EXPLORING TLR ADAPTOR PROTEIN MYDD88 AS A SENSITIVITY DETERMINANT TO HDAC INHIBITORS

Thesis submitted for the degree of

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By

Mina Elisha Bekheet

Lady Margaret Hall

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Declaration

All of the work in this thesis has been carried out entirely by myself and no part of my thesis has been accepted or is currently being submitted for any other degree or diploma in this university or elsewhere. My research was carried out under the supervision of Professor Nicholas La Thangue, Laboratory of Cancer Biology, Department of Oncology, Medical Sciences Division, University of Oxford.

Mina Elisha Bekheet

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Carcinogenesis has shown to be a result of aberrant genetic as well as epigenetic processes. Histone Deacetylase (HDAC) inhibitors, a class of therapeutic agents developed against the latter, have been shown to be efficient against certain haematological malignancies. However, the underlying molecular mechanisms are still largely unknown. An unbiased loss-of-function screen was used to identify genes that govern cellular sensitivity to HDAC inhibitors (HDIs). Myeloid differentiation primary response 88 (MyD88), a Toll-like receptor (TLR) adaptor protein, was identified as a key regulator of the anti-proliferative effects of HDIs, whereas levels of MyD88 were found to govern cells response to HDI treatment through regulating cytokines levels. Furthermore, MyD88 L265P, its most abundant loss-of-function mutation, was associated with increased cell sensitivity to HDIs. It was also found that MyD88 acetylation status contributed to its regulation via an HDAC6-dependant interaction. These results suggested that through scrutinising the underlaying molecular pathways that become altered during tumourigenesis there could be important relative prognostic and diagnostic values for cancer treatment in clinical settings; whereas levels of MyD88, its acetylation status, or the expression of its L265P mutant; could represent a valuable set of indicators to identify subsets of patients who are more likely to respond positively to HDI treatment.

TABLE OF CONTENTS

CHAPTER 1

GENERAL INTRODUCTION 18
1.1 Personalised cancer medicine. 19
1.2 Epigenetics in tumourigenesis. 22
1.2.1 Chromatin remodelling and transcription control. 24
1.2.2 Methylation. 27
1.2.3 Histone acetylation and cancer. 29
1.3 Histone Deacetylases. 33
1.3.1 Introduction. 33
1.3.1.1 Class I HDACs. 37
1.3.1.1.1 HDAC1 and 2. 37
1.3.1.1.2 HDAC3 and 8. 38
1.3.1.2 Class II HDACs. 38
1.3.1.2.1 HDAC4, 5, 7 and 9. 39
1.3.1.2.2 HDAC6 and 10. 39
1.3.1.3 Class IV. 41
1.3.1.3.1 HDAC11. 41
1.4 HDAC inhibitors. 41
1.4.1 HDI classes. 45
1.4.1.1 The short chain fatty acids. 45
1.4.1.2 Hydroxamic acids (hydroxamates). 45
1.4.1.3 Benzamides. 47
1.4.1.4 Cyclic tetrapeptides. 47
1.4.1.5 Sirtuin inhibitors. 47
1.4.2 The use of HDI in combination with other chemotherapeutics agents. 48
1.4.3 HDAC inhibitors and clinical outcomes 49
1.4.4 HDIs impact on cellular processes. 52
1.4.4.1 Modulating gene expression. 52
1.4.4.2 DNA repair 52
1.4.4.3 Protein turnover. 53
1.4.4.4 Activation of apoptotic pathways. 54
1.4.4.5 Cell cycle progression. 55
1.4.4.6 Inhibition of angiogenesis. 55
1.4.4.7 Cytokine production and immunity 56
1.5 Bromodomains and bromodomain inhibitors 58
1.6 Biomarkers 60
1.6.1 Types of Biomarkers 60
1.6.2 Properties of a good biomarker 61
1.6.3 Biomarkers in cancer epigenetics 63
1.6.4 Genome-wide loss-of-function screen 66
1.7 MyD88 68
1.7.1 MyD88: a toll like receptor (TLR) adaptor 68
1.7.2 MyD88 in cancer 70
1.7.2.1 MyD88 L265P somatic mutation 73
1.7.3 TLR-MyD88 dependent cytokines signalling 73
1.7.3.1 Interleukin 6 74
1.8 Hypothesis and aims of project 76

CHAPTER 2

MATERIALS AND METHODS. 78

2.1 Cell culture. 79
2.2 Plasmids, transfections and transductions. 80
2.3 Drug Treatments. 80
2.4 Antibodies. 80
2.5 Fluorescence-activated cell sorting (FACS) 81
2.6 Immunoblotting and Immunoprecipitation. 81
2.7 Immunofluorescence. 82
2.8 Colony formation assay (CFA). 83
2.9 Cytokine level analysis. 83
2.10 Immunohistochemistry pathology examination. 84

CHAPTER 3

MyD88 expression regulates cell sensitivity to HDI treatment. 85

3.1 Introduction. 86
3.2 Effects of different HDIs across different cell lines. 89
3.3 MyD88 expression regulates cell sensitivity to HDI treatment. 92
3.4 MyD88 KD reduced cells sensitivity to SAHA-associated apoptosis. 96
3.5 MyD88 changes cell cycle profiles. 99
3.6 MyD88 cellular localisation. 102
3.7 Conclusion. 105

CHAPTER 4

IL-6 and L265P-MyD88 sensitise cells to HDAC inhibition. 106

4.1 Introduction. 107
4.2 Impact of MyD88 on cytokine expression upon HDI. 109
4.3 IL-6 neutralisation reduces MyD88-dependent HDI-mediated apoptosis. 113
4.4 Effect of IL-6 neutralisation on cell cycle progression. 116
4.5 MyD88-L265P mutation sensitises cells to HDI. 119
4.6 L265P influence on cytokines levels. 122
4.7 Conclusion. 125

CHAPTER 5

MyD88 acetylation. 126

5.1 Introduction. 127
5.2 MyD88 is acetylated. 128
5.3 MyD88 acetylation enhances HDI cellular apoptosis. 131
5.4 MyD88 acetylation alters its protein stability. 134
5.5 Recognition of acetylated MyD88 by bromodomain proteins. 137
5.6 Conclusion. 141

CHAPTER 6

MyD88 interplay with HDAC6. 143

6.1 Introduction. 144
6.2 HDAC6 moderates MyD88 expression. 146
6.3 HDAC6 interacts with MyD88. 149
6.4 HDAC6 promotes MyD88 protein stability. 151
6.5 Mechanism of MyD88 protein control by HDAC6. 155
6.6 Conclusion. 158

CHAPTER 7

Discussion..... 160

7.1 Conclusions. 161
7.1.1 MyD88 expression levels regulate sensitivity to HDIs. 161
7.1.2 IL-6 sensitises cells to HDIs through a MyD88-dependent mechanism. 162
7.1.3 Somatic mutation in MYD88 influences HDAC inhibitor sensitivity. 164
7.1.4 MyD88 is acetylated. 165
7.1.5 Acetylated MyD88 is recognised by a bromodomain reader. 166
7.1.6 MyD88 is an HDAC6-substrate. 166
7.2 Project limitations. 169
7.2.1 Cell line system. 169
7.2.2 Measuring apoptosis. 170
7.3 Future directions. 170
7.3.1 MyD88, a biomarker for HDI-treatment. 170
7.3.2 MyD88-HDAC6 interplay via ubiquitination and Hsp90. 172
7.3.3 HDAC6 in immuno-oncology. 173
7.4 Project Summary. 174

References. 177

List of Figures

FIGURE 1.1 SCHEMATIC REPRESENTATION OF PERSONALISED MEDICINE 21
FIGURE 1.2 INTERPRETING CHROMATIN MODELLING 26
FIGURE 1.3 SCHEMATIC ORGANISATION OF CLASSIC HDACS. 36
FIGURE 1.4 SUMMARY OF CELLULAR OUTCOMES AFFECTED BY HDIS. 57
FIGURE 1.5 SCHEMATIC OF SHRNA SCREEN 67
FIGURE 1.6 SIGNAL TRANSDUCTION DOWNSTREAM OF MYD88-DEPENDENT AND INDEPENDENT PATHWAYS. 72
FIGURE 3.1 INITIAL ANALYSIS OF THE EFFECTS OF DIFFERENT HDIS ACROSS DIFFERENT CELL LINES. 91
FIGURE 3.2 MYD88 SENSITISES CELLS TO HDI-TREATMENT. 95
FIGURE 3.3 MYD88 KNOCKDOWN REDUCES SAHA-INDUCED APOPTOSIS. 98
FIGURE 3.4 MYD88 HAS LITTLE EFFECT ON S-PHASE. 101
FIGURE 3.5 MYD88 LOCALISATION 104
FIGURE 4.1 IMPACT OF MYD88 ON CYTOKINE EXPRESSION UPON HDI TREATMENT. 112
FIGURE 4.2 INTERPLAY BETWEEN IL-6 AND MYD88 AND ITS EFFECT ON HDI-MEDIATED APOPTOSIS. 115
FIGURE 4.3 EFFECT OF IL-6 NEUTRALISATION ON CELL CYCLE PROGRESSION. 118
FIGURE 4.4 HDI EFFECT ON WILD TYPE MYD88 VS L265P MUTANT 121
FIGURE 4.5 L265P INFLUENCE ON IL-6 EXPRESSION 124
FIGURE 5.1 MYD88 IS ACETYLATED. 130
FIGURE 5.2 K132R MUTANT SENSITIES CELLS TO SAHA TREATMENT. 133
FIGURE 5.3 MYD88 ACETYLATION AFFECTS ITS TURNOVER. 136
FIGURE 5.4 ACETYLATED MYD88 IS RECOGNISED BY BROMODOMAIN PROTEINS. 140
FIGURE 6.1 MYD88 LEVELS ARE PROPORTIONAL TO HDAC6 LEVELS.. 148

FIGURE 6.2 HDAC6 INTERACTS WITH WT MYD88 BUT NOT WITH K132R. 150
FIGURE 6.3 PROTEASOME INHIBITION RESCUES MYD88 IN ABSENCE OF HDAC6. 154
FIGURE 6.4 HDAC6 BUZ DOMAIN IS REQUIRED FOR HDAC6 INTERACTION WITH MYD88. 157
FIGURE 7.1 MODEL DEPICTING THE ROLE OF MYD88 AS A SENSITISER TO HDAC INHIBITOR TREATMENT. 164

List of Tables

TABLE 1.1. EPIGENETIC MODIFIERS AS EMERGING TARGETS FOR CANCER THERAPY..... 32

TABLE 1.2 CLASSIFICATION OF HDIS AGAINST CLASSIC HDACS AND REPRESENTATIVE EXAMPLES..... 44

TABLE 1.3. EXAMPLES OF APPROVED AND CURRENTLY TRIALED HDIS..... 51

TABLE 1.4 CRITERIA A GOOD BIOMARKER SHOULD FULFIL: INCREASING THE LIKELIHOOD THAT AN ASSOCIATION IS CAUSATIVE..... 65

Abbreviations

DAPI	4',6-diamidino-2-phenylindole
5-mC	5-methylcytosine
AML	Acute myelogenous leukemia
ATRA	All-trans-retinoic acid
AS-PCR	Allele-specific PCR
AR	Androgen receptor
Apaf-1	Apoptotic protease activating factor 1
BCL6	B-cell lymphoma 6
BCoR	BCL-6 interacting corepressor
BRCA1	Breast cancer 1
BRD	Bromodomain
BET	Bromodomain and extra-terminal
BRPF	Bromodomain and PHD finger-containing
BTK	Bruton tyrosine kinase
CREBP	cAMP-response element binding protein
CAP	Cap group
CART	Chimeric T cell receptors
CFA	Colony formation assay
CU	Connection unit
CpG sites	Regions of DNA where a cytosine nucleotide is followed by a guanine nucleotide in the linear sequence of bases along its 5' → 3' direction
CBP	CREB-binding protein
CTCL	Cutaneous T-cell lymphoma
CDKN1A	Cyclin dependent kinase inhibitor p21
CDK	Cyclin-dependent kinase
CDKN2B	Cyclin-dependent kinase inhibitor 2B
DAMPs	Danger-associated molecular patterns
DD	Death domain
DAPK	Death-associated protein kinase
DEN	Diethylnitrosamine
DLBCL	Diffuse large B-cell lymphoma

