. The first stage, however, is finding a potential lead compound, as well as finding a target worthy of having a therapeutic developed for it.

2.3.2.1 History of compound screening for anti-cancer agents

In the past, for cancer drug discovery, models for screening involved simply conducting a study and obtaining a phenotypic readout such as the ability of a compound to induce tumour cell death [373]. This was first conducted using *in vivo* tumour cell models including murine sarcomas, leukaemias and a carcinoma model [374-377]. These studies, of course, relied upon treatment of a murine tumour, rather than human and therefore there was often a mismatch between the activity seen in the clinic and in the experimental model [378].

Therefore, the human tumour stem cell assay, or clonogenic assay, came about as a method to bridge the divide between preclinical models and the clinic [373]. This method involved the growth of fresh patient-derived tumour samples and their subsequent embedding in soft agar to test their ability to form colonies upon treatment with experimental compounds [379,380]. The first issue with this method was that it was slow, labour intensive and tumour supply was an issue as well, meaning not as many compounds could be tested at once. There was also another set of stumbling blocks. The assessment of pharmacokinetics of the experimental compounds, the differences in pH and pO_2 levels between *in vivo* and *in vitro* conditions, as well as the fact that the effect of the microenvironment and blood supply in the *in vivo* situation could not be assessed *in vitro* were significant drawbacks [373]. These same considerations also followed the new *in vitro* methods, which used tumour cell lines. The national cancer institute (NCI) shifted their strategy for screening compounds using a panel of 60 tumour cell lines with the sulforhodamine B assay [381]. This assay had the advantage of being able to screen large libraries of compounds against a large number of tumour cell lines, and was one of the first high-throughput methods available for assessment, causing it to supplant the clonogenic assay using human tumour samples as a first-line screen [373].

It was at this point that a viable *in vivo* screen was required to overcome the limitations of the high-throughput *in vitro* assays. One assay that was conceived was the hollow fibre assay [382]. This allowed for a two-compartmental consideration, and thus gave an indication of compound distribution kinetics as well as allow for easy implantation of tumour cells in biocompatible hollow fibres into an immunocompromised model animal [373]. The fibres are removed following compound treatment and tested using a tetrazolium dye to assess tumour cell viability [383]. This method allows for multiple cell lines to be tested within one animal prior to conducting the next assay – a tumour xenograft. Xenografts are normally composed of a culture of tumour cells, normally of a human origin, injected subcutaneously into the flank of an immunocompromised animal such as a nude mouse, or SCID mouse [384]. The advantage of utilising a xenograft model hinges on the fact that pharmacokinetics of a compound, or at least pharmacokinetics in a murine model, can be ascertained [384]. Therefore the ability of a compound to reduce the growth of a tumour derived from a human tumour cell line can be assessed in a model where ADME is a consideration [384]. The greatest consideration with these models is getting the correct dosing and tumour exposure to make it applicable to man. Two research groups independently found that clinically relevant dosing of xenograft models led to similar responses to those seen in patients in the clinic, suggesting that if the dose is correct, the response is likely to be seen to an equal extent in humans [385]. Pharmacokinetics and toxicokinetics between mice and humans, however, can vary markedly and clinical toxicities are often masked in preclinical *in vivo* models [386]. One recent and egregious example of this was the compound troglitazone, which passed all the preclinical toxicity models but caused idiosyncratic hepatotoxicity in humans [387,388].

Many of these older screening methods are still in use today, however, our toolbox for the discovery of novel therapeutics has grown markedly in the last 10 years, as have the strategies for drug discovery.

2.3.2.2 Modern screening strategies in brief

There are a few schools of thought on this topic and numerous ways in which one can begin. For an initial screen one could conduct *in silico* modelling and docking studies of a library of compounds into a particular target protein [389]. If one has a particular target in mind, one can also screen directly against the target in a biochemical assay using a preparation of purified protein. One way in which one could do this would be to use isolated protein and study on-off rates of a panel of compounds using high-throughput surface plasmon resonance [390]. If the target has an intrinsic enzymatic activity one could run a screen using a labelled substrate or a colourimetric assay with an indicator that changes during the enzymatic reaction [391,392].

This is, of course, well and good if one has a particular target in mind, but what if this is not the strategy, instead one starts with a phenotype and then works backwards to find the target that elicits that particular desired outcome in cells? Our laboratory has used one such technique that relies upon the use of a forward chemical genetic screen to examine a phenotype induced by screened compounds – the ability to activate p53 transcriptional activity [393-395]. This screen was used in **paper I** for the discovery of the HZ compounds and also for the discovery of the tenovins utilised in **papers II and III**. Using a phenotypic screen allows for a target-blind approach to drug discovery and throws up the potential to yield novel and interesting targets [396]. Not only this, but it also presents the opportunity to screen compounds in cells rather than in cell-free assays, bringing the screen one step closer to the situation *in vivo* [397-399]. Finally, this strategy shifts the focus from a single target focus to a multitarget approach [400]. This strategy, however, makes the identification of the actual target of your molecule very difficult and requires a lot of knowledge about the pathways involved [396,401]. The knowledge-based approach was utilised as part of **paper I** where we took advantage of the extensive literature and plethora of reagents available to dissect the p53 signalling pathway, and mechanisms by which p53 can be activated to narrow down the prospective target for the HZ compounds. The differences in the timelines between molecular target screening and phenotypic screening are summarised in fig 8.

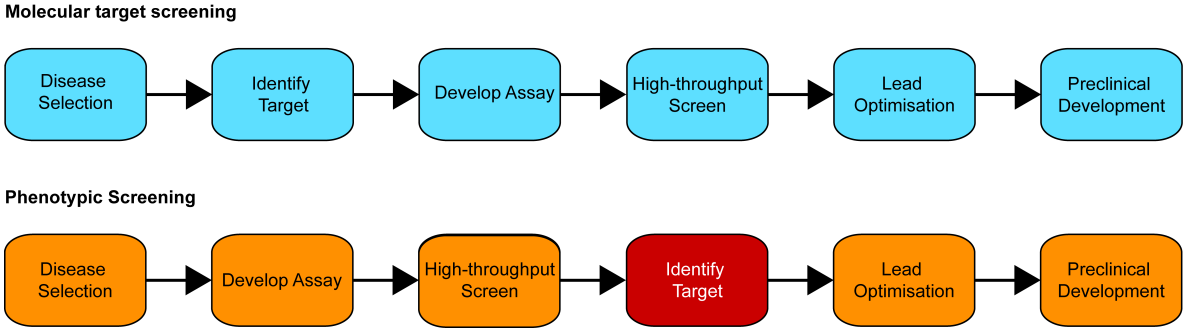

Fig 8. Differences between the timeline in screening against a known target (molecular target screening) and phenotypic screening. Diagram adapted from Zheng *et al* [396]. Reproduced with permission and all copyright (2013) retained by Elsevier.

There are a number of tools one can utilise to conduct target identification following the discovery of a lead compound from your initial phenotypic screen. One can conduct a biochemical assay, of which there are many. Affinity chromatography whereby one isolates ones compound on a column and then attempts to "capture" targets from cellular lysates is useful for "fishing" targets from a milieu [402,403]. This has been successfully used to identify numerous targets, however, it relies upon the target having a high affinity to the molecule in question, for the target to be present in relatively high abundance, for the target protein to be in the correct conformation, for the small molecule to be soluble in the buffers used, and for the washing steps to be appropriate so as to not lead to false positives [404]. One way to get around this problem has been to modify the lead compound with a chemical or UV-activated crosslinker followed by affinity purification [405,406]. Another innovative method that doesn't require compound immobilisation that is remarkable in its simplicity is based on the finding that proteins appear to be more resistant to proteolytic cleavage upon small-molecule binding [407].

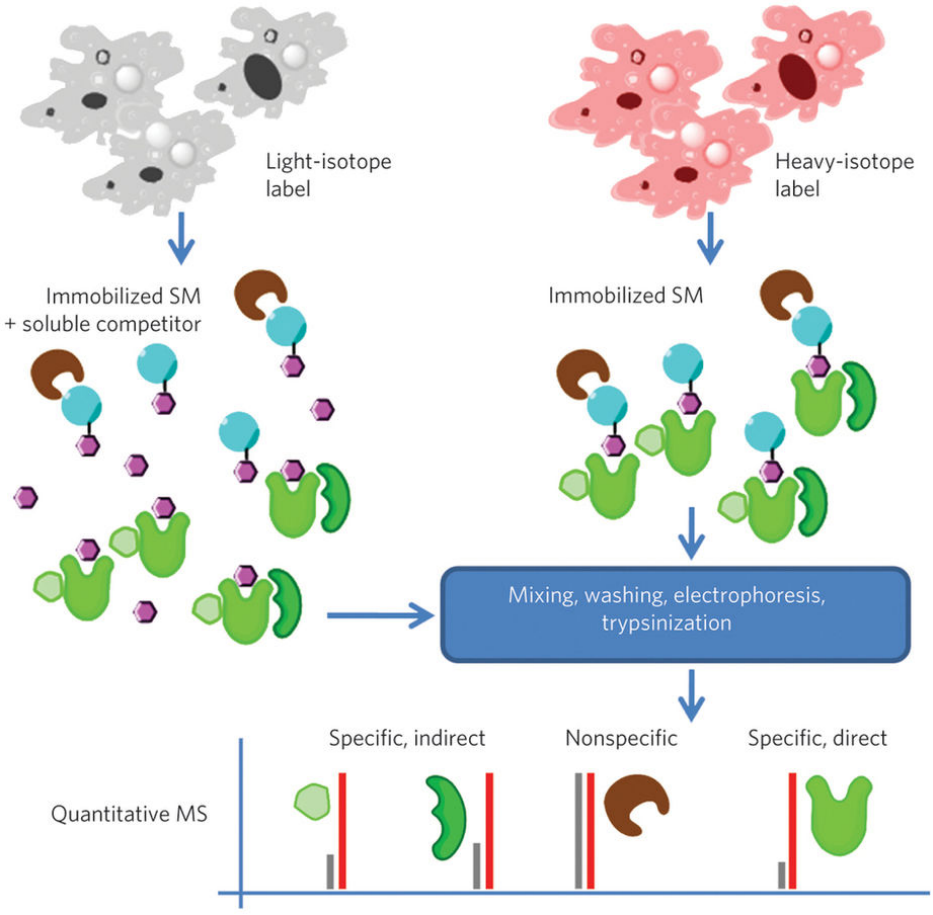

Fig 9. Diagram of SILAC labelling of cells taken from Schenone *et al* [404]. In brief, cells are labelled with either heavy or light isotopically labelled amino acids. One sample has a bead-immobilised small molecule (SM) added and the other has the same bead-immobilised SM added, but in the presence of a competitor. The samples are mixed, trypsinised and then the peptide fragments analysed by quantitative mass spectrometry. Diagram taken from [408] Copyright (2009) National Academy of Sciences and reproduced with permission.

With the rise of mass spectrometric techniques to study biological systems, target identification has become more global in its approach. In fact, by using metabolic labelling of the entire proteome using stable-isotope labelling by amino acids in cell culture (SILAC) one can potentially, using soluble competitor preincubation of lysates, discover multiple targets in one experiment [407]. Other labelling strategies for proteins include iTRAQ [409] and TMT [410], which are presently used for semi-quantitative proteomic experiments presently. See fig 9 for a diagram of SILAC as a target fishing strategy.

Another approach for target identification is to use linkage studies by examining similarity between a known small molecule inhibitor or RNAi [404,411]. Whilst these studies don't necessarily lead to finding the direct target, these studies may allow for the discovery of multiple targets as well as less abundant targets, or in the least provide a "fingerprint" of the particular class of compound studied. With the improvements in CRISPR-Cas9 technology, this can now be used in parallel with the existing RNAi library screens to provide a further target confirmatory measure [412].