DSBs	DNA double-strand breaks
DNMTs	DNA methyltransferases
DDR	DNA-damage response
E2F	E2 promoter binding factor
EMA	European Medicines Agency
eNOS	Endothelial nitric oxide synthase
ERα	Estrogen receptor alpha
FADD	Fas-associated protein with death domain
FACS	Fluorescence-activated cell sorting
FCS	Foetal calf serum
FL	Follicular lymphoma
FDA	Food and Drug Administration
GBM	Glioblastoma multiform
GSTP1	Glutathione S-transferase gene
GO	Gene ontology
HRAS	Harvey rat sarcoma viral oncogene homolog
HDIs	HDAC inhibitors
Hsp90	Heat shock protein 90
HATs	Histone acetyl transferases
HDAC	Histone Deacetylase
HRP	Horseradish Peroxidase
HER2	Human epidermal growth factor receptor
HR23B	Human ultraviolet excision repair protein RAD23 homologue B
HS	Hydrophobic spacer
HIF-1α	Hypoxia-inducible factor 1 alpha
IgM	
MGUS	IgM monoclonal gammopathy of unknown significance
IRAK	IL-1 receptor-associated kinase
IRF	Interferon regulatory factor
IFNγ	Interferon-γ
IL-6	Interleukin-6
JNKs	JUN N-terminal kinases
KRAS	Kirsten rat sarcoma viral oncogene homolog

LPS	Lipopolysaccharide
LINE	Long interspersed nuclear elements
MHC	Major histocompatibility complex
miRNA	MicroRNAs
MLL	Mixed-lineage leukemia
MEFs	Mouse embryonic fibroblasts
MLH1	MutL homolog 1
MDS	Myelodysplastic syndrome
MyD88	Myeloid differentiation primary response 88
MEF2	Myogenic transcription factor 2
NKG2D	Natural killer cell protein group 2D
NB	Neuroblastoma
NAD+	Nicotinamide adenine dinucleotide
NES	Nuclear export sequence
NF-κB	Nuclear factor-κB
NLS	Nuclear localization signal
N-CoR	Nuclear receptor co-repressor
NuRD	Nucleosome remodelling and deacetylating
PAMPs	Pathogen-associated microbial patterns
PG	Pediatric glioma
PGN	Peptidoglycan
PBMCs	Peripheral blood mononuclear cells
PTCL	Peripheral T cell lymphoma
PBST	Phosphate buffered saline
PBST	Phosphate buffered saline with Tween 20
PARP	Poly-ADP ribose polymerase
PRC2	Polycomb repressive complex 2
PCR	Polymerase chain reaction
PVDF	Polyvinylidene fluoride
P-TEFb	Positive transcription elongation factor complex
PTMs	Post-transcriptional modifications
PLAU/uPA	Protease urokinase
CoREST	REST corepressor 1

Rb	Retinoblastoma
RT-PCR	Reverse transcription polymerase chain reaction
RTP	RNA-to-protein
RUNX3	Runt-related transcription factor 3
SQSTM1	Sequestosome 1
shRNA	Short/small hairpin
Stat3	Signal transducer and activator of transcription 3
SMRT	Silencing mediator for retinoic acid and thyroid hormone receptors
SIRT1	Silent mating type information regulation 2 homolog 1
Sin3A	Sin3 transcription regulator homolog A
SDS	Sodium dodecyl sulphate
SHP-2	Src homology domains containing tyrosin phosphatase-2
SAHA	Suberoylanilide hydroxamic acid
Tip60	Tat-interacting protein 60
PI3K	Phosphatidylinositide 3-kinases
Trx	Thioredoxin
TBP-2	Thioredoxin binding protein-2
TRAIL-R	TNF-related apoptosis-inducing ligand-receptor
TLR	Toll-like receptor
TSA	Trichostatin A
TNFR	Tumor necrosis factor receptor
TME	Tumour microenvironment
uPA	Urokinase plasminogen activator
VPA	Valproic acid
VEGF	Vascular endothelial growth factor
VHL	Von Hippel–Lindau
WM	Waldenstrom macroglobulinemia
WHO	World Health Organisation
XIAP	X-linked inhibitor of apoptosis protein
YY1	Yin and Yang 1
ZBG	Zn-binding group

CHAPTER 1 GENERAL INTRODUCTION

1.1 Personalised cancer medicine.

In recent years, personalised cancer medicine has risen as the approach of choice to enhance the therapeutic outcomes and minimise the side effects associated with traditional chemotherapeutic regimens, whereby a patient-tailored treatment allows a more individualised approach to overcome the challenges posed by the patients variability and tumour subsets; steering away from non-specific cytotoxic drugs.

The proven efficacy of the approach across different classes and sub-classes of tumours suggests a revolutionary shift towards focusing biological and clinical research at the molecular level of the tumour rather than its morphology, improving the overall outcome of the treatment.

An example of a personalised cancer treatment is the use of trastuzumab in the treatment of breast cancer patients [1]. It was found that patients overexpressing human epidermal growth factor receptor 2 (HER2) are more likely to respond to trastuzumab, reducing the recurrence of a tumour by more than the half when used in combination with chemotherapy, in comparison to chemotherapy alone [2]. Other examples of recently developed personalised cancer treatments include the use of tyrosine kinase inhibitors; imatinib in chronic myeloid leukaemia [3], erlotinib in advanced pancreatic cancer [4], gefitinib in advanced breast cancer [5] [6]; intracellular therapeutics, such as vemurafenib, in melanoma treatment [7] and most recently the approval of tisagenlecleucel, a chimeric antigen receptor (CAR) T-cell therapy for

the treatment of B-cell precursor acute lymphoblastic leukaemia (ALL) in younger patients [8].

The rapid adaption of that approach is the result of the increased efficacy of reliable tumour characterisation methods across variable fields such as genomics, proteomics, and metabolomics, coupled with the rapid development of computational tools and enhanced computational capacity for analysing these large sets of data; all together are enabling a rapid and efficient identification of key oncogenic signature profiles across molecularly distinct subtypes of cancer and the subsequent development of more personalised therapeutic approaches [9 10].

However, substantial hurdles withstand the widespread application of a universal personalised cancer care. Foremost among these are restrictions in our understanding of tumour molecular biology and ability to identify targets that are essential for tumour proliferation, in addition to the scarcity of biomarkers that can reliably identify subsets of patients who are likely to respond to treatment.

One explanation to that scarcity is that it was not until recently that researchers started to establish a better understanding of the biological heterogeneity of different types of cancer [11 12 13], whereby a better understanding of the molecular profile of the tumour becomes a necessary tool to guide the development of a personalised therapy driven by the development of the relevant class of biomarkers.

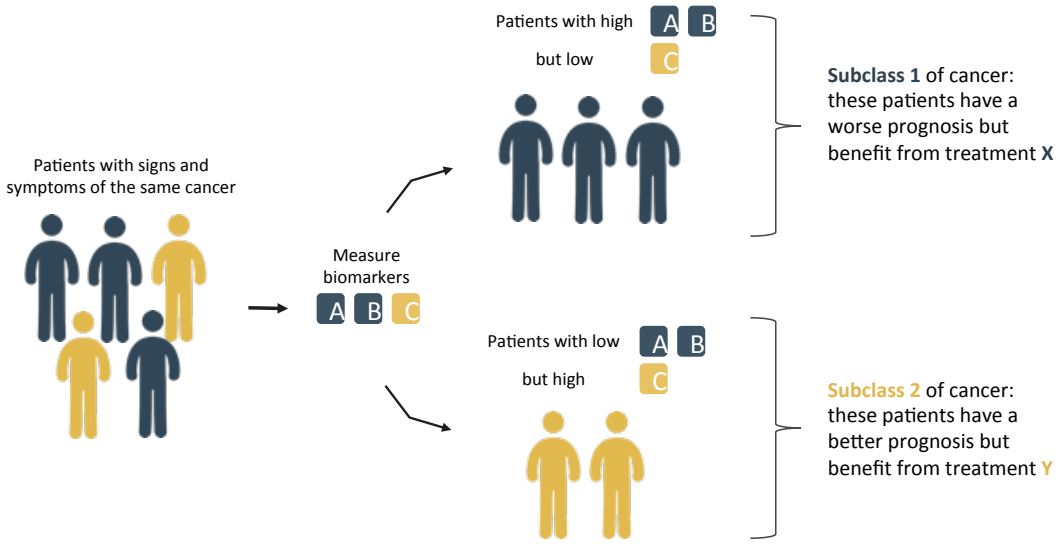


Figure 1.1 Schematic representation of personalised medicine. A diverse patient group that have the same diagnosis, but may respond differently to treatment. Representation adapted from Vargas et al., 2016 [14].

1.2 Epigenetics in tumourigenesis.

Hanahan and Weinberg's introduction of the hallmarks of cancer provided an overview of the biological framework that a tumour exhibits to successfully proliferate at both primary and metastatic tumour sites [15] which suggested that the development of therapeutic approaches to target these hallmarks require a comprehensive understanding of the encompassed complex network of signalling pathways. Tumours procurement of multiple hallmarks depends mainly on a cascade of alternations in the genomics of malignant cells; whereby, from a genetic perspective, a diverse array of defects affecting various components of the DNA-maintenance machinery, often referred to as the "care takers of the genome [16]", result in tumourigenesis. Such defects are usually introduced via silencing mutations or epigenetic modifications [17] [18].

To that end, epigenetic modifications; alternations in gene expression that are independent of the DNA sequence of a gene, represent a near-universal feature of human tumourigenesis [19].

Epigenetics is commonly defined as changes that affect gene activity and expression, as well as transgenerational heritable changes in gene expression, which do not involve changes in the underlying nucleotide sequence of DNA. Heritable changes can denote alternations that pass on from one generation of an organism to another (organism-organism) or those within the same organism (cell-cell), where gene expression patterns that can be clonally inherited might differ among different cell types and their generations [20]. When passed on from one cell generation to the

next, covalent modifications on the chromatin histone proteins as well as those occur on the DNA itself are referred to as epigenetic modifications and can result in heritable gene expression modifications [21]. Various internal and external changes in the biological environment, such as oxidative and nitrosative stress, may lead to these epigenetic modifications [22 23].

Epigenetic modification mechanisms include DNA methylation [24], histone modifications [25], chromatin remodelling and microRNAs (miRNA) [26]. The most commonly described among them is DNA methylation. The first described hypomethylation signature was investigated within the context of differentiating genes of human cancers vs their wild counterparts [27]. That was then followed by the identification of tumour suppressor genes that promote hypermethylation [28 29] and identifying the DNA methylation modifications at the miRNA level [30]. Chromatin remodelling, DNA methylation and histone acetylation are discussed in more details in the following sections.

These epigenetic mechanisms regulate gene expression together with a subset of various cellular and biological functions related to disease progression [31 32]. Examples include autoimmune disorders [33], neurodegenerative diseases [34] and cancer [35 18 36].

Recent developments in whole genome sequencing and computational biology have introduced great advancement towards our understanding of the human epigenome and how epigenomic modifications affect cancer development and progression [37 38]. Therefore, a better understanding of carcinogenic epigenetic modifications became a vital tool towards developing more novel biomarkers and personalised cancer

medicines based upon understanding the resultant epigenetic patterns and their effects on tumourigenesis [39].

1.2.1 Chromatin remodelling and transcription control.

Protein and mRNA expression levels are controlled by gene transcription; a process that is made possible by allowing or restricting transcription factor complexes access to the DNA scaffold. That access depends on how the DNA is packaged into dynamic chromatin structures, where DNA methylation and histone post-translational modifications together form an interdisciplinary arrangement that is involved in chromatin dynamic status, and alterations in epigenetic regulators leading to aberrant chromatin regulation have been shown to play an important role in the initiation and progression of cancer [40].

Chromatin is made up of DNA, histone- and non-histone proteins packaged in a highly complex dynamic structure. Chromatins exist in two functional conformational states: hetero- and eu-chromatin. The former usually represents the compact status and therefore less transcription is likely to occur at that state. On the contrary, euchromatin is the decondensed, form, where most transcription is likely to take place [38]. That dynamic remodelling in between the compact and decondensed chromatin status is considered to be a "switch" for the majority of gene transcription, DNA replication, DNA repair, and apoptosis processes [35 38 41].

The basic repeating structural and functional unit of chromatin is the nucleosome, which contains approximately 147 DNA base pairs wrapped around an octamer of core histone proteins H2A, H2B, H3 and H4. The addition of one H1 protein wraps another 20 base pairs, resulting in two full turns around the octamer; forming a chromatosome. Nucleosomes cores are separated by a linker DNA. Early observations by electron microscopists showed chromatin to appear in a structure similar to "beads on a string"[42] and chemical biologists cross-linking histones in chromatin[43] provided early evidence that nucleosomes exist.