One other method for both target confirmation as well as target elucidation is to take advantage of an intrinsic property of many proteins – their thermostabilisation upon binding a ligand [413,414]. This property can be exploited in the cellular thermal shift assay (CETSA) [415]. This assay relies upon an incubation of either a cellular lysate or whole cell with an experimental compound for a short time followed by a liberation of cellular contents, usually from snap freezing-thawing, heating across a temperature gradient and then centrifugal separation of the insoluble fraction [416]. The samples can then either be run on an SDS-PAGE gel and probed for the target of choice, or processed for bottom-up whole proteome profiling [416,417]. This technique was utilised in **paper II** to demonstrate SirT1 target interaction with various tenovin analogues.

2.3.2.3 Taking the molecule further and preclinical work

After these steps to ascertain the phenotype and target as detailed in fig 8, the next step in any good drug discovery project is to optimise the lead compound. This is the point at which pharmacology and being "drug-like" becomes important. The Lipinski rule of five formed the basis for this assessment [418]. These four rules that mark a compound as potentially not drug-like are as follows:

1. Greater than 5 H-bond donors.
2. Greater than 10 H-bond acceptors.
3. A molecular weight greater than 500
4. A calculated logP greater than 5

The eagle-eyed reader will note that though it is referred to as "the rule of five" there are in fact four rules. The origin of the name is due to the fact that each of the rules is a multiple of five. As with all "rules", these are more guidelines for likely success rather than being hard and fast laws, and in fact there are many successful therapies that do not conform to these rules, nor do most natural products [419-421]. The next consideration for a lead-like

compound is to not be one of the flagged pan-assay interference compounds (PAIN) or to possess any PAIN-like features (see fig 10) [422,423]. These compounds possess structures that often make them turn up in chemical screens due to their ability to cause off-target effects that may yield a positive hit [423].

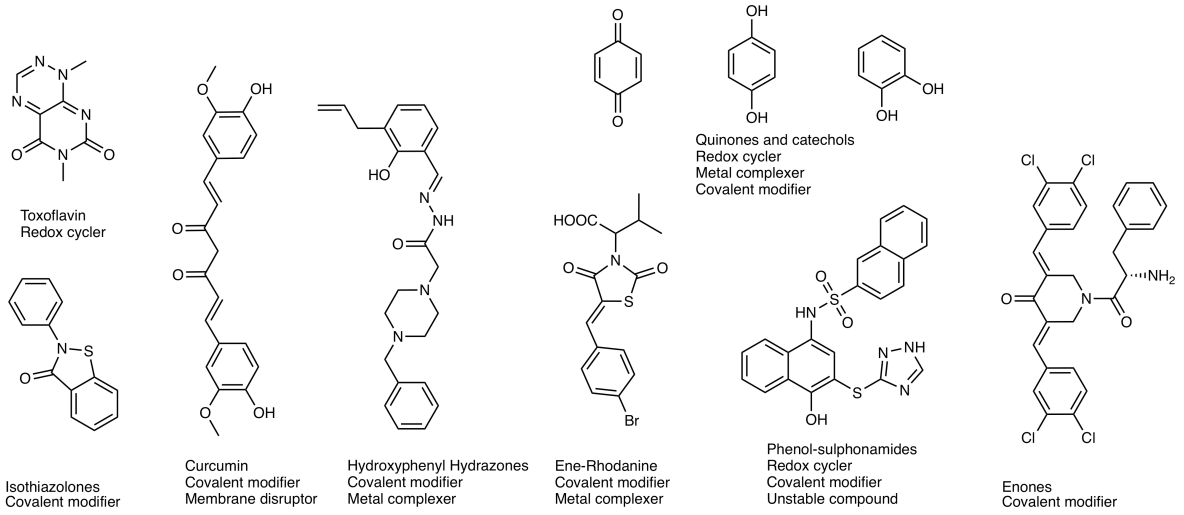

Fig 10. Some of the most common PAIN compounds that appear in screening assays. Reproduced with permission from Nature, with the figure adapted from Baell et al [424] All Copyright retained by author and publisher (2014).

Whilst there are a number of strategies on when to carry out various preclinical tests, there are a few "gold standard" tests that should be carried out for the compound to proceed to clinical trial [425]. One test that all compounds should go through is the aforementioned Ames test (see chapter 2.1.2.3) [98-100] as well as the micronucleus test to assist in ruling out a mutagenic effect [103]. Another essential test is to examine whether it is a hERG inhibitor. The human ether-à-go-go related gene (hERG) channel is an essential ion channel that transports potassium ions out of the cardiomyocytes and inhibition of this channel by a compound may cause long QT syndrome [426]. There are a number of assays to assess hERG inhibition, though the gold standard assay is the patch clamp [427-429]. There is also the possibility of running an *in silico* predictive model for hERG inhibition [430]. A further consideration is that of reproductive toxicity. Teratogenicity is notoriously difficult to test, though the OECD does offer guidelines for a one-generation reproductive toxicity test in model organisms [431,432]. There are a number of other tests including the limb bud culture assay, the hydra assay, FETAX and whole embryo culture [433-437]. There is hope, however, that new assays such as the zebrafish teratogenicity assay may simplify and streamline the workflow for testing teratogenicity in preclinical models [438]. Hand-in-hand with these assays is the necessity to conduct preclinical *in vivo* toxicity testing both single dose acute toxicity studies as well as chronic repeated dose toxicity studies usually in either mice or rats [439]. For these studies, endpoint histopathological analysis is conducted, primarily of the liver and kidney, though the brain, eyes and other organs can also be examined [439]. These preclinical studies are not perfect, however, and interspecies

differences can often miss toxicities with fatal consequences, as alluded to earlier with the example of troglitazone leading to idiosyncratic hepatotoxicity in humans [387,388].

Other factors that are highly important aside from toxicological considerations are pharmacokinetic and pharmacodynamic parameters. One of the biggest reasons, aside from unwanted toxicity, for drug attrition is a lack of efficacy, and this may stem from poor pharmacokinetic properties of the compound in question [440]. Thankfully, due to sufficient pharmacokinetic testing, this reason for attrition has dropped off markedly leading to only around 5% of drugs failing for this reason [441]. The first and easiest preclinical test to conduct is a simple liver microsome metabolism test to determine the half-life of the compound, that is the time it takes for the initial concentration of the compound to half [442]. Microsomes, vesicle-like artifacts formed from pieces of the endoplasmic reticulum following centrifugation at forces greater than 100 000 *g*, are prepared from a liver homogenate and contain the key phase I metabolic enzymes present in the liver [443]. This assay can be conducted using murine, rat and human microsomes to give an impression of the half-life in both the model organisms and in humans as there are often significant differences in the substrate specificity and rate of metabolism of the CYP450 enzymes between species [444,445]. After establishing the rate of metabolism, another consideration is the formation of dangerous metabolites or highly reactive intermediaries [446]. Much like with proteomics and target ID studies, mass spectrometry is the workhorse of metabolite identification studies [447]. These studies can be conducted using individually isolated CYP450 or UGTs, using microsomal incubations with NADPH and/or UDP-glucuronic acid to show oxidative metabolism or glucuronidation respectively, or incubations with cultured hepatocytes to determine conjugation reactions and reactive metabolite trapping [448]. For more specific metabolite trapping, one can add glutathione or cysteine to trap “soft” nucleophiles or cyanide for “hard” nucleophiles following microsomal incubation [449,450]. The most complex metabolism studies are from animal models, though they are also highly informative especially when one uses newer humanised or chimeric mice that possess human hepatocytes as they take into account distribution and excretion as well as the metabolism [451,452]. These studies are often conducted by taking the blood of the animal in question at regular time points following dosing. For a complete study, a metabolism cage can be used to also collect the urine and faeces to monitor excretion of the compound [453]. The next consideration for drug metabolism is whether the compound is capable of inhibiting or upregulating drug metabolising enzyme activity [454]. This is especially important when it comes to the possibility of drug-drug interactions and the risk this may pose to patients [455,456]. Inhibition of the pathway could have the effect of potentiating a second treatment that utilises that detoxification pathway leading to overdose, whereas activation of a metabolic pathway may lead to loss of efficacy of a second compound.

One can see that the drug discovery pipeline to the point at which preclinical tests of efficacy and toxicity are being conducted is a long and arduous road. Compound attrition due to lack of efficacy and/or toxicity has meant that many of the compounds found in early stages are unsuccessful. Often, during the journey of discovery, these failed compounds play the role of

biological tools to uncover new mechanisms by which to target disease, as well as perhaps highlighting potential new avenues to mitigate toxicities of current therapies. This makes these early drug discovery programmes vital both for the potential for creating new treatments, but also for increasing the understanding of basic biology.

2.4 NUCLEOTIDES AND URIDINE

2.4.1 The role of pyrimidines in the cell

The first pyrimidine was isolated by Italian scientist Brugnatelli (1761–1818) in the year of his death [457]. Pyrimidines are heterocyclic, six-membered, nitrogen-containing carbon ring structures with uracil, cytosine and thymine being the base structures of ribose-containing nucleosides [458]. Pyrimidines, along with purines, are the bases that form the fundamental structures for all RNA and DNA within the body. Pyrimidines have extensive biological roles beyond their role in RNA and DNA, as they are also responsible for polysaccharide and phospholipid synthesis, glucuronidation in detoxification pathways, and glycosylation of proteins and lipids [458].

Glucuronides are of particular interest as they are often the final phase of drug metabolism, and therefore a dearth of pyrimidines affects this particular pathway [459]. Many different chemicals, both endogenous and exogenous, are capable of accepting glucuronic acid, with the only feature being an appropriate functional group, normally R-OH, Ar-OH, R-NH_2, Ar-NH_2, R-COOH or Ar-COOH [460]. The conjugation reaction is mediated by the UDP-glucose glycosyltransferase superfamily, of which there are nine isozymes in humans [461]. There are glucuronide conjugates that play an incredibly important role in toxicology. The first class of reactive glucuronides are *N*-*O*-glucuronides of hydroxamic acids [462]. One such conjugate that is reactive towards cellular nucleophiles is the *N*-*O*-glucuronide of *N*-hydroxy-2-acetylaminofluorene [462]. A second class of reactive glucuronides are the acyl glucuronides, which exhibit electrophilic reactivity [463]. These conjugates have a much greater impact on the body, as many commonly used drugs, including NSAIDs like paracetamol, undergo conjugation in this manner and can be a key reason for unwanted toxicity [464].

The second system that is of interest is glycosylation of proteins and lipids. *O*-linked β-*N*-acetylglucosamine (*O*-GlcNAc) modifies many different nuclear and cytoplasmic proteins including p53 and c-Myc transcription factors [465-467]. An interesting observation in cancer is that due to the Warburg effect and a shift from oxidative phosphorylation to glycolysis, there is often an increased level of *O*-GlcNAc levels in cancer, and thus a subsequent increase in glycosylation of proteins [468]. In fact, it is possible that this increased glycosylation in tumours may even be linked to secondary consequences of the disease such as cachexia, or skeletal muscle wasting. Tumour-induced changes in muscular dystrophy–associated dystrophin glycoprotein complex (DSG), whereby proteins in the DSG are hyperglycosylated by tumour cells, have been implicated in cachexia [469].

2.4.2 DHODH and *de novo* pyrimidine synthesis

Unlike the *de novo* synthesis of purines, pyrimidines are not built from the ribose-phosphate, instead there is a series of five enzymes that synthesise UMP from bicarbonate, ATP and glutamine (fig 11) [470]. The first enzyme, aspartate transcarbamoylase, is the only enzyme committed solely to pyrimidine biosynthesis and is responsible for the formation of carbamoyl aspartate from aspartate and carbamoyl phosphate [471]. All steps of the *de novo* synthesis occur in the cytoplasm except the step between dihydroorotate and orotic acid, which is catalysed in the mitochondria [472]. The enzyme catalysing this reaction is dihydroorotate dehydrogenase (DHODH) [473]. The final product of the pathway shown in fig 11 is the molecule UMP, with the reaction from OMP to UMP catalysed by uridine monophosphate synthetase. This molecule is the key nucleotide from which all uridine nucleotide di- and triphosphates are formed by ATP-dependent kinases [474].

There are two different classes of DHODH, the class I and class II enzymes. The class I enzymes are a cytosolic family, whereas the class II enzymes are membrane-associated [475]. DHODH in mammals is a member of the class II DHODHs and located on the outer surface of the inner mitochondrial membrane [473]. As shown in fig 11, DHODH catalyses the stereospecific reaction of (*S*)-dihydroorotate to orotic acid, the only redox step in the entire pathway [476]. To accomplish this redox reaction DHODH relies on the presence of a cofactor – flavin mononucleotide (FMN), which is located in the core of the protein [476]. This cofactor requires regeneration to its oxidised form, and for this to occur, coenzyme Q10 enters the portion of the enzyme known as the quinone tunnel and undergoes reduction to ubiquinol before exiting the tunnel [476,477]. Therefore, this reaction catalysed by DHODH is not a simple one-step process, but a number of redox steps as shown in fig 12.

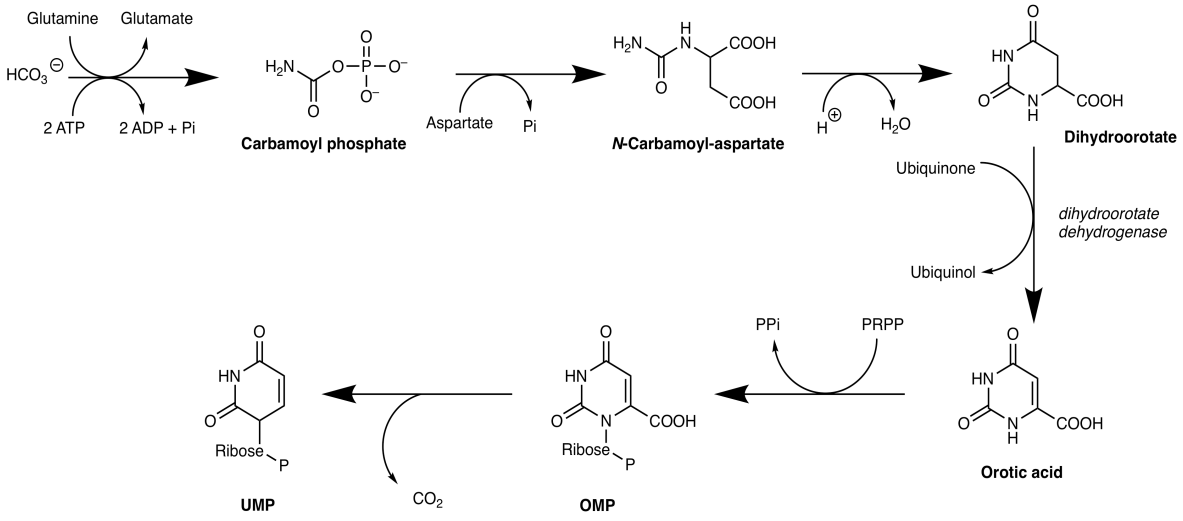

Fig 11. Reaction scheme of the *de novo* pyrimidine synthesis pathway in humans.

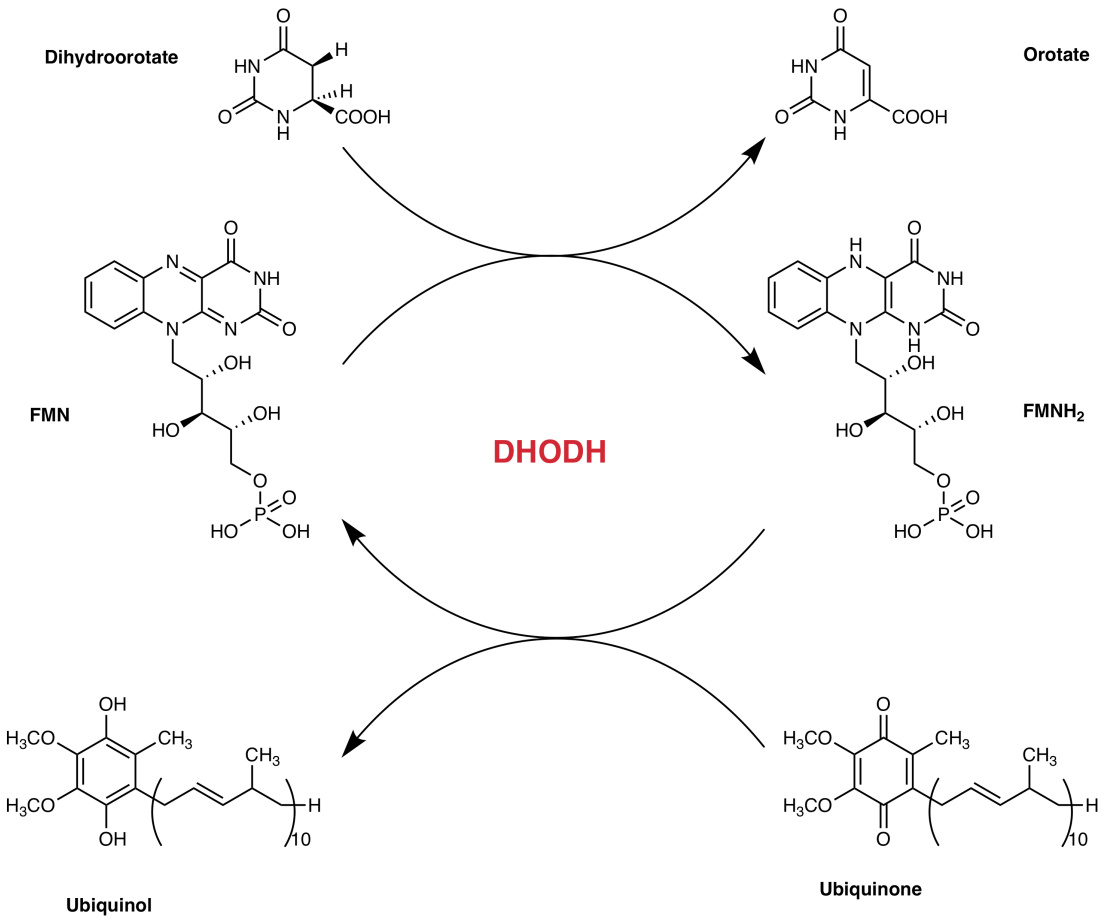

Fig 12. The class II DHODH redox reactions.

The structure of the enzyme itself is of interest. Firstly, the N-terminal of the human DHODH appears to contain a putative mitochondrial localisation signal, a transmembrane helix and two amphipathic helices [476,478,479]. Deletion of the cationic portion of the N-terminal sequence from the rat DHODH blocked its importation into mitochondria, but deletion of the hydrophobic region in the N-terminus led to importation into the mitochondrial matrix, suggesting that the dual signal of each portion of the enzyme is responsible for its correct cellular localisation in the mammalian cell [478]. Secondly, the enzyme has two distinct redox sites. The first is the site where FMN oxidises dihydroorotate to orotate, and the other site is where $FMNH_2$ is back-oxidised by ubiquinone [477]. The ubiquinone binding area, otherwise referred to as the distal redox site or quinone tunnel, is formed by the space between α1 and α2 in the hydrophobic portion of enzyme, allowing for the insertion of the hydrophobic ubiquinone with a number of polar sidechains located at the end of the tunnel (Gln-47, His-56, Thr-360 and Arg-136) [476]. This area of DHODH is the most important when considering inhibition of the enzyme as it is the site which the two most-published inhibitors, brequinar and terifluonomide (A77 1726) occupy [476], as well as being the site that the HZ compounds (**paper I**) and tenovins (**paper III**) bind.

2.4.3 Targeting DHODH for clinical benefit

There are currently a number of potential therapies targeting DHODH [476,480]. The first treatment that I will consider is the clinically-approved pro-drug, leflunomide.

2.4.3.1 Leflunomide and teriflunomide

Leflunomide is a small-molecule pro-drug that, upon metabolism, inhibits DHODH and has been approved for rheumatoid arthritis [481]. Teriflunomide, also known as A77 1726, is the active metabolite of leflunomide and has been approved for treatment of multiple sclerosis [482].

Firstly, a small background to rheumatoid arthritis (RA), which is an autoimmune disorder characterised by chronic inflammation. The disease involves a complex pathogenesis with contributions from the genotype of the patient, environmental triggers as well as presently unknown factors [483]. One aspect of the pathogenesis that is overwhelmingly clear is that a disturbance in the immune regulatory factors drives the disease [484]. It is thought that these disruptions takes the form of a perturbation in T-cell repertoire selection, antigen presentation, or alteration in peptide affinity, thus promoting the autoreactive adaptive immune response leading to the disease [483].

Multiple Sclerosis (MSC), much like rheumatoid arthritis, also appears to have an immune component to its pathogenesis [485]. The disease is characterised by chronic demyelination and immune cell infiltration into the central nervous system (CNS) [486]. Dysregulation of effector-suppressor cell interactions is thought to be the largest driver in the disease which leads to autoreactive adaptive immune cells infiltrating the CNS [486].

So, how do leflunomide/teriflunomide, lead to a clinical improvement in RA/MSC? To answer this question, we will first examine how leflunomide binds to DHODH. As leflunomide is a pro-drug, it requires metabolism prior to target interaction. Leflunomide is actually quite rapidly metabolised to its active metabolite, teriflunomide (A77 1726). This metabolism is both spontaneous in the small intestine and in plasma, but there is also evidence for first pass liver metabolism of the compound [487,488]. Teriflunomide has been shown to occupy the quinone tunnel, the site at which ubiquinone exchanges electrons with FMN [476]. This reversibly inhibits DHODH by preventing the back-oxidation of the FMN cofactor, thus preventing the redox reaction, the production of orotate, and therefore disrupting the *de novo* pyrimidine synthesis pathway [476].

Leflunomide is thought to inhibit the formation of specific antibodies, and in particular reduce circling levels of autoantibodies in animal models of autoimmune disease [489-491]. There is evidence that leflunomide accomplishes this by suppressing expansion of double negative T cells and inhibiting B cell activation. Interestingly, regulatory $CD4^{+}Foxp3^{+}$ T cells are increased markedly by teriflunomide treatment, as are inducible regulatory T cells ($CD4^{+}CD25^{+}Foxp3^{+}$) [492]. This may account for some of the activities of leflunomide/teriflunomide in autoimmune diseases, but the exact link between DHODH

inhibition and effects on the immune system is not well understood at present beyond reducing proliferation of activated T and B cells [482].

It is worth noting that leflunomide following meta-analysis has shown a clinically profound effect on RA, with 53% patients on leflunomide reporting an ACR20 (20% according to the American College of Rheumatology) as compared to 26% of placebo, and an ACR50 (50% improvement according to the American College of Rheumatology) in 34% of patients as compared to only 8% of placebo [493]. Teriflunomide, was, however, not quite as effective at reducing disease progression in multiple sclerosis and was not as effective as other therapies available, but still demonstrated efficacy and did slow disease progression [494].