Core histones contain a histone-folding domain and N-terminal tails, knows as histone tails, of variable lengths, which are subject to a variety of post-transcriptional modifications (PTMs) as shown in Figure 1.2 [44]. PTMs can introduce changes to DNA and its connected proteins, whereby these epigenetic modifications play a role in regulating gene expression and DNA chemical modifications without resulting into DNA sequence changes [38] [41]. PTMs can include acetylation, methylation, ubiquitination and phosphorylation among others [45] [46], and although that it was believed that the N- and C-terminal tails are the main targets for such modifications, yet it is now established that changes occur at the histone core level as well [47].

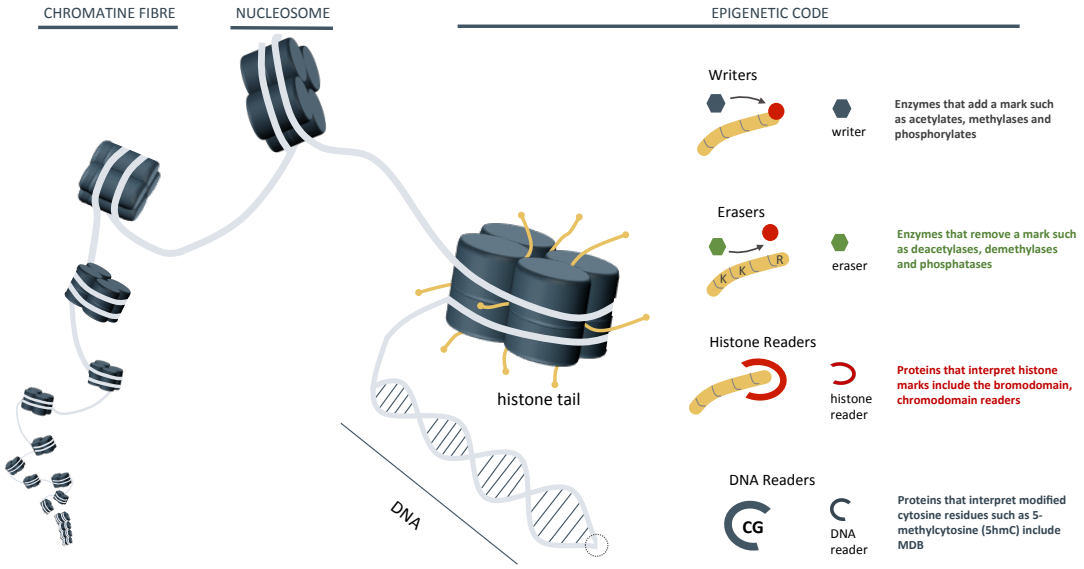


Figure 1.2 Interpreting chromatin modelling. Chromatin regulates gene structure and function and regulates various functions including transcription, repair, replication, and condensation. The nucleosome is comprised of 147 DNA bp comprising an octamer of 2 copies of each of the 4 core histone proteins. The histone tail is subject to several modifications by writing enzymes, erasing enzymes and interpreted by histone and DNA reader proteins that identify histone tail modifications. Adapted from Keating et al, 2016.

1.2.2 Methylation.

DNA methylation, not to be confused with histone methylation, is a process via which a methyl (-CH3) group is enzymatically added to the cytosine ring of the DNA resulting in 5-methylcytosine (5-mC), known as “the fifth base”, by DNA methyltransferases (DNMTs). In somatic cells, 5-mC is mostly coupled with symmetrical methylation of a CpG site; a location within a DNA sequence in which a cytosine and guanine appear consecutively. However, in embryonic stem cells, 5-mC occurs largely in non-CpG regions. The majority of CpG sites are heavily methylated, yet CpG islands, sites of CpG clusters, in germ-line tissues, which are found mainly near promoters regions, remain unmethylated, thus allowing gene expression to occur.

When DNA is aberrantly hypermethylated in the CpG islands around promoter region of genes, where transcription is initiated, genes become inactivated, allowing the subsequent silencing of tumour suppressor genes [35] [48] [49]. Tumour suppressor genes and those encoding cell adhesion molecules and growth-regulatory proteins are often silenced in hematopoietic malignancies by DNA hypermethylation, with examples including MutL homolog 1 (MLH1) mismatch-repair gene in colorectal, the von Hippel–Lindau (VHL) gene in renal cell cancer, the p16/CDKN2A cell-cycle control gene in various malignancies, and breast cancer 1(BRCA1) in early breast cancer [50] [28] [51] [52]. Moreover, other un-mutated tumour suppressor genes still encounter hypermethylation during the course of carcinogenesis. An example is

cyclin-dependent kinase inhibitor 2B (CDKN2B), encoding the cell-cycle regulator p15 [53].

Aberrant hypermethylatoin in cancer has been shown to happen via various mechanisms that recruit chromatin-modifying activities to methylated DNA; where silencing in general is thought to be either due to direct inhibition of transcription factor binding by DNA methylation [54]; as in the case of E2 promoter binding factor (E2F) regulation [55], modulating proteins that bind selectively to methylated DNA or the expression level of DNMTs [56 57 58].

On the contrary is hypomethylation. Although it occurs concomitantly with CpG island hypermethylation, hypomethylation is an independent process, which targets different process at different stages in tumourigenesis [27 59]. It is proposed that hypomethylation can contribute to tumouregenesis via three possible mechanisms; activation of proto-oncogenes such as Harvey rat sarcoma viral oncogene homolog (HRAS) and Kirsten rat sarcoma viral oncogene homolog (KRAS) and MYC proto-oncogene protein [60 61], prompting chromosomal instability via global hypomethylation [62 63], and increasing metastasis as the case in the differential regulation of Urokinase plasminogen activator (uPA) expression in different stages of prostate cancer [64]; a serine protease protein that catalyses the transformation of plasminogen to plasmin, resulting in the degradation of the extracellular matrix and basement proteins and subsequent metastasis.

Examples of oncogene overexpression due to hypomethylation include protease urokinase (PLAU/uPA) in breast cancers [65 66] and prostate cancers [67]. Examples of global hypomethylation include long interspersed nuclear elements (LINE)-1

repeats in ovarian cancer[68], whereby LINE1 hypomethylation is significantly more pronounced in metastatic prostate cancer [69].

Another recently identified DNA epigenetic modification is DNA hydroxymethylation, where the hydrogen atom at the C5-position in cytosine is replaced by a hydroxymethyl group, forming 5-hydroxymethylcytosine (5-hmC), a reaction that was shown to be catalysed by 2-oxoglutarate and Fe(II)-dependent oxygenases of the ten-eleven translocation (Tet) proteins [70]. DNA hydroxymethylation was shown to be involved in gene regulation, for example in genomic DNA on human and mouse brain, neurons and from mouse embryonic stem cells [71] [72] [73], where levels of 5-hmC were found to be decreased in a several types of solid tumors, including melanoma for example [74].

Overall the interplay between hyper- and hypo-methylation is now widely regarded as a viable target for biomarker development with both diagnostic and prognostic relevance, as well as targets for epigenome-directed therapies.

1.2.3 Histone acetylation and cancer.

Gene transcription is largely controlled by the balance of histone acetylation and deacetylation, whereby 2-10% of genes' transcription were found to be regulated by acetylation [38] [75] [76]. Histone acetylation is induced by histone acetyl transferases (HATs) via acetylating lysine amino acids on histone proteins by transferring an acetyl group from acetyl-CoA to form ε-N-acetyllysine, rendering a more relaxed DNA that becomes subject to transcription. On the contrary, histone hypoacetylation is induced by histone deacetylase (HDAC) activity and is

associated with gene silencing, whereby acetyl groups are removed from the ε-amino groups of lysine residues on histones [77 78 35 40].

HATs and HDACs have shown to play a role in regulating driving solid as well as haematological malignancies via an epigenetic-dependant mechanism [79 41], whereby aberrant expression profiles and mutations of genes that encode HDACs have been linked to several cancers, making HDAC-inhibitors (HDIs) attractive cancer therapeutics. [80 81].

Mechanisms that could result into HAT deregulation include chromosomal translocations, aberrant recruitment of epigenetic modifiers as well as genetic mutations, [35 46]. For example, CREB-binding protein (CBP), a HAT and bromodomain-containing protein and a regulator of chromatin remodelling, becomes aberrantly recruited to genes promoter regions involved in tumourigenesis. Additionally, there are instances where structural modifications of CBP and p300 have resulted into reducing the acetylation-mediated inactivation of the B-cell lymphoma 6 (BCL6) oncoprotein and activation of p53 in follicular lymphoma (FL) and diffuse large B-cell lymphoma (DLBCL) [82]. Other examples of CBP/p300 mutations in solid tumours include breast, gastric, colorectal, cervical and lung cancers [83 38 84 85].

Another HAT example is tat-interacting protein 60 (Tip60), a co-regulator of transcription factors, such as Myc and p53, via acetylation, [86 87]. The monoallelic loss of Tip60 was found to result in dysregulation of p53 and reduced transcription of pro-apoptotic target genes in lymphomas, breast, head and neck cancers due to loss of oncogene-induced DNA damage response [41 87]. On the contrary, Tip60 was

found to regulate DNA-damage response (DDR) signalling, where activated the later can counteract tumour progression [88] [89].

On the contrary, HDACs are not generally mutated in cancer, but rather over-expressed or aberrantly localised [41].

Table 1.1. Epigenetic modifiers as emerging targets for cancer therapy. Adapted from Sheikh et al., 2016.

Epigenetic modifier	Epigenetic protein example	De-regulation in cancer	Tumour type	Example of targeted agents
DNA methyltransferases (DNMT)	DNMT1 DNMT3A DNMT3B	Mainly over-expression, some mutations	Multiple solid and haematologic (eg colorectal cancer, acute myeloid leukaemia)	**1st generation:** • Azacitidine • Decitabine **2nd generation:** • MG98 (DNMT1 inhibitor)
Histone acetyltransferases (HAT)	p300 CBP pCAF MOZ Tip60	Mutations, translocations, deletions	Multiple solid and haematologic (eg colorectal cancer, acute promyelocytic leukaemia)	**Bisubstrate inhibitors:** • Lys-CoA 1 (specific p300 inhibitor) • H3-CoA-20 (pCAF inhibitor) **Natural products:** • Curcumin • Garcinol **Synthetic inhibitors:** • CTK7 • LTK-14 (selective inhibition of CBP/p300)
Histone Deacetylases (HDAC)	HDAC 1-11 Sirtuins 1-7	Mainly over-expression, some mutations, deletions	Multiple solid and haematologic (eg glioblastoma, prostate, breast)	**Hydroxamic acids:** • Vorinostat • Belinostat • Panobinostat **Cyclic tetrapeptides and depsipeptide:** • Romidepsin **Benzamides:** • Entinostat • Mocetinostat **Aliphatic acids:** • Valproic acid
Histone methyltransferases (HMT)	DOT1L EZH2 BLIMP1	Amplification, over-expression, some mutations	Multiple solid and haematologic (eg breast, gastric, acute myeloid leukaemia, lymphoma)	**DOT1L enzyme/ H3K79 modification:** • EPZ004777 **Set7/9 enzyme/ H3K4me1/Tat/ER modifications:** • PDB 4e47 **EZH2 enzyme/ H3K27me1/2/3 modification:** • GSK343
Histone Demethylases	LSD 1 JmJD3	Amplification, overexpression	Breast cancer, leukaemia	**Selective LSD-1 inhibitor:** • GSK-LSD1 **Selective jumonji H3K27 inhibitor:** • GSK-J1
BET/Bromodomain proteins	BRD2 BRD4	Mutation	Prostate cancer, leukaemia	**Pan-BET/BRD inhibitor:** • OTX015 **BRD-4 inhibitor:** • JQ1

1.3 Histone Deacetylases.

1.3.1 Introduction.

In humans, eighteen HDAC enzymes have been identified so far divided into four classes based on domain organisation, DNA sequence and subcellular location [90]. The classic family; that includes classes I, II and IV, comprises 11 HDACs that are categorised based on their protein homology to yeast as illustrated in Figure 1.3 [91]. All of the classical family members contain at least one catalytic domain, besides HDAC6 and HDAC10 that contain two, a zinc-dependent active site chelated by aspartate and histidine residues that encompasses an eight-stranded β –sheet located over twelve α-helices [79]. It is worth noting that the myocyte enhancer-binding factor 2-interacting transcriptional repressor (MIRT) a splice-variant of a similar amino acid homology to HDAC9, lacks such catalytic domains [92].