Leflunomide/teriflunomide do have some caveats when considering them as therapies. Firstly, their activity is not just confined to DHODH inhibition. In fact, they appear to target epidermal growth factor receptor (EGFR) [495], $p56^{lck}$, $p59^{fyn}$ [496] and the Janus kinases Jak1 and Jak3 [497]. Whilst multiple targeting is not necessarily intrinsically bad as it may even contribute to the beneficial therapeutic outcome of leflunomide/teriflunomide, it does increase the risk of unwanted toxicities from a lack of specificity. Leflunomide also demonstrates interesting pharmacokinetics. Peak concentration of leflunomide is reached after around 6–12 hours [498]. In fact, the half-life of teriflunomide is around 2 weeks, with detection in plasma being possible up to two years following cessation of treatment without using a washout using the bile acid binding resin, cholestyramine [499,500].

2.4.3.2 Brequinar as an inhibitor of DHODH

Brequinar was first discovered as part of the NCI developmental therapeutics programme and given the name NSC 368390 / DuP-785 [501]. Brequinar demonstrated good *in vivo* activity against a number of different tumour cells *in vivo*, reducing tumour growth in murine models very successfully [501]. Given these promising results, brequinar was quickly selected for a phase I clinical trial. Through systematic experimentation it was found that the anti-tumour activity of brequinar could be prevented by addition of 1 mM of uridine or orotic acid, which through deductive reasoning assisted in the isolation of its target, DHODH [502,503]. Unfortunately the phase I clinical trials demonstrated dose-limiting toxicites for brequinar not limited to severe desquamative maculopapular dermatitis and thrombocytopoenia [504,505]. From the phase I studies, brequinar demonstrated a median half life of around 10 h and exhibited high levels of plasma protein binding (>98%), however the relationship between unbound and plasma bound fractions of brequinar and the patient pharmacokinetic data demonstrated that plasma protein binding was not a major determinant of brequinar disposition in cancer patients [506,507]. Three phase II clinical trials in cancer patients showed only a modest response, and not one that justified the dose-limiting side effects [508-512]. Therefore, brequinar was shelved as a therapeutic due to the narrow therapeutic window and only modest outcomes it appeared to exhibit [513].

But despite its failure as a therapeutic, brequinar proved to be a highly useful molecular tool. Brequinar was still a very potent inhibitor of DHODH though, with a reported IC_{50} of 10 nM

against the purified enzyme [502,514]. It also occupied the same compound binding site as leflunomide and exhibited more interactions with the protein, perhaps hinting at a tighter binding mode [476,480]. Thus, due to its potency, brequinar found its use in uncovering a novel biological response to DHODH inhibition. Inhibition of DHODH in acute myeloid leukaemia (AML) cells caused differentiation of these tumour cells [515]. As discussed above, depletion of pyrimidine nucleotides could be theorised to interfere with glycosylation [516]. Given certain proteins exhibit a gain of function or stabilisation upon glycosylation, it stands to reason that their lack of glycosylation due to nucleotide depletion may lead to a loss-of-function in a driver protein [516]. Therefore, it has been theorised that alterations in glycosylation allow the AML cells to overcome the differentiation blockade upon DHODH inhibition [515].

Targeting DHODH is a hot-topic at the moment, and multiple inhibitors are in development as it is seen as a relatively benign target with a promising therapeutic potential [517-519].

2.5 AUTOPHAGY AND CANCER

2.5.1 Self-ingestion and mechanisms of autophagy

> *"Of design he created thus; his own waste providing his own food, and all that he did or suffered taking place in and by himself."*
>
> *– "Description of Self-sufficiency" Plato, Timaeus (33c-)[520]*

Much like the representation of Oroborous, the snake eating its own tail from mythology, the word autophagy is derived from the Ancient Greek αὐτόφαγος (autóphagos), meaning "self-devouring" [521]. Autophagy is a process whereby the cell catabolises its own contents to rid itself of old proteins or organelles to provide the necessary building blocks to produce new proteins or organelles [522]. Autophagy is a highly conserved survival mechanism in all eukaryotic cells to cope with external stresses, such as nutrient deprivation, allowing cells to survive for a limited time during hardship [523]. Autophagy has three main forms: macroautophagy, microautophagy, and chaperone-mediated autophagy (fig 13) [524].

2.5.1.1 Macroautophagy

Macroautophagy is the major inducible pathway for cellular turnover of organelles and other cytoplasmic components [525]. The process can be summarised as starting with formation of double-membrane vesicles, termed autophagosomes, which gather up cytoplasmic contents and then fuse with lysosomes whereby lysosomal acidic hydrolases break down the contents into amino acids, free fatty acids and nucleic acids [524]. Each step of this process is under an exquisite control all the way from formation to fusion. The formation of the autophagosome occurs at what is known as the phagophore assembly site (PAS) on the endoplasmic reticulum, and is regulated by phosphatidylinositol 3-kinase (PI3K) [522,526]. Under normal conditions during formation, PI3K associates in a complex with Atg15, Ambra1, Vps15,

Vps34 and Beclin1 (Atg6) at the PAS, but requires a further phosphorylation of Beclin1 to proceed. The ULK1 (Atg1) complex regulates the phosphorylation of Beclin1, and this in turn is under regulatory control of AMPK and mTORC1 [527].

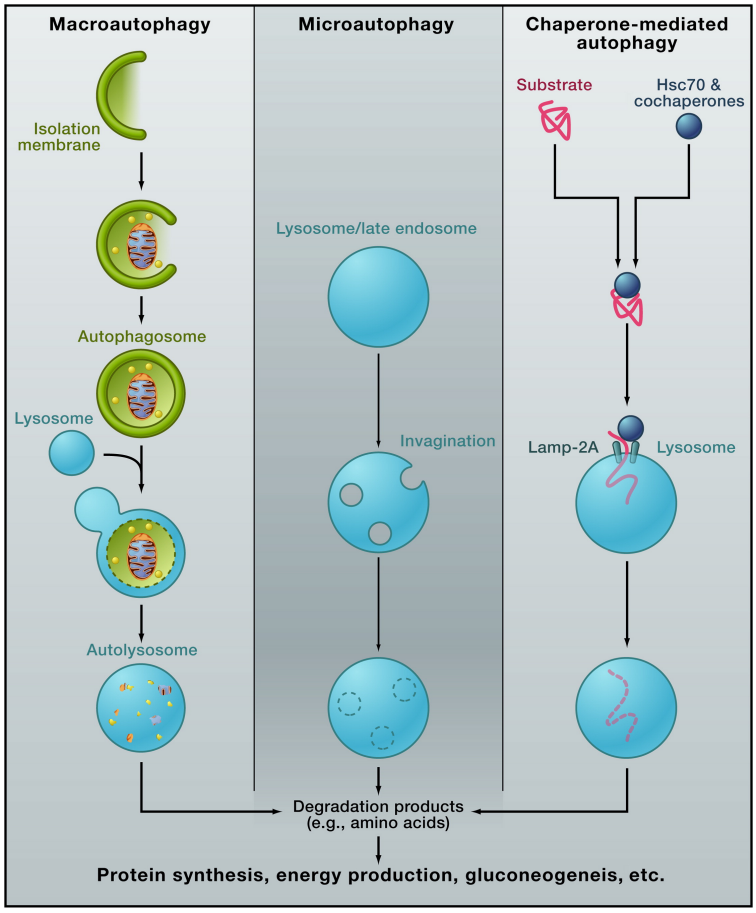

Fig 13. The three primary types of autophagy. Diagram taken from Mizushima and Komatsu [522]. Reprinted with permission from Elsevier with all Copyright retained (2011).

Under conditions of high glucose, mTORC1 becomes active and phosphorylates ULK1 at Ser-757, preventing the interaction with AMPK [524,528]. In low ATP conditions, AMPK is capable of phosphorylating ULK1 at Ser-317 and Ser-777 [528]. Upon phosphorylation of ULK1 at Ser-317 and Ser-777, the ULK1 complex, which consists of ULK1, Atg13, FIP200 and Atg101 primarily, is able to induce formation of the autophagasome after ULK1 phosphorylates FIP200 [524]. After activation, the ULK1 complex is able to phosphorylate Beclin1 at Ser-15 to activate the PI3K complex and start the formation of the autophagosome [529]. There are two key ubiquitin-like conjugation systems that play a role in the elongation step of autophagosome formation; the Atg5, Atg12, Atg7 complex and the Atg8, MAP-LC3, GABARAP, GATE-16 complex [524]. Next in the chain of events, LC3 is cleaved from MAP by Atg4, allowing for an exposed glycine at the C-terminus of the protein. Upon cleavage from MAP, LC3 is referred to as LC3-I [530]. It is Atg7 that, through its E1-like activity allows the E2-like Atg3 to transfer phosphatidylethanolamine to LC3-I to convert it to LC3-II [530]. LC3-II is then recruited to the phagophore by Atg9 [531].

The direction of substrates for degradation depends on two different mechanisms. One mechanism, the receptor-mediated process, relies upon the Atg8 interacting motif being placed on the surface of the target organelle [532,533]. The second mechanism is a ubiquitin-mediated process involving polyubiquitination of target proteins. Factors such as NBR1 and p62 bind to these and act as an adaptor protein to interact with LC3, which can ultimately lead to autophogosome recruitment to the target [534]. Fusion of the autophagosome with its cargo to the lysosome involve Ras-like GTPase Rab proteins, which traffic vesicles [535]. One other set of factors capable of reacting to different metabolic conditions in the cell are the sirtuins [536]. Glucose deprivation leads to increased expression of SirT1 and FoxO1 [537]. Not only is there a general increase in expression of both proteins, but SirT1 leads to deacetylation of FoxO1 allowing FoxO1 to increase expression of Rab7, one of the proteins responsible for autophagosome-lysosome fusion [537].

2.5.1.2 Microautophagy and Chaperone-mediated autophagy

Despite sharing a name with the autophagosome, these processes both do not use an autophagosome as a means of trafficking the cargo to lysosomes [524]. Microautophagy is not very well studied. It has been theorised that it works in a similar manner to endosomal sorting complex required for transport (ESCRT)-dependent multivesicular body (MVB) formation [538,539]. On the other hand, in chaperone-mediated autophagy a protein called heat shock cognate 71kDa protein (HSC70/HSPA8) mediates the selective degradation through endosomal microautophagy [538,540]. It turns out that a specific pentapeptide (KFERQ) is the motif that determines whether HSC70 can bind to that region [541,542]. A phosphorylation event within this motif at Ser, Thr or Tyr allows for binding to HSC70 and subsequent degradation [543-546].

2.5.2 Autophagy in cancer

The role of autophagy in tumourigenesis is often dichotomous as, depending on the context, autophagy can be both protective against the development of disease, but it can also lead to survival of tumour cells due to the pro-survival functions autophagy exhibits [547].

The primary function of autophagy is to sustain survival during nutrient stress [548]. But it isn't just sustenance that autophagy is important for, it's also vital for clearing defective or damaged organelles and macromolecules [549]. If autophagy is defective, the presence of defective organelles and macromolecules can cause oxidative stress, leading to DNA damage and chromatin instability [550]. Adding to the weight of evidence that defective autophagy can be tumour permissive, it has been found that Beclin1 and Atg6 is often deleted in breast, ovarian and prostate cancers [551-553]. Constitutive activation of PI3K-Akt-mTOR is a common characteristic of cancer and is known to suppress autophagy whilst promoting tumour cell growth, proliferation and survival through other downstream signalling in the mTOR pathway [554-556]. On the flip side, autophagy is also intrinsically linked to p53 through the protein damage-regulated modulator of autophagy (DRAM), which causes increased expression of Beclin1 as well as mediation of p53-induced apoptosis [557,558].

p53 also upregulates TIGAR, which perturbs glycolysis and lowers ROS, though the effect of this on autophagy is unclear [559]. Additionally, autophagy has been shown to elicit an antiviral effect through the Atg5-Atg12 conjugate [560].