Class III enzymes are a family of nicotinamide adenine dinucleotide (NAD^+)-dependent proteins, namely sirtuins, and have a different mechanism of action that makes them not sensitive to the classic small molecule HDIs [93]. Recently, there has been growing interest in class III HDACs role as transcriptional regulators. An example is silent mating type information regulation 2 homolog 1 (SIRT1) upregulation in cancer cells that promotes aberrant methylation and therefore silencing of tumour suppressor genes [94].

HDACs are known not only to regulate acetylation level of histones in chromatin, but also that of a variety of non-histone substrates. HDAC substrates include

transcription factors (Runt-related transcription factor 3 [RUNX3], p53, E2F, c-Myc, Nuclear factor-κB [NF-κB]), hypoxia-inducible factor 1 alpha (HIF-1α), estrogen receptor alpha (ERα) and the androgen receptor (AR), MyoD, Hsp90, signaling mediators (Signal transducer and activator of transcription 3 [Stat3] and Smad7), DNA repair proteins (Ku70), and many others [95]. Moderating these proteins can in return affect a number of cellular processes including cell cycle control, apoptosis, angiogenesis, cell invasion and tumour progression [96].

Although, as previously mentioned, somatic mutations of genes encoding HDACs are rare, yet it has been shown in few studies. One example is driver mutations in HIST1H3B and H3F3A in pediatric glioma (PG) patients, which encode histones H3.1 and H3.3, respectively [97] [98]. Another example is HDAC2 truncating mutations in human epithelial cancer cell lines [99].

HDACs-mediated deacetyaltion is by large their key contributor to regulate cellular activities. HDACs usually interact via forming multi-protein complexes containing corepressor DNA-binding proteins, such as silencing mediator for retinoic acid and thyroid hormone receptors (SMRT), nuclear receptor co-repressor (N-CoR) co-repressors and Sin3 transcription regulator homolog A (Sin3A) [100] [101], with the aberrant increase in HDACs activity resulting in transcriptional repression of tumour suppressor genes and subsequently tumouregensis. However, it is believed that HDACs influence a range of other cellular outcomes in ways other than transcriptional regulation as suggested by a range of cDNA arrays studies that showed as many as 7–10% of genes were altered in their expression across different cell lines by HDAC inhibition [102] [103] [104], with examples of other processes

influenced by HDACs include regulating acetylation of histone and non-histone substrates as mentioned above.

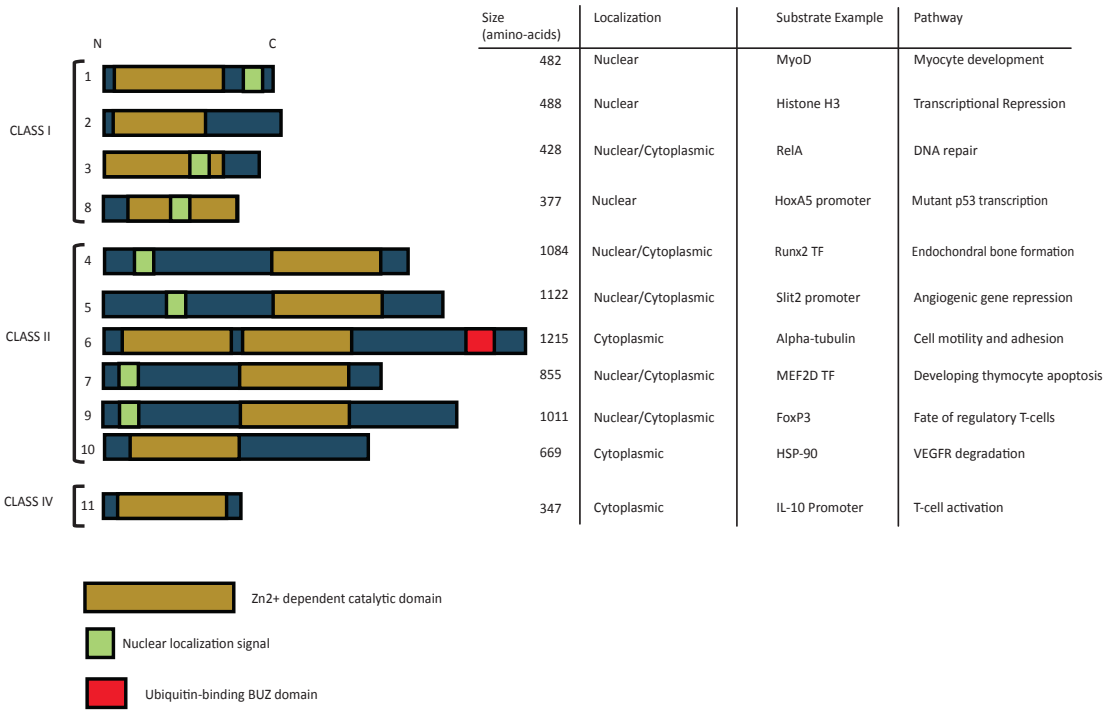


Figure 1.3 Schematic organisation of classic HDACs. Class I, II and IV showing their domain composition, size, cellular localization, target proteins and the cellular processes which are consequently regulated. The HDAC deacetylase catalytic domains are shown in beige, nuclear localization targeting-sequences in light green, and the ubiquitinin binding BUZ domain of HDAC6 is in red. A single substrate example is given alongside each enzyme, as well as the process in which this substrate participates. Adapted from New et al, 2012.

1.3.1.1 Class I HDACs.

The class I HDAC family consists of HDAC1, 2, 3 and 8, which are homologues with Rpd3 — a *Saccharomyces cereisiae* yeast transcriptional regulator. They are mainly nuclear enzymes and expressed ubiquitously.

1.3.1.1.1 HDAC1 and 2.

HDAC1 and HDAC2 are found to bind to repressive protein complexes such as the Sin3, nucleosome remodelling and deacetylating (NuRD), REST corepressor 1 (CoREST) and polycomb repressive complex 2 (PRC2) complexes [105]. These complexes mediate the recruitment of HDACs to the promoters of genes and assist in modulating their deacetylase activity.

Additionally, HDAC1 and HDAC2 are found to exert post-transcriptional modifications, regulated by phosphorylation, via binding directly to DNA binding proteins such as retinoblastoma (Rb) binding protein-1, Sp1 and Yin and Yang 1 (YY1), a cellular nuclear matrix regulatory protein [106] [107] [108].

Examples where HDAC1 and 2 was associated with tumouregensis include their aberrant overexpression in aggressive colorectal cancers. In vitro, HDAC inhibition as well as shRNA knockdown of HDAC1 and 2 was shown to be effective at inhibiting cellular proliferation of cancer cells [109].

1.3.1.1.2 HDAC3 and 8.

HDAC3 is structurally most similar to HDAC8 [110] [106]. Although HDAC3 is known to mainly interact on its own, yet it is also able to form oligomers in vitro and in vivo with HDAC4 [111] and also to co-precipitate with HDACs 4, 5, and 7 through forming complexes with SMRT and N-CoR [111] [112].

HDAC8 consists mainly of the catalytic domain with a nuclear localisation signal (NLS) in the centre and can be found ubiquitously expressed and localised primarily in the nucleus and less often in the cytoplasm [113]. Although no complex has been described for HDAC8 [114], yet it is known to have different expression patterns compared to other Class I HDACs [115]. Clinically, selective HDAC8 inhibitors are likely to be relevant in treatment of T cell leukemia and in neuroblastoma (NB), where HDAC8 found to be the only correlated HDAC [116].

1.3.1.2 Class II HDACs.

Class II HDACs are sub-divided into Class IIa and IIb depending on their cellular localization. The class IIa HDAC, HDAC 4, 5, 7, and 9, has a distinctive C-terminal deacetylase catalytic domain homologous to yHda1 and an N-terminal domain with limited similarity to any other classes of HDAC. Class IIa HDACs are tissue-specific and are found to present both in the cytoplasm and the nucleus. Conversely, class IIb HDAC, HDAC 6 and 10, are mainly cytoplasmic.

1.3.1.2.1 HDAC4, 5, 7 and 9.

HDAC4, HDAC5 and HDAC7 are a subgroup of Class II HDACs, where HDAC4 and HDAC5 are almost structurally similar and HDAC7 is closely related. All three HDACs have their catalytic domain located at the C-terminal [110], unlike those of Class I. Their N-termini are found to repress transcription via interacting with the myogenic transcription factor 2 (MEF2), blocking muscle cell differentiation [117]. It was also found that that HDACs 4, 5, and 7 can interact with N-CoR, SMRT, similar to class III HDACs, in addition to interacting with BCL-6 interacting corepressor (BCoR) as well. It is also found that the catalytic domain of HDAC4 can interact with HDAC3 via N-CoR/SMRT but not with Sin3 [118].

HDAC9 also has structural similarity to HDACs 4, 5 and 7, although its catalytic domain is located at the N-terminal, similar to those of HDAC I class. So far, HDAC9 is known to have three splice variants, HDAC9a, HDAC9b and HDRP/HDAC9c, with certain cell types expressing one isoform while others express another [119]. Similar to other class IIa HDACs, HDAC9 is also known to play an important role in muscle differentiation via interacting with MEF2 [100].

1.3.1.2.2 HDAC6 and 10.

HDAC6 has unique structural features and substrate specificity. HDAC6 is the only HDAC that is mainly found in the cytoplasm as a result of a nuclear export signal and SE14 motif [120].

The first identified substrate of HDAC6 is tubulin. HDAC6 is known to be responsible for the deacetylation of α-tubulin, which destabilises the microtubular network, a function that was found important in tumour cell metastasis via regulating micro tubule-dependent cell motility [121] [122]. HDAC6 is also associated with the ubiquitin-proteasome pathways whereby it's ubiquitin-binding domain, located at it's c-terminus, associates with the p97/VCP, resulting in the proteasomal degradation of misfolded proteins, and the formation of polyubiquitinated aggregates [123] [124].

Another HDAC6 substrate is Hsp90. HDAC6 is known to modulate a number of heat shock protein 90 (Hsp90)-mediated regulatory processes via deacetylating Hsp90 and the subsequent recruitment of chaperone complexes [125] [126]. Additionally, it is known that HDAC6 plays a role at cell signalling transduction via β-catenin, another substrate. HDAC6 associates with and deacetylases β-catenin, a process that is mainly induced via HDAC6 translocation by EGF, promoting Wnt signalling and c-Myc activation [127].

Additionally, HDAC6 can also control activities of several transcriptional regulators; such as Runx2 and NF-κB [128] as well as modulating the acetylation of Ku70, an apoptosis-related protein, in neuroblastoma [129].

HDAC10 is the most recently discovered member of the class II HDACs. To date two different splice variants are known, with a slight difference in mRNA length [130]. HDAC10 has a catalytic domain on its N-terminus, and a nuclear export sequence (NES) and a putative second catalytic domain on its C-terminus. Also, two Rb binding domains have been found on HDAC10, which reflects upon a potential role

in regulating cell cycle progression. However, it is worth noting that HDAC10 is found largely in complex with most of the other HDACs, suggesting that it might function as a recruiter rather than a deacetylator on its own [131] [132] [130] [131].

1.3.1.3 Class IV.

1.3.1.3.1 HDAC11.

HDAC11, the most recently discovered HDAC, is the sole member of class IV and is located in the nucleus; although it is also known to co-precipitates with HDAC 6 cytoplasmically [133] [134]. Structurally, it is most closely related to HDAC3 and HDAC8, yet it does not share the same homology in the catalytic motif. HDAC11 contains a catalytic domain at its N-terminus, and to date, HDAC11 is not known to complex with any other HDACs, which might highlight a different function [134]. HDAC11 was found to be mainly overexpressed in NB and rhabdomyosarcoma [135].

1.4 HDAC inhibitors.

HDIs are a class of naturally occurring and synthetic compounds, which primarily exhibit their functions via modulating HDACs Zn^{2+} dependant sites [136]. They can target transcription, positively or negatively, by restraining the activity of HDACs, whereby they can promote acetylation of lysine residues on histone as well as non-histone proteins such as chromatin remodelling proteins, DNA-binding nuclear receptors, transcription co-regulators and others. Nevertheless, the exact mechanisms they exert their effects through are often more complex and cannot be

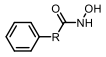
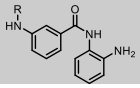
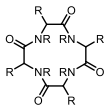
solely attributed to regulating genetic transcription as previously discussed. Therefore, enhanced gene expression determined by HDI is not always increased even if the chromatin structure is relaxed.