Since autophagy occurs during cellular stress, and upregulation of autophagy can, depending on other cellular factors, allow cells to survive under conditions that could otherwise cause death, autophagy can allow tumour cells to survive selection pressures that could cause cells to otherwise die. These adverse conditions often result in the production of ROS, which can lead to protein and DNA damage [561]. In cells with defective apoptosis, autophagy allows for prolonged survival, and a potential to accumulate additional tumourigenic stress [562,563]. Autophagy also allows tumour cells to survive nutrient starvation that can often occur once solid tumours reach a particular size and poor vasculature leads to insufficient nutrients and oxygen in the hypoxic core [562]. One particular aspect whereby autophagy can lead to tumour survival is during tumour dormancy. This phenomenon is the method by which residual tumour cells survive in a dormant state following treatment or surgical resection, and this may in fact be mediated by autophagy [564-566].

Thus it can be seen, depending on the cellular context, autophagy can be either tumour permissive or tumour suppressive. During tumour initiation, evidence thus far suggests that autophagy may be tumour suppressive, however, following malignant transformation, autophagy is often upregulated as it helps cancer cells to survive in adverse conditions [560]. See fig 14 for a summary.

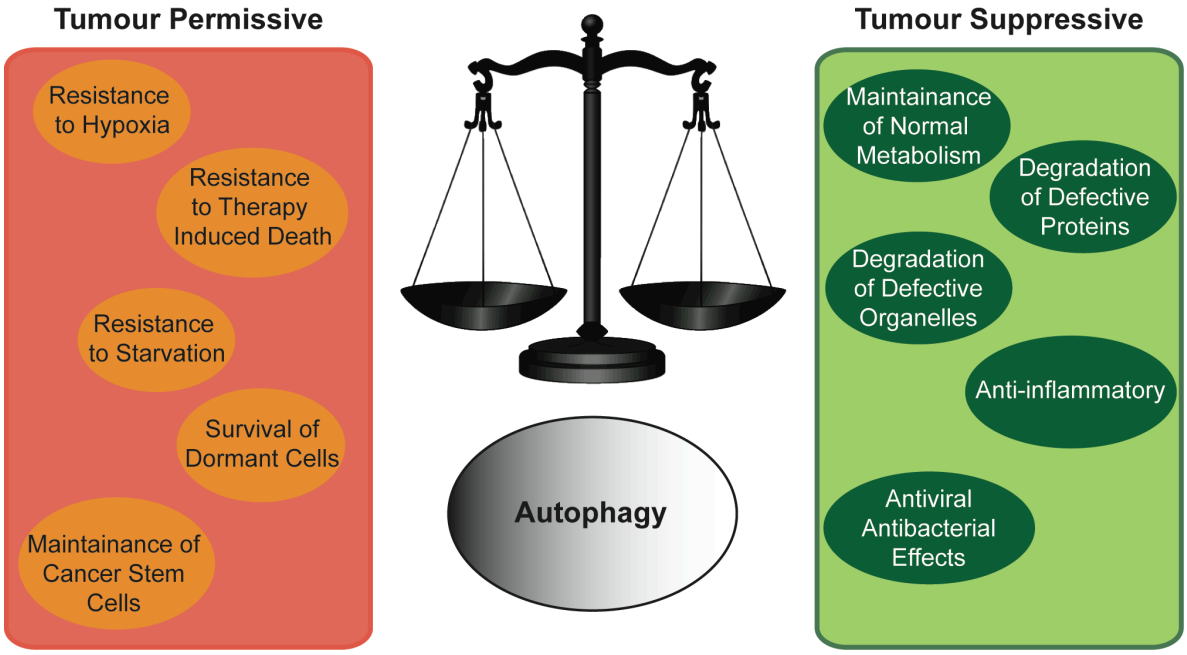

Fig 14. The delicate balancing act between tumour permissive and tumour suppressive functions of autophagy.

2.5.3 Modulation of autophagy using small molecules

As we have explored, autophagy exhibits a duality whereby it can be beneficial or deleterious depending on the cellular context. There are currently therapies that modulate autophagy in a positive and a negative manner.

2.5.3.1 Pharmacological upregulation of autophagy

One of the mechanisms by which one can induce autophagy is by blocking mTOR. One such compound capable of doing so is rapamycin, which mTOR (mammalian target of rapamycin) was named for as it was discovered when attempting to isolate the cellular target of rapamycin [567]. Another method by which to increase autophagy is to activate SirT1. The compound resveratrol has been shown to upregulate SirT1 and, by the mechanism outlined in chapter 2.5.1.1, induces autophagy [568]. Resveratrol is also capable of inducing AMPK, which can lead to inhibition of mTOR and induction of autophagy [569]. Metformin is a well-studied inhibitor of complex I in the mitochondrial respiratory chain [570]. Metformin has also been shown to induce AMPK, and can thus lead to upregulation of autophagy through this pathway [571]. In addition to this mechanism, metformin can also inhibit mTOR through two different pathways – REDD1 and by signalling through Rag GTPases [572,573].

2.5.3.2 Pharmacological downregulation of autophagy

Many inducers of autophagy act at the beginning of the pathway, however, two of the most well studied inhibitors prevent the final step in autophagy – fusion of the autophagosome to the lysosome to form the autolysosome [574]. Bafilomycin A1 (BafA1) is one such compound that acts on the lysosome, rather than the autophagosome [575]. BafA1 binds directly to one of the key proteins, vacuolar-type H^+-ATPase (V-ATPase) [576]. The inhibition of the V-ATPase causes an increase in the pH within the lysosome [575]. In concert with the blockage of V-ATPase, BafA1 has also been shown to inhibit Ca-P60A/SERCA-dependent fusion between the lysosome and the autophagosome, thus leading to a complete blockage of autophagic flux [576,577]. A second well-studied inhibitor of autophagic flux that acts in a similar manner to BafA1 is chloroquine (CQ). CQ is a weak base and is, therefore, sequestered in acidic organelles such as the lysosome [578]. This rise in pH within the lysosome prevents the fusion of the autophagosome to the lysosome, therefore blocking autophagy and leading to an accumulation of autophagosomes in the cell [579].

3 AIMS OF THE THESIS

The overall aim of this thesis was to elucidate mechanisms of action of small molecules found using a phenotypic screen for p53 transcriptional activity. There are two families of molecules studied as part of this thesis. The first family of molecules, the tenovins, were first discovered by Laín et al in 2008 [342], and the second family, the HZ series of compounds, were discovered in our laboratory and published in **paper I**.

The specific aims of each paper are as follows:

Paper I: Characterisation of the HZ compounds – novel activators of p53, and the elucidation of their mechanism of action. Following the elucidation of the target of the HZ series, we studied the therapeutic implications of DHODH inhibition.

Paper II: Uncovering the mechanism by which tenovins inhibit autophagy.

Paper III: Examining new targets of the tenovins and the multiple routes of p53 activation by tenovins despite differing in their target profile.

Paper IV: Studying lipid binding to intact DHODH by mass spectrometry and modelling the interaction between the protein and the inner mitochondrial membrane.

4 MATERIALS AND METHODS

All methods have been detailed in each respective paper. Whilst this is so, there is one more interesting technique I would like to draw attention to – the first is the use of imaging flow cytometry to quantify lysotracker staining that was included as part of **paper II**

Imaging flow cytometry is a relatively new technology. It has garnered attention due to combining the ability to localise signals within the cell that one can achieve with microscopy with single-cell analysis of a large population one can accomplish with flow cytometry. In **paper II**, imaging flow cytometry is used to analyse the levels of lysotracker seen in two different cell lines upon treatment with disruptors of autophagy. Lysotracker is an acidophilic dye that fluoresces at low pH. This means that it selectively localises to acidic cell compartments, primarily the lysosomes, and fluoresces under normal conditions. If the pH of the lysosome rises, then the fluorescence of the lysotracker dye is diminished, thus indicating a disruption in one of the pathways responsible for acidification of the lysosomes. The suggested use of lysotracker is to use it either for microscopy, or for flow cytometry, however in this paper we adapted it for the use in imaging flow cytometry. This allowed us to ensure that it was not a change in background fluorescence, but a change in the intensity of fluorescent puncta, which one can use as a proxy for lysosomes. See fig 15 for a workflow and result of this technique.

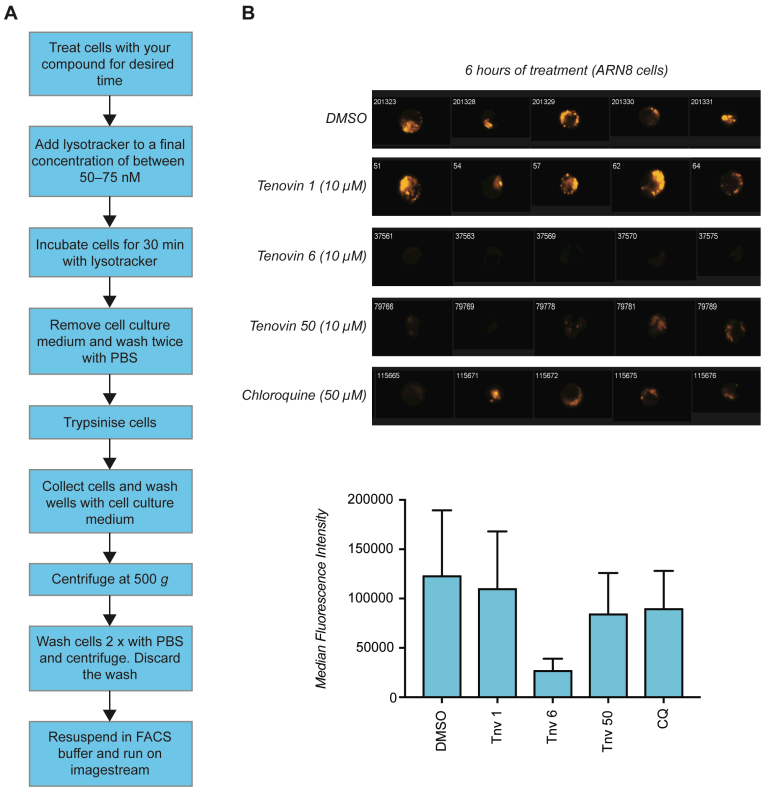

Fig 15. (A) Workflow for lysotracker imaging flow cytometry. (B) Example of preliminary results obtained for imaging flow cytometry during a pilot study for **Paper II** whereby cells were treated for 6 hours with compound before being processed and imaged. The median fluorescence intensity of each treatment was graphed below.

5 RESULTS AND DISCUSSION

5.1 DISCOVERY OF A NOVEL INHIBITOR OF DHODH (PAPER I)

5.1.1 Background

Discovery of novel therapies that activate p53 function in tumours containing wild-type p53 has been a focus of our laboratory since the first use of a phenotypic screen to identify a series of compounds called the tenovins [342]. This project used a modified version of the screen used previously in our drug discovery project and included a further selection criteria. Previously, murine fibroblasts were stably transfected with the pRGC-ΔFosLacZ to give the T22 cell line, with this cell line being used as the primary screen for the discovery of tenovins. This time, human melanoma A375 cells stably transfected with the same construct, yielding the ARN8 cell line, were used as another cell line in the screen. We were interested in compounds that would activate the transcription factor activity of p53 in the ARN8 tumour cells, but not in the T22 murine fibroblasts. 10,000 compounds from the DIVERSet and 10,000 additional compounds from the CombiSet libraries from ChemBridge were screened in both cell lines. Twenty compounds were shown to activate p53 in ARN8 melanoma cells >1.5-fold and did not activate or did so below 1.5-fold in T22 fibroblasts. One tetrahydroindazole that passed the screen was of particular interest from a medicinal chemistry standpoint; it was not planar, it did not possess any PAIN-like moieties [424], and it also possessed a chiral centre. We named this compound HZ00, a name derived from its key functional group (tetra**H**ydroinda**Z**ole).

5.1.2 HZ00 Mechanism of Action

Now that we had a compound of interest from the screen, it was important to see whether or not it would be a viable lead compound. First, we tested HZ00 in a large kinase panel and found no activity there, meaning that it was unlikely that HZ00 was a kinase inhibitor as well as demonstrating a non-promiscuous targeting profile for the molecule. Next we tested whether these compounds were activating p53 through DNA damage. We tested whether p-ATM, p-ATR, p-chk1, or p-ser-15 p53 were induced by HZ00, however we saw no evidence of induction of DNA damage pathways nor stabilisation of p53 due to phosphorylation at serine 15, a phosphorylation normally associated with DNA damage [580].

Now that HZ had displayed an apparent lack of genotoxicity, it was time to see whether it would be therapeutically viable. HZ00 displayed a decent therapeutic window when comparing the effect on cell viability using an SRB assay [381,581]. We also started digging deeper into the mechanism of p53 induction by HZ00 by comparing it to the known HDM2 binder, nutlin 3, which has been described previously in section 2.2.3.2. A thermal shift assay in cells, the CETSA [415-417], was conducted to compare the ability of HZ00 to thermally stabilise HMDX or HDM2. Unlike nutlin 3, which was capable of thermally stabilising HDM2 but not HDMX, HZ00 was unable to stabilise either protein. This suggested that

HZ00 did not act to prevent the interaction between p53 and its main binding partners. The final test in this series of characterisations was to see whether p53 protein was stabilised through another mechanism by HZ00. Cycloheximide is an inhibitor of protein synthesis, and thus makes it an ideal tool for studying protein degradation when used in a chase experiment [582,583]. To this end, we treated cells for six hours with HZ00 and then added cycloheximide to determine whether p53 protein degraded at a different rate compared to control. At this particular time point there was no evidence of stabilisation of p53 by HZ00. We also conducted a pulse-chase experiment using ^{35}S labelled methionine and cysteine to examine the incorporation of these labelled amino acids into newly synthesised protein [584]. This experiment demonstrated very clearly that HZ00 led to an increased incorporation of labelled amino acids into p53 after only five hours of treatment, whereas nutlin 3 led to no discernable increase in incorporation at this timepoint. Following a qRT-PCR experiment of cells treated with HZ00, it was also clear that p53 mRNA was only very marginally affected upon HZ00 treatment. This suggested to us that the possible reason for increased p53 is down to altered increased translation. After these investigations, it appeared that HZ00 was unable to interact with negative regulators of p53, was not demonstrated to increase p53 protein stability, nor did it lead to a rise in p53. Instead, HZ00 promoted an increase in p53 synthesis.

Prior to engaging in the long and arduous process of target identification, we carried out one final series of tests with HZ00. We formulated the hypothesis that combining HZ00, a compound that we found to increase p53 synthesis, with nutlin 3 a disruptor of p53/HDM2 binding, we could increase the levels of p53 in cells to a greater extent and perhaps tip the balance in favour of killing the tumour cells. Firstly, we showed by western blot that combining nutlin 3 with HZ00 increased total p53 levels. Secondly, the combination of nutlin 3 and HZ00 markedly increased the sub-G1 population of ARN8 cells in a propidium iodide flow cytometry experiment, whereas it induced a G1 arrest in HNDF cells. Third, we tested each enantiomer of HZ00 and found that only *R*-HZ00, but not *S*-HZ00 was capable of inducing p53 transcriptional activity, giving the compound an enantiomer-specific effect on cells. With this evidence in our pocket, we conducted a preliminary experiment in an ARN8 xenograft model in NOD/SCID mice. Whilst nutlin 3 and *R*-HZ00 were both capable of slowing tumour xenograft growth, the combination of both compounds was significantly more effective. These data strongly affirmed to us that HZ00 was a compound that was worth taking to the next, and most difficult stage of this drug development project – target elucidation.

During our initial studies with HZ00, we noted that in BrdU/PI bidimensional flow cytometry experiments that HZ00 resulted in an accumulation of cells in S-phase. This accumulation of cells in S-phase was, however, not an S-phase arrest, more a slowdown in progression through S-phase. We theorised this was linked to fewer origins of replication by cdc6 [585,586] as cdc6 protein expression is downregulated by HZ00. We began to speculate about factors that may lead to this reduction. One change within the cell that can lead to slowed replication is nucleotide depletion [587]. Concomitant with this change in cdc6 levels, general RNA levels are reduced with HZ00 treatment, hinting again at a scarcity of

ribonucleotides. More telling again is that depletion of the ribonucleotides GTP, CTP or UTP was reported to result in p53 activation and cell cycle arrest [588]. It was for these reasons that we began to add ribonucleosides to the cell culture medium of cells treated with HZ00. We noted that only one ribonucleoside recovered the viability of cells treated with HZ00, and that was uridine.

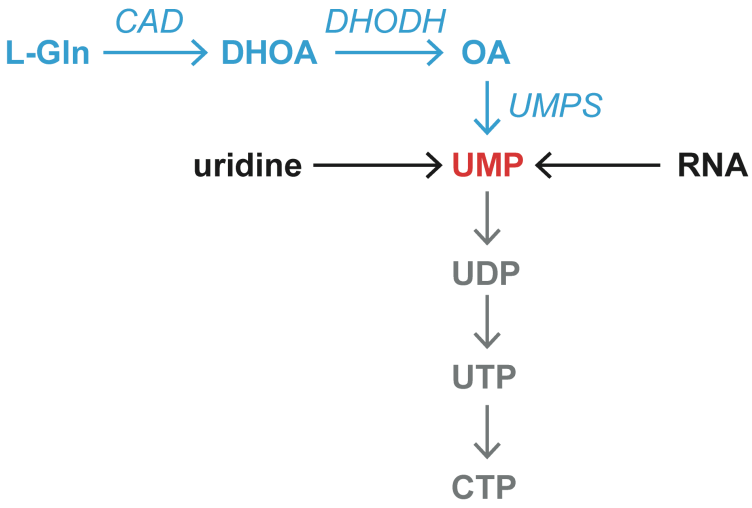

Fig 16. A schematic of the pyrimidine nucleotide synthesis pathway. The *de novo* pyrimidine pathway is denoted in light blue. The pyrimidine salvage pathway is denoted in black.

Finally, after this essential piece of the puzzle fell into place, and we knew we were most likely affecting the synthesis of pyrimidine nucleotides, we looked to see which enzymes made up the pyrimidine synthetic pathway (see fig 16). From supplementing with both the substrate and product of the enzymes we found that we were able to rescue cell viability of cells treated with HZ00 by the addition of orotate, but not dihydroorotate, giving us the strong indication that DHODH was our target. We then attempted to ablate p53 induction by the addition of uridine, orotate or dihydroorotate to HZ00 treated cells. Reassuringly we saw that induction of p53 transcriptional function was completely ablated by orotate and uridine, but not dihydroorotate, pointing us towards the conclusion that the depletion of pyrimidine ribonucleotides was the primary reason for the induction of p53. We then tested two known DHODH inhibitors, the highly potent inhibitor brequinar; and active metabolite of the clinically approved inhibitor leflunomide, teriflunomide [480]. Both of these compounds induced p53 and reduced ARN8 culture growth, and both compounds had their activity on cells reversed upon supplementation with either uridine or orotate. Following these phenotypic studies, we carried out a direct enzyme activity assay using purified DHODH. We found that (*R*)-HZ00, but not (*S*)-HZ00, was capable of inhibiting DHODH, thus confirming that the enantiomer-specific effects we noted earlier were mirrored in this assay.

5.1.3 The search for more potent HZ00 analogues

We now had a compound that had a known and interesting target, enantiomer-specific effects, good medicinal chemistry properties, specificity towards tumour cells in culture, activity in an *in vivo* xenograft model, and a novel way to induce levels of p53 protein. Therefore, we needed to improve one last feature of the compound series – potency. HZ00, whilst possessing many qualities of a lead compound exerted an effect on tumour cells only at higher concentrations. Therefore, we screened a number of HZ00 analogues that possessed similar medicinal chemistry properties as well as maintaining the key pharmacophore. We tested a number of analogues in the DHODH enzymatic activity assay and came across a compound that was very potent and that maintained the specificity between its two enantiomers. We named this compound HZ05. HZ05, much like HZ00, had its ability to induce p53 transcriptional activity completely ablated upon the addition of uridine and its effect on cell viability almost totally prevented by uridine addition except at doses over 20 times the IC_{50} for DHODH inhibition. To further elucidate the mechanism by which HZ05 inhibited DHODH, we incubated purified human DHODH with racemic HZ05. We obtained a crystal structure of (*R*)-HZ05 bound to DHODH within the quinone tunnel, further cementing the enantiomer-dependent specificity of HZ05.

It was at this point in the project that we decided to also return to the same compound library used in the Cancer Cell paper in 2008 and rescreen them in ARN8 cells and also conduct DHODH assays on all compounds in both screens as we had noticed discrepancies in the ability to activate p53-dependent transcription between the screen in the murine T22 and ARN8 [342]. Here we found that DHODH is a remarkably frequent target for compounds able to increase p53 transcriptional activity, and further identified 12 other chemotypes as DHODH inhibitors.

5.1.4 Novel therapeutic mechanism behind HZ compounds

We had observed previously that HZ00 was capable of increasing the proportion of ARN8 melanoma cells in S-phase. We found that HZ05 was also able to increase the proportion of ARN8 cells in S-phase upon treatment. We also found that HZ05 synergised with nutlin 3a, the active enantiomer of nutlin 3, to kill ARN8 cells further demonstrating that this synergy is a feature preserved through the compound series. We noted that S-phase accumulation also occurred in a number of tumour cell lines, but not HNDF cells. U2OS cells appeared to be particularly susceptible to S-phase accumulation upon HZ05 treatment, but only after 72 hours. Interestingly, we noted that p53 levels were raised in U2OS cells that had slipped into S-phase. Further to this, we wondered if the U2OS cells needed to accumulate in S-phase with p53 for nutlin 3 to be effective. Therefore we tested co-treatment of HZ05 and nutlin 3a against a 72h pre-treatment with HZ05 followed by co-treatment with HZ05 and nutlin 3a. We noted in the pre-treatment condition that HZ05 and nutlin 3a cooperated to kill the U2OS cells, however, co-treatment resulted in an ablation of killing and an arrested phenotype. To

this end, we proposed the final model shown in fig 17 whereby cells that have been treated with an inhibitor of DHODH slip through into S-phase and accumulate there with elevated p53 levels. If an inhibitor of p53 degradation, such as nutlin 3a, is added on top of this situation, we propose that this acts to cause p53 to switch from inducing cell cycle arrest to promoting tumour cell death.

Fig 17. A diagram of our proposed model for the synergy seen between DHODH inhibition and an inhibitor of p53 degradation.

5.2 EXPLORING THE TARGETING PROFILE OF THE TENOVINS (PAPERS II & III).

5.2.1 Background

Prior to the discovery of the HZ compounds, in the distant past of 2008, and as described in section 5.1.1, a compound screen was conducted using murine fibroblasts stably transfected with the pRGC-ΔFosLacZ construct to give the T22 p53 reporter cell line [394,395]. A 30,000 compound screen of drug-like molecules from the chembridge DIVERSet yielded the tenovins, or more specifically a compound that came to be known as tenovin 1 [342]. Tenovin 1 was capable of raising p53-dependent transcriptional activity in the T22 cells and raised p53 levels in MCF7 cells within 2 hours of treatment [342]. Tenovin 1 had problems with its aqueous solubility, limiting it in terms of testing in biochemical assays. For this reason, new analogues of tenovin 1 were synthesised and tested, with tenovin 6 coming out as the compound that maintained the ability to induce p53 as well as being markedly more water-soluble [342]. Now that there was a water-soluble tenovin, it was time to conduct a yeast genetic screen to attempt to pick out strains sensitive to tenovins. It turned out that SIR2 was identified as a potential target [342]. The human homologue for SIR2 actually consists of a series of NAD^+-dependent class III histone deacetylases and/or ribosyl transferases named the sirtuins [589,590]. From using biochemical assays for sirtuin enzyme activity, it appeared that tenovin was capable of inhibiting both SirT1 and SirT2 with an IC_{50} of 21 μM and 10 μM respectively [342]. Following on, the generation of further tenovin analogues by our group and our collaborators has focused on either improving medicinal chemical properties [591,592], or examining their targeting profile [593]. One peculiar finding did arise in the last few years – that tenovin 6 perturbs autophagy [594,595]. This finding opened up a research

avenue for us as we were in possession of a bank of tenovin analogues to attempt to pinpoint the mechanism by which tenovins perturbed autophagy.