HDIs are mainly developed as cancer therapeutics against HDAC classes I, II and IV, with recent increasing interest in developing inhibitors against the class III sirtuin family as well [136]. Recently, there has been growing interest in using HDIs as the treatment-of-choice for different types of cancers, either as a single agent treatment or in combination with other anti-cancer therapeutics, taking into account their ability to promote a range of anti-tumour activities, including induction of cell-cycle arrest, stimulation of differentiation as well as promoting the expression of several apoptotic genes [137].

The pharmacophoric features of HDIs usually include: a cap group (CAP) allowing them interact with the catalytic pocket of the enzyme; a polar connection unit (CU) connected to a hydrophobic spacer (HS) that enables the inhibitor docking into the catalytic pocket, and a Zn-binding group (ZBG) to complex with the enzyme's Zn^{2+} [138]. An example is trichostatin A (TSA). The crystal structure of TSA docked into an HDAC analogue shows that the tubular pocket in the HDAC harbours the aliphatic long chain of TSA, and the enzymatic Zn^{2+} is coordinated by the carboxyl and hydroxyl groups of the hydroxamic group of the compound [139]. Another relevant example with similar mechanism is vorinostat (SAHA), although it yields weaker overall inhibitory effect since its aliphatic chain makes fewer van-der-Waals contacts with HDACs tubular pockets compared to TSA [139].

HDIs can be developed against all HDAC classes, or alternatively they can inhibit a specific class(s) or isoform(s) of HDAC(s). They can be chemically classified under four classes of compounds as indicated in Table 1.2: short chain fatty acids, hydroxamic acids (hydroxamates), benzamides, cyclic tetrapeptides in addition to sirtuin inhibitors.

Table 1.2 Classification of HDIs against classic HDACs and representative examples.

Group and Structure	Compounds	*In vitro* IC_{50} range
Short-chain fatty acids 	Butyrate Phenylbutyrate Valproic acid	mM mM mM
Hyrdoximic acids 	TSA SAHA	nM µM
Benzamides 	N-acetyldinaline MS-275	µM µM
Cyclic tetrapeptides  	Trapoxin Chlamydocin Depsipeptide	nM nM nM

1.4.1 HDI classes.

1.4.1.1 The short chain fatty acids.

Butyric acid was the first short chain fatty acid recognised as an agent that modulate histone acetylation. Other short chain fatty acids, with lower potency, include phenyl butyrate and phenyl acetate, known to be weak inhibitors of HDAC class I and IIa and I and II, respectively.

Valproic acid (VPA), a short chain fatty acid that has been used as anticonvulsant for seizures and bipolar disorders, has also shown to hyperacetylate histones. VPA has shown activity as a monotherapy in a limited number of patients [140], and was also clinically trailed in combination with other agents. An example is its combination with all-trans-retinoic acid (ATRA) in either acute myelogenous leukemia (AML) or myelodysplastic syndrome (MDS) [141 142].

1.4.1.2 Hydroxamic acids (hydroxamates).

TSA was first developed as an anti-fungal agent after its isolation from *Streptomyces hygroscopicus*. Later it was identified as a HDI. However, due to its high toxicity, it is used only in laboratory experiments.

Vorinostat (suberoylanilide hydroxamic acid [SAHA]) is the first HDAC inhibitor that has been approved by Food and Drug Administration (FDA) for clinical use in the treatment of relapsed and refractory cutaneous T-cell lymphoma (CTCL). It was

originally isolated as an active inducer of differentiation of murine erythroleukaemia cells [143] and later on identified as an HDI based on recognition of the similarity of its structure to that of TSA [144].

SAHA is a pan-HDI; inhibiting all HDAC I and II enzymes at about 50 nm. Its cellular potency against arresting cellular growth of a wide variety of transformed cells in culture was found to be in the range of 2–5μM [145] [146] [147]. Along with its effects on tumour growth, it was also found to selectively alter the transcription of expressed genes in tumour cells such as p21 associated with TF complex[148] and the thioredoxin (Trx) binding protein-2 (TBP-2) gene [149].

Other hydroxamic acid-based pan-HDIs include belinostat (PXD101); a HDI efficient at nanomloar concentrations in various cell lines developed by Topotarget Prolifix, approved for peripheral T cell lymphoma (PTCL) and panobinostat (LBH589); approved for multiple types of myeloma. Additionally other pan-HDIs currently going into clinical trials include givinostat (ITF2357), quisinostat (JNJ-26481585) and resminostat (4SC201)

HDAC class-specific inhibitors in this group include CXD101; a selective Class I inhibitor acting as an immune-regulator that has shown promising results as a monotherapy in lung and colon xenograft models as well as rocilinostat (ACY1215); a selective inhibitor of HDAC class II, practinostat (SB939), all of which are currently under clinical studies.

1.4.1.3 Benzamides.

A number of benzamide-based HDIs currently under clinical trials, including entinostat (MS-275-SNDX-275), tacedinaline (CI994) and 4SC202, all of which are selective inhibitors of class I HDACs. Mocetinostat (MGCD0103) though is a selective benzamide acts against both HDAC classes I and IV.

1.4.1.4 Cyclic tetrapeptides.

The most prominent of which is the bicyclic depsipeptide romidepsin (FK228, FR901228), a compound that has been approved by FDA and EMA (European Medicines Agency) to treat CTCL. Romidepsin acts as a prodrug that when reduced intracellularly it releases a zinc-binding thiol, which then chelates the zinc ions in the active center of the HDAC of class I [150]. Other cyclic peptides include the epoxyketones trapoxins A and B that were isolated from *Helicoma ambiens* and were shown HDI efficacy at the nanomolar level. Due to the later effect being irreversible, their therapeutic applications have been of limited interest.

1.4.1.5 Sirtuin inhibitors.

That class includes nicotinamide; a pan-HDI, sirtinol; a specific inhibitor against SIRT1, and cambinol, a specific inhibitor against SIRT2 [151] [152]. They are also thought to act against different types of neurodegenerations and cancers [153].

1.4.2 The use of HDI in combination with other chemotherapeutics agents.

Several in vivo and in vitro investigations have highlighted the synergetic effects of combining HDIs with other cancer therapeutics as well as in combination with radiotherapy[154] [155]. One important approach is the combination of HDIs with proteasome inhibitors. Examples include studies showing increased cellular apoptosis in patients with multiple myeloma demonstrated when SAHA was used in combination with bortezomib, a proteasome inhibitor [156], and in vitro pancreatic cancer cells when SAHA or entinostat where administered in combination with marizomib, another protesosome inhibitor [157].

Another important approach is the combination of HDIs with DNA damaging agents, such as inhibitors of topoisomerases, inhibitors of DNA synthesis, DNA-intercalators and agents covalently modifying DNA. In vitro, treatment of UKF-NB-4 neuroblastoma cells with DNA-damaging chemotherapeutics cisplatin or etoposide, combined with VPA resulted in synergistic antitumour effects [158]. In vivo, vorinostat was found to enhance the efficacy of karenitecin, a topoisomerase inhibitors, in phase I/II melanoma clinical studies [159].

Additionally, HDIs were also found to promote the antitumour activity of several radiotherapeutics [160] [161], which is thought to be mainly due to the expression of ATM, p53 and BRCA1 [162] [163]. An example is the enhanced antitumour activity of VPA when combined with temozolomide in the radiotherapy of glioblastoma patients [164].

1.4.3 HDAC inhibitors and clinical outcomes

So far, four HDIs have been approved for clinical use by the FDA and many others are currently under clinical trials as indicated in Table 1.3. Namely; Vorinostat, approved in 2006 for the treatment of CTCL; romidepsin approved in 2009 for the treatment of CTCL, and in 2011 for the treatment of PTCL; belinostat, approved in July 2014 for the treatment of patients with relapsed or refractory PTCL, and in 2015, panobinostat was approved as the first HDI for the treatment of multiple myeloma.

Vorinostat was shown to have modest clinical efficacy when used as a single agent, with response rates between 10-20% in AML and myelodysplastic syndrome, compared to 30% efficacy when used in combination with 5-azacitidine [165]. On the contrary, it has shown 30% efficacy when used on its own in patients with refractory CTCL, providing it with the FDA approval based on the objective response rate determined by direct evidence of clinical benefit [166].

Similarly, romidepsin FDA approval as a single agent for the treatment of relapsed or refractory PTCL was based on an objective response rate of 25% across patients with all major types of PTCL [167]. Belinostat was FDA approved based on a multi-centre single-arm BELIEF trial of 120 patients with relapsed or refractory PTCL, showing a 25.8% response rate [168].

After deferring the initial accelerated application of panabinostat due to associated disease progression, it was later re-evaluated as third-line therapy in a trial with a subgroup of 193 patients who had previsouly received both bortezomib and an

immunomodulatory drug and who had a median of two prior therapies. Panobinostat was shown to increase the median progression-free survival to 10.6 months compared 5.8 months in the control treatment, with an overall response rate of 59% versus 41%, respectively; granting panobinostat accelerated approval for patients who received at least two prior lines of therapy, including the previously mentioned bortezomib and an immunomodulatory drug [169].

Overall, several HDIs agents have shown high efficacy in pre-clinical and clinical trials, with several already being the first line of anti-cancer treatment as discussed above. However, the underlying molecular mechanisms that harness their anti-proliferative activities are still to be resolved, making it more challenging to adapt a more patient-tailored approach where the right HDI is administered to the right subsets of patients.

Table 1.3. Examples of approved and currently trialed HDIs. Adapted from Sheikh et al, 2016.

	Proposed Indication	Target HDAC	IC50
Approved			
Vorinostat	Cutaneous T cell lymphoma (CTCL)	HDAC1, HDAC2, HDAC3, HDAC8, HDAC9	10 nM
Romidepsin (trade name Istodax)	Cutaneous T-cell lymphoma (CTCL)	HDAC1 HDAC2	36 & 47 nM
Panobinostat	Cutaneous T cell lymphoma (CTCL)	Pan HDACs	5-20 nM
Belinostat (PXD101)	Relapsed ovarian cancer, reported good results for T cell lymphoma		27 nM
Started phase III clinical trials			
Valproic acid (as Mg valproate)	Cervical cancer, ovarian cancer	HDAC1, HDAC2, HDAC3	0.7-20 mM
Started phase II clinical trials			
Mocetinostat (MGCD0103)	Examples include follicular lymphoma, Hodgkin lymphoma, acute myeloid leukemia	HDAC1, HDAC2, HDAC3, HDAC11	0.15-10µM
Abexinostat (PCI-24781)	Sarcoma and another for lymphoma	HDAC1, HDAC3, HDAC2, HDAC6, HDAC8	0.15µM- 3.09µM
Entinostat (MS-275)	Hodgkin lymphoma, lung cancer and breast cancer	HDAC1, HDAC2, HDAC3, HDAC9	0.5 mM
Pracinostat (SB939)	Recurrent/Metastatic Prostate Cancer	pan-HDAC	40-140 nM
Resminostat (4SC-201)	Hodgkin lymphoma, hepatocellular carcinoma	HDAC1, HDAC3, HDAC6, HDAC8	42.5 nM-877 nM
Givinostat (ITF2357)	Refractory leukemias, myelomas	HDAC2, HDAC1B and HDAC1A	7.5-16 nM
Quisinostat (JNJ-26481585)	Myeloma	Modest potent to HDACs 2, 4, 10, and 11; greater than 30-fold selectivity against HDACs 3, 5, 8, and 9 and lowest potency to HDACs 6 and 7	0.11 nM
Started phase I clinical trials			
CUDC-101	Lymphoma, also inhibits EGFR and HER2.	Class I and class II HDACs	2.4-17.7 nM
AR-42	Relapsed or treatment-resistant multiple myeloma, chronic lymphocytic leukemia or lymphoma		30 nM
4SC-202	Advanced hematological indications	Class I	1µM
CG200745	Solid tumours	Pan- HDAC	
Rocilinostat (ACY-1215)	Multiple myeloma in combination with bortezomib (Velcade, Takeda Millennium) and with lenalidomide (Revlimid, Celgene)	HDAC6	5 nM
Preclinical			
Kevetrin	Selective for HDAC2		

1.4.4 HDIs impact on cellular processes.

Among those cellular responses that HDIs modulate are gene expression, DNA repair, protein stability, apoptosis, cell cycle progression, angiogensis as well as cytokine production and immunity, as summarised in Figure 1.4.