5.2.2 Accumulation of LC3B-II upon treatment with tenovins (paper II)

One of the first things we noted when we commenced this study was that the ability to cause alterations in autophagy was not universal for all tenovins. It immediately appeared to us to be a structural change, but not necessarily a targeting profile change, that resulted in a switch in the ability to perturb autophagy. Following previous studies, we had expanded our library of tenovin analogues to use (see fig 18).

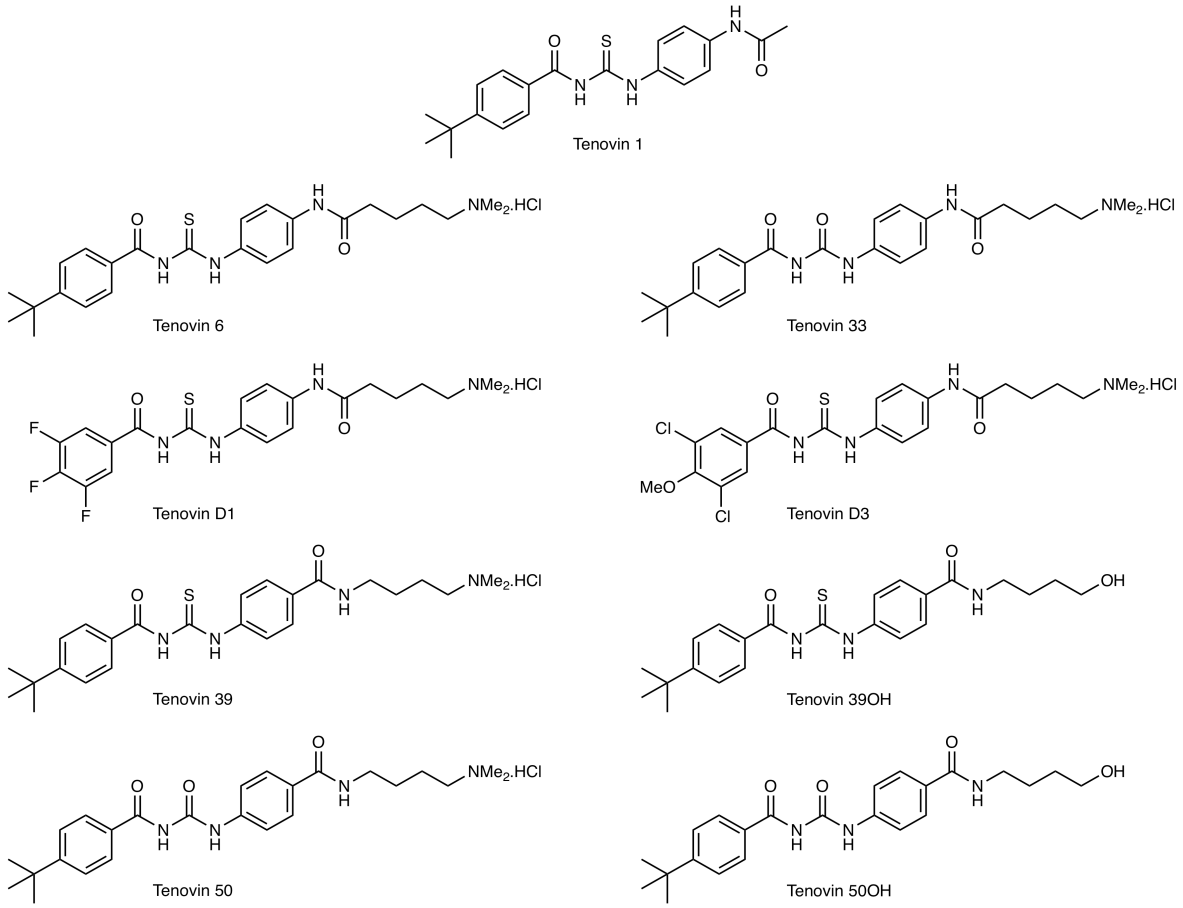

Fig 18. Analogues of tenovin 1 synthesised by our laboratory or our collaborators.

To commence in a more rational manner, we first examined the ability of each tenovin in our library to affect tumour cell growth in culture. First by looking at tenovins 6, D1 and D3 we noted that all tenovins were capable of killing tumour cells. This was in spite of the fact that tenovin D3 only inhibits SirT2 and tenovin D1 was incapable of inhibiting either SirT1 or SirT2. Therefore, their ability to kill tumour cells was independent of their ability to inhibit sirtuins. Next, we noted that between tenovin 50 and tenovin 50OH that tenovin 50 was capable of reducing tumour cell growth, but tenovin 50OH was completely unable to affect tumour cell growth. These studies looking at tumour cell viability upon tenovin treatment

pointed to two factors that would become important later. Firstly, that without sirtuin inhibition tenovins are still able to reduce tumour cell growth; and secondly, that within two very related compounds activity can be lost by substituting the tertiary amine at the end of the aliphatic chain of the tenovin.

We then decided to dissect the effects of each compound on tumour cells and fibroblasts. One way in which we did this was look at the cell cycle of these cells. Thereupon we noted greater discrepancies between the tenovins. What we noticed was that, at high doses, tenovins that lacked a tertiary amine at the end of an aliphatic chain (tenovin 1, 39OH and 50OH) resulted in a mixed killing and cytostatic effect on tumour cells, whereas on the HNDF cells, these tenovins were more cytostatic in nature. In contrast, the tenovins with a tertiary amine (tenovin 6, 33, 39, 39OH, 50) were showed a mixed cytostatic/killing effect on both cell lines.

To further understand whether the ability to perturb autophagy was the reason for this difference we saw, we examined one of the key markers for autophagosomes, LC3 (summarised in detail in section 2.5.1). We noted that, in a dose dependent manner, there was an increase in LC3B-II upon treatment with tenovins in possession of a tertiary amine, but not those lacking a tertiary amine. This was evident in both a p53 wild-type cell line (ARN8) and also a p53 mutant cell line (MDA-MB468). The reason for the use of a p53 mutant cell line hinges on the role that p53 plays on the induction of autophagy through DRAM [557], and therefore if we saw effects on autophagy in a p53 mutant line, it would be highly unlikely that the effects on autophagy were due to p53. To also confirm the type of perturbation of autophagy, we conducted a co-treatment of either tenovin 39, 39OH, 50 or 50OH with and without a saturating dose of chloroquine, a well-studied blocker of autophagic flux [596-599]. This experiment showed quite categorically that there was no change upon combination of any tenovin tested with chloroquine; and that the tenovins unable to induce an increase in LC3B levels (tenovin 39OH and 50OH) did not prevent the induction of autophagy. This strongly hinted that it was autophagic flux that was blocked by the tenovins rather than them causing induction of autophagy [526,599,600]. It was also further evidence that, unlike previously thought, SirT1 inhibition was not linked to the effects of tenovins on autophagy. Therefore, this is very much in agreement with a study in 2017 that showed that the effect of tenovin 6 on autophagy was not linked to SirT1 inhibition [601].

Having established that it was blockage of autophagic flux that tenovins with a tertiary amine achieved, it was time to investigate the mechanism by which these tenovins blocked autophagy. There were two theories – did it act on the V-ATPase that maintains the pH gradient of lysosomes in a similar manner to bafilomycin A1 [575,576,599], or did it raise the pH of lysosomes in a similar manner to chloroquine [597,599]? Firstly, we had to test whether the pH of the lysosomes was altered upon tenovin administration, as this would occur in both mechanisms. We set about achieving this using lysotracker, a lysosomotropic dye that fluoresces at low pH, ergo if we were to raise the pH, the fluorescence would be lost. Interestingly, all tenovins that were capable of increasing levels of LC3B-II were capable of

reducing lysotracker staining assessed by both light microscopy and by imaging flow cytometry. This confirmed that the tenovins were most likely acting on the lysosomes in a similar manner to chloroquine or bafilomycin A1. It is at this point we descend into the hypothetical when separating the mechanism by which lysosomal pH is raised. One point that counts in favour of the V-ATPase theory is that in the initial yeast genetic screen highlighted VCX1; a gene encoding vcx1p, a vacuolar H^+/Ca^{2+} exchanger that carries out a similar function to the V-ATPase responsible for lysosomal acidification in humans, conferred hypersensitivity to tenovin 6. One point that counts against this theory is that hypersensitivity does not necessarily equal target interaction with tenovin 6. In fact, one would imagine that if tenovins were able to accumulate in the lysosomes or the yeast lysosome-like vacuole, a loss of vcx1p would actually potentiate the basification of that vacuole due to the loss of the concentrative transporter, and one would see almost a synergistic effect on the yeast from this knockout. Another point that disfavours this theory is that tenovins with a tertiary amine are weakly basic. Chloroquine and ammonium chloride, two well-studied inhibitors of autophagic flux, accumulate in acidic compartments as they, too, are weakly basic. The protonation of these weak bases alters their membrane permeability, thus trapping them in the lysosome to a greater degree than would be predicted from pH partitioning theory [602-604]. It is for this reason I favour the theory that tenovins work in a similar manner to chloroquine and ammonium chloride.

A final consideration for this particular project is examining the contribution of the blockage of autophagic flux on the therapeutic application of the tenovins. Firstly, it was very evident that tenovins without a tertiary amine exerted a far milder effect on non-tumour cells at high doses. However, this also translated to a milder effect on tumour cells. By carrying out clonogenic regrowth assays it was evident that tenovins lacking a tertiary amine were unable to eliminate tumour cells despite initial impressions from the SRB assay used for the initial viability assays. This is in stark contrast to tenovins in possession of a tertiary amine which, universally, were able to eliminate tumour cells in culture. These data suggested strongly that to eliminate tumour cells with the tenovins one needs to block autophagy as well as inhibit the sirtuins. As a final therapeutic application, tenovin 50 was combined with vemurafenib to demonstrate that even melanoma cells arrested in G1 by vemurafenib could be eliminated by addition of tenovin 50. This revealed that even arrested or quiescent tumour cells may be eliminated by blocking autophagic flux.

5.2.3 The “real” targets of the tenovins (paper III)

Since their first description in 2008 [342], the tenovins have subsequently been revealed to also target autophagy, most likely through their basic moiety at the end of the aliphatic chain as described in section 5.2.1. With our recent work examining DHODH inhibitors, we noted that the tenovins possessed many of the phenotypes we saw with the HZ compounds. To this end, we decided to test whether the tenovins were capable of inhibiting DHODH enzyme activity in a simple biochemical assay. We were highly surprised to note that a number of

tenovins, namely tenovin 1, tenovin 6 and tenovin 39OH were capable of inhibiting DHODH. We then conducted a thermal shift assay to see whether the tenovins could stabilise DHODH across a temperature range, and indeed the same tenovins that were capable of inhibiting enzyme activity also stabilised DHODH in the thermal shift assay. It was following these biochemical assays that we decided to use tenovin 6, as it is more water-soluble than tenovin 1, to attempt to obtain a crystal structure of a tenovin bound to DHODH. DHODH did indeed co-crystalise with tenovin 6, and tenovin 6 was found to bind in the same quinone tunnel that HZ05, teriflunomide and brequinar inhabit [338,475,476]. This quinone tunnel is so named as it is where the electron donor coenzyme Q10 binds to transfer electrons to the FMN to allow the conversion of dihydroorotic acid to orotic acid, therefore occupying this site prevents electron transfer to FMN and halts the reaction [475].