1.4.4.1 Modulating gene expression.

HDIs are known to regulate genes expression via the induction of histone and non-histone proteins acetylation, including the induction of transcription factor acetylation. Several studies showed evidence of NF-κB acetylation by HDI. For instance, the treatment of leukemia cells with SAHA and MS-275 induced hyperacetylation of p65, its accumulation in the nucleus, and NF-κB transcriptional activation [170]. Another example of regulating transcription factor acetylation is cyclin-dependent kinase (CDK) inhibitor p21 (WAF1/CIP1), which was shown to be a target of HDI in a p53-independent manner. Examples include SAHA modulating transcription at the proximal promoter of the p21 via changing the acetylation status of its histones H3 and H4 in ARP-1 mylenoma cells [148].

1.4.4.2 DNA repair

HDIs can induce or enhance DNA damage when used as monotherapies or in combination with DNA damaging agents through transcriptional downregulation or impaired function of DNA repair proteins, impaired recruitment of DNA repair proteins to sites of DNA damage, promoting an increase in ROS or via inducing

DNA double-strand breaks (DSBs) [171] [172]. For example, SAHA was found to induce ROS and DNA damage in acute myeloid leukemia cells [173]. Interestingly, it was also found to induce DSBs in normal and cancer (LNCaP, A549) cells via accumulation of acetylated histones H3 and H4, whereas, unlike malignant cells, normal cells managed to repair these DSBs despite continued exposure to SAHA [174]. These effects gave rise to various combination therapies whereas both DNA-damaging agents and HDIs are used synergistically. An example is the use of TSA and VPA to induce sensitisation of multidrug-resistant cancer cells to the topoisomerase II inhibitor etoposide/VP16 [175].

1.4.4.3 Protein turnover.

Lysine acetylation, a PTM that is largely in control by HDACs, has an important direct regulatory potential in the control of protein stability. Several studies suggested that this can be due to either the lysine "locking" activity of acetylation that blocks protein ubiquitination and leads to protein stabilization or due to modulating protein–protein interactions [176]

A classic example is the hyperacetylation of Hsp90 through HDAC6 inhibition that leads to the release and subsequent degradation of several oncogenic proteins substrates. BCR–ABL, an oncogenic protein that induces the t(9:22) driver mutation in chronic myeloid leukaemia cells, was found to decrease when BRC-ABL rich cells were treated with SAHA resulting in the subsequent increase of Hsp90 hyperacetylation, degradation of BCR–ABL and tumour cell death [177].

1.4.4.4 Activation of apoptotic pathways.

HDIs can influence cellular apoptosis through activating a variety of extrinsic and/or intrinsic pathways [116]. Extrinsically, TNF superfamily death receptors such as Fas, tumour necrosis factor receptor (TNFR), TNF-related apoptosis-inducing ligand-receptor 1 (TRAIL-R1) and TRAIL-R2 bind to their relevant TNF receptor ligand, whereas the consequent downstream cascade of events results in the induction of apoptosis by direct cleavage of caspases such as caspase-3. HDIs were shown to affect that pathway through enhancing the sensitivity of many tumours to activators of this pathway. An example is inducing expression of TRAIL by directly activating the TNFSF10 promoter that triggered tumour-selective death signalling in AML samples upon treatment with MS275 and SAHA.

Intrinsically, a cascade of events initiated by the release of cytochrome c, from the mitochondrial intermembrane space to the cytoplasm, where it binds to apoptotic protease activating factor 1 (Apaf-1), results eventually in the activation of caspase-3 and caspase-7. This initiation of this process is mainly controlled through the interplay of pro- and anti-apoptotic Bcl-2 proteins levels, whereas HDIs exert apoptotic effects through reducing the expression of the later or increasing the expression of the former. For example, TSA treatment repressed the transcription of Bcl-2 and Bcl-xL, anti-apoptotic Bcl-2 proteins, in myeloma cells [178], whereas SAHA treatment resulted into increased X-linked inhibitor of apoptosis protein (XIAP), a pro-apoptotic protein, levels in some pancreatic cancer cell lines [179].

1.4.4.5 Cell cycle progression.

HDIs can induce cell cycle arrest through a variety of mechanisms; the most prominent of which is increasing the p53-mediated expression of cell cycle genes such as Cyclin dependent kinase inhibitor p21 (CDKN1A) [180] [151]. p53 competes with HDAC1 to bind with p21 promoter and thus decreasing its expression. Conversely, HDI can influence that process through releasing HDAC1 [181], or inducing p53 stability via inducing its acetylation, both resulting in improved interaction with the p21 promoter [182]. Moreover, Sodium Butyrate, SAHA and TSA were found to induce NB cells apoptosis via a two-step process, with the first step being induction of cell cycle arrest in the G2/M phase, followed by the activation of apoptotic pathway [183].

1.4.4.6 Inhibition of angiogenesis.

HDIs anti-angiogenic effects could be mainly attributed to their ability to down-regulate pro-angiogenic genes expression, such as the genes of the vascular endothelial growth factor (VEGF), endothelial nitric oxide synthase (eNOS), (HIF-1a) and others [184]. For instance, treating neuroblastoma cells with TSA and SAHA was shown to decrease VEGF receptors levels, which illustrates their function as potent anti-angiogenic agents [184].

1.4.4.7 Cytokine production and immunity

HDI treatment was shown to modulate a variety of pathways that affect several immune functions [185]. HDI treatment was shown to modulate several immune functions in cancers either by directly enhancing immune recognition of cancer cells, via increasing major histocompatibility complex (MHC) class I and II proteins, and co-stimulatory/adhesion molecules CD40, CD80, CD86 levels, or by moderating immune activity and cytokine production [185]. An example of modulating cellular immune-vulnerability is the VPA-associated upregulation of MHC class I chain-related molecules MICA and MICB on the surface of tumour cells via a-natural killer cell protein group 2D (NKG2D) mediated mechanism in hepatocellular carcinoma cells [186]. Another example is depsipeptide enhancement of the expression of the tumour antigen gp100 in murine melanoma cells [187].

Although various studies showed that HDI treatments at different time intervals, different concentrations and against different tumour subsets could have different effects at regulating both co-stimulatory and cytokine signals, yet in large these effects were rather inhibitory. An example is inhibition of the cytokines interleukin-6 (IL-6) and TNF in human macrophages in cells treated with tubastatin [188]. Also treating lipopolysaccharide (LPS)-stimulated human peripheral blood mononuclear cells (PBMCs) with ITF2357, a synthetic HDI, showed reduced release of TNFα, IL-1α, IL-1β and interferon-γ (IFNγ) [189].

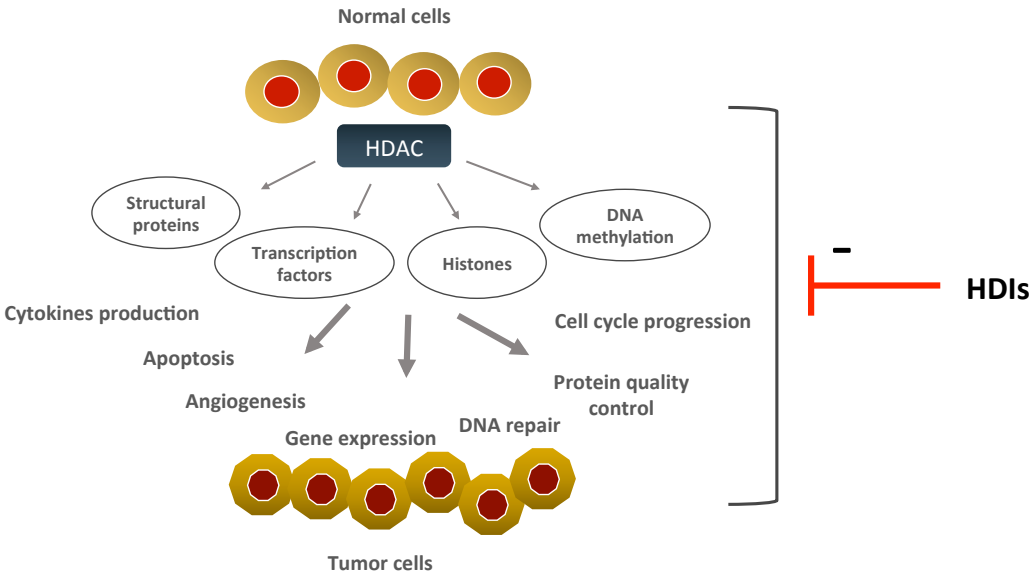


Figure 1.4 Summary of cellular outcomes affected by HDIs.

1.5 Bromodomains and bromodomain inhibitors

As previously discussed, lysine acetylation occurring in histone tails plays a vital role in chromatin modelling and proteins assembly. Subsequent to their acetylation, chromatins need to be identified to regulate their transcription. Bromodomain (BRD), often the term used to describe bromodomain and extra-terminal (BET) bromodomain proteins (BRD2, BRD3, BRD4), can principally identify acetylated chromatins and bind via their conserved BRD activity to mono-acetylated lysine residues, where as they act as "readers" of this post-translational modification.

To date, a total of 61 bromodomains, classified into eight subfamilies on the basis of their structure, were found across 46 different proteins in humans. Bromodomains were found to share a highly conserved structure that encompasses a pocket that contains four α-helices (αZ, αA, αB, αC), linked by flexible loop regions known as ZA and BC loops, which vary in length and charge; forming the acetyl binding site. The hydrophobic nature of this pocket made bromodomains an interesting target against the development of small inhibitory molecules.

Several studies have shown bromodomains involvement in a variety of biological processes [190]. Example include BRD4 and BRD2 role in transcription elongation by recruiting the positive transcription elongation factor complex (P-TEFb) [191], regulating expression of c-MYC [192], BRD4 regulation of NF-κβ-dependent genes [193], bromodomain and PHD finger-containing (BRPF) family regulation of HAT assembly [194], and BRD7 role as a tumour suppressor gene in several types of cancer [195]. Bromodomains were also found to play a role in cAMP-response element

binding protein (CREBP)-mediated p53 transcriptional activation of p21 in cell cycle arrest [196] and p300/CBP-associated factor PCAF (also known as KAT2B) acetylation of histones H3 and H4 [197].

Altogether, the sum of their functions in addition to their structural properties raised interest in developing therapeutics against them. The efficacy of the first bromodomain inhibitor, JQ1, developed at the Structural Genomics Consortium at the University of Oxford and the Dana Farber institute [198], has already been investigated into several types of haematological and solid cancers [190]. JQ1 acts by competing against BRD4 and binding to acetyl-lysine binding motifs thus displacing BRD4 fusion oncoprotein from chromatin and promoting cellular apoptosis [199].

Another example is OTX015 (MK-8628), a more potent BRD2, 3 and 4 inhibitor, which is currently going under clinical trials since it has shown stronger anti-proliferative effects in glioblastoma multiform (GBM) models [200]. Moreover, it was found to modulate the above mentioned expression of c-Myc in leukaemia which rationaled its use in acute leukaemia and other haematological malignancies Phase I clinical trials [201] and also explained its synergetic effects in combination with vorinostat and romidepsin B-cell tumour models [202]. Also I-BET151 (GSK1210151A) has shown efficacy in promoting cellular apoptosis via inhibition of BRD3 and 4 in mixed-lineage leukemia (MLL) [203].

1.6 Biomarkers

1.6.1 Types of Biomarkers

In recent years, clinicians have been able to stratify patients according to the likelihood of their response to epigenetic drugs by means of examining changes in a single gene/protein; biomarkers, or in multiple genes/proteins; molecular signatures, as a mean of evaluating changes in their biological processes and their potential response to a particular therapeutic regime. A biomarker is widely defined as, according to the World Health Organisation (WHO), "any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease" [204]. According to Aronson et al, “a biomarker that is intended to substitute a clinical end point” is a surrogate endpoint [205] [204], where the former is a characteristic or variable that reflects “how a patient feels, functions, or survives”, and the later is substituted for a clinical endpoint that can predict clinical outcomes. Within the context of cancer treatment practice, a biomarker can therefore be identified as “biological molecule produced either by the tumour cell or by human tissues in response to cancer that is objectively measured and evaluated as an indicator of cancerous processes within the body” [206].