It was at this point that we realised that there was most likely a component of DHODH inhibition to the mechanism of action of the tenovins – especially for the most potent inhibitors of DHODH, tenovin 1 and tenovin 6. We, therefore, conducted some phenotypic assays using supplementation with uridine and orotate to recover the effects of DHODH inhibition. When it came to p53 transcriptional activation by tenovins, tenovin 1 and tenovin 33 had their ability to induce p53 completely ablated by addition of orotate and uridine. Even tenovin 6 demonstrated an ablation at low doses ($\leq$ 2.5 μM) and had the maximal activation of p53 transcriptional activity all the way up to 10 μM. No other tenovins responded at all to supplementation. The next step was to ascertain whether this supplementation with orotate or uridine could rescue any effect that the tenovins may exert on the cells. Interestingly, only tenovin 1 and tenovin 33 exhibited a recovery in cell viability until high doses ($>$ 5 μM). The effect of tenovin 6 on viability was not recovered, showing that the inhibitory effect on DHODH is masked by the effect of tenovin 6 on its other targets. No other tenovins demonstrated any recovery at all upon supplementation.

As a consequence of our previous findings, we engaged our collaborators to model the interaction between DHODH and the tenovins to see whether the results from the enzymatic and phenotypic experiments could be explained by the interactions between the protein and compound. Using the crystal structure of tenovin 6 interacting with DHODH as a template, the interactions of a panel of tenovins with DHODH were modelled. These models favoured binding of tenovins 1, 6 and 39OH with the other tenovins being disfavoured, thereby confirming what we saw with our biochemical and phenotypic studies. Another important consideration that is be touched upon in **paper IV** in more detail is the interaction of DHODH with the inner mitochondrial membrane. As DHODH is a peripheral membrane protein that possesses a transmembrane domain that anchors it to the inner mitochondrial membrane with the quinone tunnel sitting directly over the membrane itself, there is a distinct possibility that there are interactions with the charged polar lipid heads as well as the hydrophobic membrane interior itself for any compound binding to DHODH. This is essential as coenzyme Q10, the cofactor for the DHODH reaction, is freely available in the lipid bilayer of the membrane [605]. The Q10, therefore, has to enter the quinone tunnel from the lipid membrane, and must have favourable conditions to do so due to its hydrophobicity

[605]. In the case of many tenovins, they possess an aliphatic tertiary amine and therefore may interact with the membrane. Therefore, using molecular dynamic simulations of DHODH interacting with the lipid bilayer obtained during **paper IV** as a reference, our collaborators modelled the interaction of DHODH with our compound and the membrane. This confirmed that the aliphatic chain of tenovin 6 protruded from DHODH and interacted with the phospholipid layer of the inner mitochondrial membrane serving to increase its binding affinity. The substitution of the terminal tertiary amine for a hydroxyl group led to less favourable interactions with the membrane.

Further to their effect on DHODH, the tenovins also yielded another striking secondary target – the inhibition of uridine uptake from the extracellular environment. This is of particular importance given an extracellular pool of uridine can either partially or wholly rescue the phenotype arising from DHODH depending on its concentration. Therefore, one way to potentiate a therapy targeting DHODH is to also block uridine uptake. There are two different types of transporters that are responsible for maintaining uridine levels in cells – the equilibrative nucleoside transporters (ENT) and the concentrative nucleoside transporters (CNT) [606,607]. Using ^{3}H-labelled uridine, we tested to see whether the tenovins were capable of inhibiting its uptake into cells after only 15 minutes of incubation with tenovins. It appeared that tenovins 6, 33, 39, 39OH and 50 were capable of blocking uridine uptake to some degree after this short period of incubation. Furthermore, when we tested whether the effect of tenovins on uridine uptake was persistent after 24 hours of incubation, we found that the effect was stronger upon longer periods of incubation, and was dose-dependent. We also found that none of the tenovins tested affected protein levels of either ENT1 or ENT2 after 24 hours. These findings were particularly interesting as it seemed that there was no relationship between the ability to activate p53 or inhibit DHODH with the ability to block uridine uptake.

This study is particularly important for a number of reasons. Firstly, it highlights that the main target of certain tenovins may in fact be DHODH, as this is the protein that tenovin 1 and 6 display the lowest IC_{50} for out of all the currently identified targets of these molecules. Secondly, that p53 activation by the tenovins may occur via multiple mechanisms depending on their targeting profile. Finally, that a one-atom change in the structure of a small molecule can completely switch the targeting profile of the molecule without changing the phenotypic readout. If we were to judge the tenovins on their ability to activate p53 transcriptional activity alone using the CPRG assay and a reporter cell line, we would believe that there were few differences between each of the analogues, thus highlighting once again the plethora of mechanisms by which p53s activity can be modulated in cells.

5.3 EXPLORING THE BINDING PARTNERS OF DHODH IN THE GAS PHASE AND ITS INTERACTION WITH MEMBRANE LIPIDS (PAPER IV)

5.3.1 Background

Studying the interaction between peripheral membrane proteins and the lipids of the membrane holds great interest for drug development, as unlike soluble proteins in the cytosol,

the membrane can intrinsically affect the structure of the protein [608]. This structural difference between the membrane-anchored and free protein can affect the binding of compounds to the protein in question and thus lead to discrepancies between free protein *in vitro* activity assays and the true biological situation. Thus, further research into the structural properties of membrane-associated proteins holds an intrinsic value in a number of fields. Non-denaturing nano-electrospray ionisation mass spectrometry (nESI-MS) has been previously used to study integral membrane protein complexes [609,610], and therefore the extrapolation of this technique to study peripheral membrane proteins is explored in **paper IV**. By marrying an experimental mass spectrometry technique with molecular dynamic modelling to examine the characteristics of the peripheral membrane protein, DHODH, **paper IV** sought to better understand the determinants of its structure when associated with the inner mitochondrial membrane.

5.3.2 Making protein complexes fly in a vacuum

One of the most important considerations in studying intact proteins in the gas phase is maintaining their native structure after what is a comparatively harsh process to transfer sufficient charge to a protein to allow it to fly through the mass spectrometer. Electrospray ionisation, unlike techniques such as electron impact, is a softer ionisation technique that imparts charge onto liquid leaving a capillary needle causing it to aerosolise. For large macromolecules like intact proteins, there is a well-defined model for the impartation of charge onto the analyte through a process called the charge residue model [611], though this model is primarily theoretical as it is not wholly proven at present as to how charge is imparted.

For these studies a DHODH lacking the transmembrane domain, but which still retains enzymatic activity as well as the requirement for detergent to be present for the enzyme to be soluble was employed. The requirement for detergent was highly important as it demonstrated that the hydrophobic, membrane-interacting portion of the enzyme was intact in the truncated protein. The choice of detergent for this study was pivotal, and importantly, thanks to studies on transmembrane/integral membrane proteins, the detergent lauryldimethylamine oxide (LDAO) was the first choice as it is MS compatible. The next step was to attempt to spray the protein, ionise it and introduce it to the MS. Upon using settings appropriate for soluble proteins, the proteins sprayed with a broad charge state, however, it was possible to isolate a high m/z range that corresponded to the molecular weight of the enzyme + the bound cofactor FMN. It was at this point we conducted ion mobility mass spectrometry (IM-MS) to examine the cross sectional area of both the holo- and apo-DHODH. Firstly, it was clear that the holo-DHODH corresponded to a compact conformation, whereas the apo-DHODH appeared be unfolded. This was highly important as this suggests that folded DHODH with FMN present can be preserved in the gas phase.

5.3.3 The association of lipids and drugs with DHODH

The next stage of this study was examining the effect of lipids present in the human mitochondrial membrane by analysing their interactions in the gas phase. The three lipids, phosphatidyl ethanolamine (PE), phosphatidyl choline (PC) and cardiolipin (CDL) were added individually to DHODH and an enzyme activity assay was conducted. It showed that the reaction rate increased between 30–60% relative to the detergent solubilised protein alone in the presence of the lipids. Further to this, MS studies examining the interaction of DHODH with the lipids to see which lipids bind more favourably. With PE and CDL, one can see a clear association, whereas complexes with PC was not as easily detected. The fact that there are only a few lipids associated with DHODH demonstrated that the association must involve only a small portion of the protein, confirming the peripheral nature of DHODH. Even addition of up to 360 μM PE only demonstrated a small number of additional lipid adducts. Interestingly lipid binding was found to be resistant to DMSO-induced protein unfolding, suggesting that the interaction sites are located outside the globular domain of DHODH.

Another highly interesting result came when the DHODH inhibitor, brequinar, was incubated with the enzyme and then analysed by nESI-MS. Firstly, only minor peaks of the apo-DHODH with the inhibitor were noted, suggesting that the intact protein-cofactor complex is required for brequinar to bind, and thus confirming that the binding of brequinar to DHODH is not random, but requires a properly structured DHODH protein, and/or that the interaction between brequinar and DHODH is fairly weak and more easily disrupted in the gas phase with the unfolded protein. Previously obtained crystal structures of a brequinar analogue (PDB: 1D3G) the inhibitor sits within the quinone tunnel, a tunnel created by two helicies that form part of the membrane binding domain [476]. We performed MD simulations of solvated ligand-free DHODH in the absence of lipids and detergents, which demonstrated a large degree of conformational freedom in the membrane-binding domain. This region was more stable upon ligand binding.

Following these experiments, we conducted MD simulations using both the full length and the truncated protein associated with a model PE bilayer. Pulling the protein towards the centre of the membrane whilst monitoring the opposing force exerted allowed for the positioning of the protein on the membrane. This positioning study confirmed once again that any insertion resulted in unfavourable energetics, thus suggesting that the protein itself sits loosely on the surface of the lipid bilayer. Leading on from this finding, we conducted further MD simulations on the full-length and truncated forms of the protein. It was found that the soluble domain of the protein forms additional contacts with the membrane and that this was largely unaffected by the presence of the transmembrane helix. What the transmembrane helix appears to do, however, is anchor the protein to the membrane to prevent the complete detachment of the membrane-binding domain from the bilayer. Finally, and most importantly for the function of the enzyme, the interactions with the lipids of the bilayer appears to shape the configuration of the membrane-binding domain, thus allowing insertion of coenzyme-Q10 into the quinone tunnel of DHODH. This structure is highly conserved across species including in bacteria and in human complex I. We theorise this orientation to the membrane

prevents the interference of oxygen in the quinone tunnel that would otherwise interfere with the interaction between coenzyme Q10 and FMN and the resultant reduction of Q10 required for enzymatic activity.

This paper elucidates a number of important features of DHODH using a novel strategy that could be applied when studying peripheral membrane proteins. Firstly, we established that it is possible to find parameters for a peripheral membrane protein to be studied by nESI-MS in its intact and folded form. Secondly, we established that lipid binding to DHODH affects its enzymatic activity and that the lipid interaction is limited even in highly saturated conditions, as would be expected from a peripheral membrane protein. Thirdly, we were able to show that brequinar, an inhibitor of DHODH, was capable of binding to only the holo form of the enzyme, and not the apo, suggesting that the interaction is weak and preferential to the folded protein over the unfolded protein. Finally, we demonstrated using MD simulations the orientation of DHODH on the enzyme and the way in which its orientation facilitates the interaction between the nested FMN and CoQ10.

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