When several biochemical components are expressed differently in different tissues and across different disease status; they can be considered as biomarkers. Examples include DNA, RNA, proteins, hormones, metabolites, as well as biological processes such as apoptosis or angiogenesis, whereby they can be measured non-invasively in the circulation, secretions or in other biological fluids, or invasively in

biopsies for example. Current advancements into omics and large-scale microarray techniques allowed investigating the relationship between several of these components together, giving rise to the more complex and comprehensive "molecular signatures" [207]. Such an approach can be considered of vital importance in monitoring and/or predicting response to an epigenetic therapeutic, given the complex and sometime universal changes these drugs might trigger [208].

Biomarkers can be generally classified either according to their physico-chemical or physiological characteristics [209] or as per their molecular features [210]. Cancer biomarkers can be generally classified as predictive biomarkers, those that can predict the response to a particular therapeutics[211]; prognostic biomarkers, those that can inform clinicians about the likelihood of diseases progression or recurrence [212] or diagnostics biomarkers, those that can give an overview about underlying pathological conditions [213].

1.6.2 Properties of a good biomarker

The recent advancements in omics technologies [214], coupled with the previously discussed growing interest in developing a more personalised therapeutic approach that depends on understanding the molecular biology of the disease rather that its morphology [39]; these factors suggest a reduction in the cost of measuring biomarkers and new therapeutics discovery, and an increase in the former's development and application as well as the latter's specificity [39].

In order to introduce viable biomarkers; their discovery and development involves several stages starting from initial identification of changes at the molecular levels

and extends all the way to their clinical implementation [215], whereas an ideal biomarker should be developed to support the clinical decision; pre- and post-treatment. In particular, predictive biomarkers should enable the identification of patients who are more likely to benefit from a particular therapeutic agent [39].

One of the challenges underlying the development of viable biomarkers is the gap between their molecular discovery and their clinical application, where inadequate studies' design or poor reproducibility can slow their translation to the clinic. In case of diagnostic biomarkers for example, a molecular signature should enhance diagnostic efficacy in general versus in-risk populations, providing valuable clinical validity and utility, in addition to being cost-effective and ethically compliant.

In order to achieve that, a well-designed "road map", starting with identifying an "unmet clinical need", understanding molecular pathways underlying the relevant disease and the mechanism of action of relevant therapeutics should be in place. Following that initial phase, validating assay accuracy, its comprehensive reproducibility in the target group and understanding its limitations at end-points during the course of development, are detrimental in deciding the next steps in developing a clinically-valid biomarker [216]. Thereafter, pre-clinical safety assessments and conducting clinical trials should be conforming with the approval criteria, as those suggested, for example, by FDA's Center for Drug Evaluation and Research for defining the biomarker qualification process. Eventually, a biomarker approval for clinical use should be identified within the relevant context of use, level of evidence and said qualification criteria [216].

As outlined in Table 1.4, several criteria governs the development of biomarkers, where those that conform with these criteria are more likely to be useful in patients stratification and drug assignment [217].

1.6.3 Biomarkers in cancer epigenetics

The universality, widespread across the genome; stability, DNA is relatively stable, and tissue accessibility, samples can be obtained from circulating fluids, make epigenetic modifications good candidates as biological biomarkers. Several investigations identified a number of epigenetic cancer biomarkers, some of which are currently commercialised [218].

At the DNA methylation end, and as previously discussed, hypermethylation of CpG islands in cancer cells represents a major driving factor behind tumourigenesis, and therefore monitoring the relevant molecular changes is of interest. In fact, the unique profiles of hypermethylated CpG islands across different types of tumours makes them a near-universal biomarker for detecting early tumourigensis [219]. An example is death-associated protein kinase (DAPK) [220] that has been shown as a good predictive and prognostic cancer biomarker owing to its silencing via hypermethylating its promoter CpG island [219]. It was found that DAPK methylation increased during cervical cancer progression [221] and also increased the risk of relapse after therapy in bladder cancer [222]. Another classic and important example is the hypermethylation of the glutathione S-transferase gene (GSTP1) in 80–90% of prostate cancer in men, an epigenetic modification that is exclusive to malignant tissues only [223].

On the other hand, aberrant histone acetylation patterns could also serve as targets for biomarker development. In general, expression levels of HDACs have been shown to differ in specific tumour types. For example high levels of HDACs 1 and 2 were correlated with histone hypoacetylation and the subsequent tumourigensis that reduced prostate cancer patients survival [224]. Another example is CBP/p300 that were found to activate oncoproteins SV40 T and E1A, which subsequently results into the hypoacetylation of H3K18 and activation of cell growth promoters [225] [226].

According to Goossens et al, recent technologies where thousands of individual molecules can be easily interrogated supported "research hypotheses (that) are often generated in a post hoc manner, following often serendipitous discovery from unbiased mining of the genome-wide measurements (data-driven hypothesis generation)". A good example was a genome-wide loss-of-function screen previously developed in our group, which has identified human ultraviolet excision repair protein RAD23 homologue B (HR23B) as a biomarker to determine sensitivity to HDI treatment in CTCL cells [227] with further studies revealing that HDAC6 moderated this response [228] [229].

Table 1.4 Criteria a good biomarker should fulfil: Increasing the likelihood that an association is causative. Modified after Sheikh et al, 2016.

Guidelines	Class	Characteristics of useful biomarkers
High-importance		
Strength	Predictive, Prognostic	A strong association between the biomarker and the outcome
Consistency	Diagnostic	The association persists in different individuals
1.6.3.1.1 **Experimental evidence**	Predictive, Prognostic	An intervention gives results consistent with the association
1.6.3.1.2 **Temporality**	Diagnostic	The time-courses of changes in the biomarker and outcome occur in parallel
1.6.3.1.3 **Coherence**	Prognostic, Diagnostic	The association is consistent with the natural history of the disease and the biomarker
Specificity	Predictive, Prognostic	The biomarker is associated with a specific disease
Medium-importance		
Temporality	Diagnostic	The time-courses of changes in the biomarker and outcome occur in parallel
1.6.3.1.4 **Plausibility**	Predictive, Prognostic, Diagnostic	Credible mechanisms connect the biomarker, the pathogenesis of the disease, and the mode of action of the intervention
Biological gradient (dose-responsiveness)	Predictive, Diagnostic	Increasing exposure to an intervention produces increasing effects on the biomarker
Low-importance		
Analogy	Predictive, Prognostic, Diagnostic	There is a similar result from which a relationship can be concluded

1.6.4 Genome-wide loss-of-function screen

The genome-wide loss-of-function screen previously performed in our research group utilised a short/small hairpin (shRNA) library to target a wide array of oncogenes involved in human cancer under SAHA treatment in order to identify potential sensitivity determinant(s) to HDI treatment [227]. Cells were allowed to grow for 25 days under conditions of 2μM SAHA treatment, and genes were silenced via transfection with the pRETRO shRNA library, whereas surviving colonies were isolated followed by confirming a particular gene silencing in these colonies [227] (see Figure 1.5).

As previously discussed, the screen identified HR23B and follow up investigation was focussed to confirm its role in mediating cells sensitivity to HDI treatment [227]. Further investigations confirmed the previous findings in mouse embryonic fibroblasts (MEFs), where HR23B +/+ samples were found to be exhibit a higher sensitivity profile to HDI-induced apoptosis compared to HR23B -/- ones [227]. Conversely, it was found that HR23BA, an isoform of HR23B, does not mediate HDI-induced apoptosis, and that the role of HR23B in NER is not relevant to its role in mediating cells sensitivity to HDI treatment [227]. Moreover, a recent study in patients with unresectable hepatocellular carcinoma confirmed an interplay between levels of HR23B and a patients sensitivity to belinostat treatment [230].

Additionally, the same screen identified a number of genes other than HR23B that could also act as sensitivity determinants for HDI treatment, including Myeloid differentiation primary response gene 88 (MyD88), an adaptor molecule for Toll-like receptors (TLRs) [227 231].

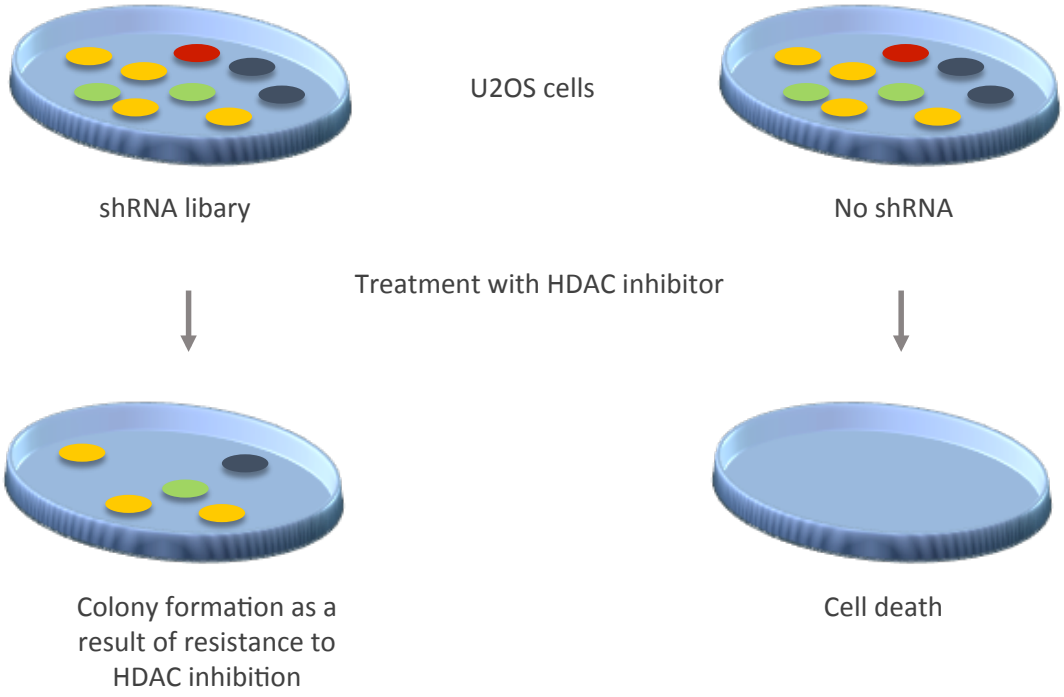


Figure 1.5 Schematic of shRNA screen. U2OS cells were transfected with a library targeting a large number of genes involved in human cancer alongside an untrasnsfected control. The cells were then treated with pan-HDI SAHA at 2µM and allowed to grow of 25 days. The colonies which survived where those in which a potential SAHA sensitivity determinant gene was silenced.

1.7 MyD88

1.7.1 MyD88: a toll like receptor (TLR) adaptor

A number of different genes have been identified as potential determinants for HDI treatment through the genome-wide loss-of function screen [227]. Although HR32B has been thoroughly investigated [227], yet another identified potential biomarker of interest was MyD88, a TLR adaptor protein, of which preliminary investigation was under its way during the start of this project.

TLRs are a class of proteins that play a key role in the innate immune response, via enhancing the ability of immune system to recognise molecules that are broadly shared by pathogens, directly and indirectly. They could be found expressed on a variety of the membranes of leukocytes including dendritic cells, macrophages, natural killer cells, cells of the adaptive immunity (T and B lymphocytes) as well as non immune cells (epithelial and endothelial cells, and fibroblasts) [232].

TLRs recognise pathogen-associated microbial patterns (PAMPs), highly conserved structural motifs, which are exclusively expressed by microbial pathogens, or danger-associated molecular patterns (DAMPs) that are endogenous molecules released from necrotic or dying cells. PAMPs include various bacterial cell wall components such as LPS, peptidoglycan (PGN) and lipopeptides, as well as flagellin, bacterial DNA and viral double-stranded RNA. DAMPs include intracellular protein, heat shock proteins for example, as well as protein fragments from the extracellular matrix. They function through one or more of their adapter proteins (see Figure 1.6) [231]. Among all TIR domain-containing adaptors involved

in TLR signalling, only MyD88 and TRIF play a role in TLR-mediated apoptosis [233].

MyD88 has a toll-like receptor interacting (TIR) domain in its C-terminal region and a death domain (DD) in its N-terminal. MyD88 dimerisation is shown to be mediated through its intermediate domain and its death domain, but not through its toll-like receptor interacting domain [234]. It interacts with the TLRs, except TLR3, through its TIR domain and, and once stimulated its death domain interacts with IL-1 receptor-associated kinase (IRAK) death domain, leading to the latter's recruitment. IRAK is then activated by phosphorylation and subsequently interacts with TRAF6 leading to the activation of two signalling pathways and the resultant TLR simulation of different other signalling pathways, including NF-κβ, IRF, MAP kinases, p38 and JUN N-terminal kinases (JNKs) [235].

Of relevant interest, TLRs act as a "double edged sword" in modulating cell survival either via directly affecting cellular apoptosis or indirectly via regulating the immune response across different subsets of cancers [236]. TLRs can induce apoptosis via affecting a downstream cascade of events that regulate a number of molecules that include Fas-associated protein with death domain (FADD), type I IFN, TNF-α, ROS and p38 MAPK [236]. TLR2 was the first to be described as a death- inducing receptor, either after transfection, or directly in macrophages, neutrophils and trophoblasts [236]. Another example is TLR3, whereby its overexpression induces PARP cleavage [237] and its siRNA knockdown was found to regulate apoptosis in breast and multiple myeloma human cancers [238].

Conversely, TLRs were also found to play a pro-survival role. TLR3 and 4 triggering was shown to overcome p27-Induced growth arrest when type I IFN signalling was blocked [239]. Also TLR9 ligation was found to induce pancreatic stellate cells (PSCs) to become fibrogenic and secrete chemokines that promote epithelial cell proliferation and to play an immuno-suppressive role in the tumour microenvironment (TME) via induction of regulatory T cell recruitment and myeloid-derived suppressor cell proliferation [240]. Another study showed that increased STAT3 activation resulted in over-expression of TLR2 to promote epithelial cell growth in gastric cancer [241]. Such opposing pro-survival and pro-apoptotic roles of TLRs remain largely unknown, with different outcomes depend mainly on cancer subsets and the associated immunological conditions.

1.7.2 MyD88 in cancer

MyD88 was shown to play a role in intrinsic oncogene-induced inflammation and in tumour extrinsic inflammation via activating the signalling of NF-κB, MAPKs, the phosphatidylinositide 3-kinases (PI3K)-Akt pathway, and IRFs 3, 5, and 7. To that end, MyD88 was shown to contribute to the development of several cancers, including skin, liver, pancreatic, and colon cancers.

Most predominantly, the clinical relevance of MyD88 was established when it was found frequently mutated in diffused large B-cell lymphoma (DLBCL) patients, particularly the activated B-cell-like (ABC) subtype [242]. RNA interference screening has also shown that MyD88 is essential for ABC DLBCL, whereby a frequent gain-

of-function mutation at amino acid 265 (L265P) leads to constitutive activation of NF-κB that subsequently promotes cell survival [243].

Other several studies have highlighted the role of MyD88 in tumouregensis and cell survival. For example, MyD88 signalling in the epithelial cells was found to play a role in skin carcinoma via the activation of EGFR–RAS–MAPK pathway in keratinocytes [244]. Additionally, Myd88−/− mice were found to be more resistant than wild type mice to the formation of papillomas, partially via a TLR4-depedant activation [245]. Besides skin carcinoma, MyD88 mediates tumourigenesis in other types of cancer as well. One study has shown the role of MyD88 in hepatocellular carcinoma, whereby Myd88−/− male mice were resistant to diethylnitrosamine (DEN)-induced carcinogensis via an IL-6 dependant mechanism [246]. MyD88 reduced the proliferation, migration, and invasion of MCF-7 cells and increased the sensitivity of MCF-7 cells to paclitaxel treatment through the inhibition of activation of NF-κB via PI3K/Akt [247]. MyD88 was also shown to contribute to TLR signalling and the subsequent development of liver fibrosis and cancer in hepatocyte-specific TAK1-deleted mice [248] and its levels have been shown to play a role in pro-tumourigenic transformation by interacting with the MAP kinase Erk, thus activating the RAS pathway [249]. Most recently, MyD88 was also found to play a role in immuno-mediated antitumour activity, whereas if induced, together with CD40, in chimeric T cell receptors (CART)-cells, results in controllable and potent antitumour activity in preclinical solid tumour models [250].

Conversely, MyD88 inhibition in vivo impaired pancreatic inflammation and tumourigensis by enhancing the capacity of DC-mediated induction of the protumourigenic TH2-type response [251].

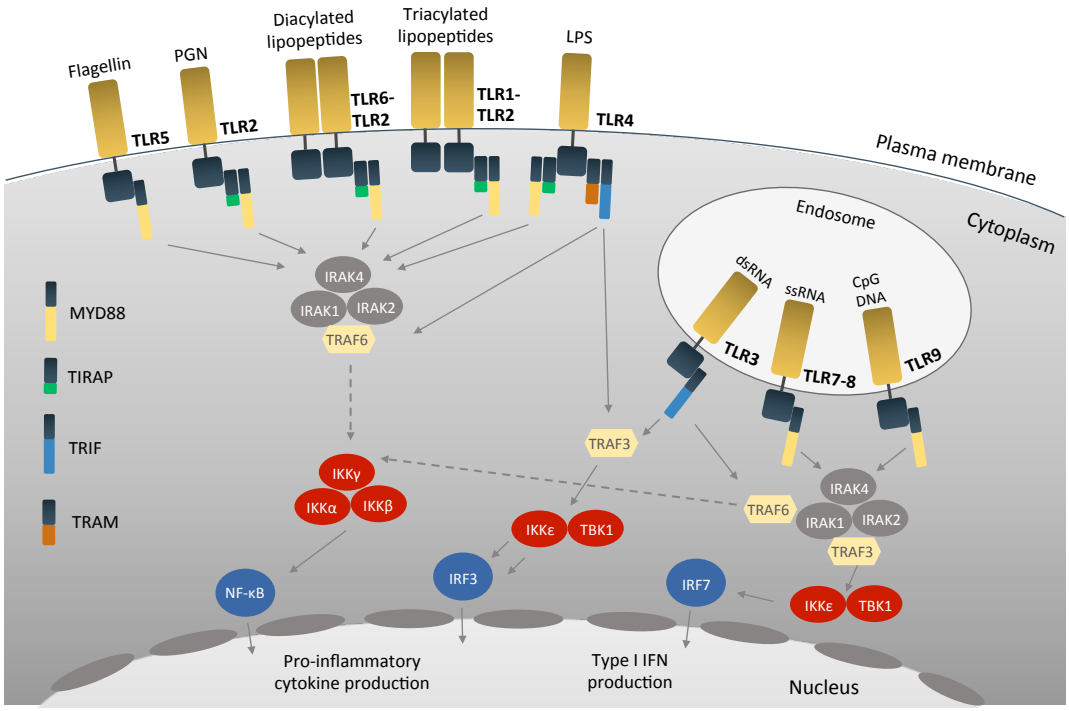


Figure 1.6 Signal transduction downstream of MyD88-dependent and independent pathways. TLRs that recognise pathogens are located on the plasma membrane (TLR1, 2,4,5,6), while those that recognise viral protein are located on the endolysosomal compartment (TLR 3,7,9) [231]. They function through one or more of the four adapter proteins: TICAM1, TIRAF, TICAM2 and MyD88 [231]. Adapted from Wang et al, 2014.

1.7.2.1 MyD88 L265P somatic mutation

Treon et al. whole genome sequencing in Waldenstrom macroglobulinemia (WM) allowed the identification of a somatic variant (T→C) at position 38182641 in chromosome 3p22.2 that results in an amino-acid change from leucine to proline (L265P) in the MyD88 gene [252]. Since then, several recent studies have confirmed the high frequency of the MyD88 L265P somatic mutation in patients with WM and IgM monoclonal gammopathy of unknown significance (IgM MGUS) by Sanger, polymerase chain reaction (PCR), and allele-specific PCR (AS-PCR) assays [253] [254].

As previously mentioned, it was found that ABC DLBCL cell lines expressing MyD88 L265P inhibits MyD88/IRAK signalling, which diminishes NF-κB signalling and promotes survival of MyD88 L265P–expressing WM cells [252]. Moreover, it was shown that inhibition of MyD88 in L265P–expressing WM cells is accompanied by decreased activation of Bruton tyrosine kinase (BTK), whereas overexpression of MyD88 L265P enhances BTK phosphorylation [255].

1.7.3 TLR-MyD88 dependent cytokines signalling

One of the major MyD88-depdenant effects on TLR signalling, that is known to play an important role in tumourigenesis and consequent cellular outcomes, is the release of inflammatory cytokines [256] [235]. Cytokines are small signalling molecules that regulate cell-to-cell communication and are secreted by specific cells of the immune system. They play a role in cellular growth, activation and differentiation whereby their local secretion becomes aberrant in tumourigenesis [257].

It was shown that the TLR-MyD88-dependent pathway activates the transcription of NF-κB activator protein 1 and Elk-1 as well the induction of proinflammatory ctyokines such as TNF-α, IL-6 and cyclooxygenase-2 [258] [259]. Activation of the NF-κB p65 and activator protein 1 transcription factors was found to be reduced in endotoxin-tolerant cells [260] . TLR7, -8 and -9 were also found capable of inducing IFN production in a MyD88-dependent manner, involving the activation of the interferon regulatory factor 5 (IRF-5) and IRF-717 transcription factors [261].

As previously discussed, it was also observed that the inhibition of the TLR-MyD88 signalling pathway in pancreatic tumour microenvironment is pro-tumourigenic owing to the DC-mediated TH2-polarization of CD4+ T cells [262]. Conversely, it was shown that IL-10 produced by DC induces ubiquitination and subsequent protein degradation of MyD88-dependent signalling molecules, such as IL-1 IRAK4 and TRAF6, to moderate MyD88-dependent, but not MyD88-independent signalling pathway [263].

1.7.3.1 Interleukin 6

IL-6 is a cytokine that acts as both a pro-inflammatory cytokine and an anti-inflammatory myokin, acting as a prosurvival as well as a protumourigenic. It is released as a result of NF-κB signalling, and affects a variety of biological functions related to inflammation. In classic signalling, interleukin-6 stimulates target cells via a membrane bound interleukin-6 receptor, which upon ligand binding associates with the signalling receptor protein gp130. Gp130 is then dimerised, activates JACK, resulting in the subsequent phosphorylation of tyrosine residues within the cytoplasmic part of gp130. This leads to the engagement of phosphatase Src

homology domains containing tyrosin phosphatase-2 (SHP-2) and activation of the ras/raf/MAPK pathway.

Downstream effects of this signalling pathway include mediating STAT3, which exhibits various effects on tumour cells and tumour microenvironment [264]. STAT3 is strongly associated with development of tumours of various etiology[265]. Although IL-6 is predominantly known as a cytokine that promotes proliferation, yet there has also been evidence of an "inverse" role for IL-6, whereby IL-6 enhances Fas-induced apoptosis in normal lung fibroblasts for example [266].

Anti-IL-6 treatment was originally developed for treatment of autoimmune diseases [267], but due to the role of IL-6 in chronic inflammation; its depletion was also considered for cancer treatment, with examples including colorectal cancer [268] and multiple myeloma [269].

Of interest, there have been also several studies that highlighted the interplay between MyD88 and IL-6 in tumouregensis. A recent study has showed that Polymyxin B, a cyclic cationic polypeptide antibiotic widely used to counteract the effects of endotoxin contamination, attenuates LPS-induced death but aggravates radiation-induced death via TLR4-MyD88-IL-6 pathway [270]. Another study showed that the suppression of MyD88 expression blocked erlotinib (EGF-receptor inhibitor)-induced IL6 secretion in vitro and increased the antitumour activity of erlotinib in head and neck cancer cells [271].

1.8 Hypothesis and aims of project

HDIs were shown to have good potency as therapeutic agents for certain haematological malignancies. However, the particular molecular pathways that regulate HDIs activities in cells are still largely unknown. One of the sensitivity determinants identified by the previously performed shRNA genome-wide screen was MyD88 [227].

Based on the loss-of-function screen (section 1.6.4), and in agreement with the literature (section 1.7), the main hypothesis of this study was that MyD88 is a predictive sensitivity determinant for HDI treatment via a TLR-dependant mechanism.

In order to address the hypothesis, the initial aim of the project was to verify that levels of MyD88 alter cell fate on HDI treatment via a series of altering both endogenous and ectopic MyD88 levels. Subsequently, it was of interest to identify mechanistically how MyD88 alters cell fate on HDI treatment, through investigating it's role as a TLR signalling adaptor, and the role of its gain-of-function mutation, L265P, coupled with monitoring changes in cellular cycle profiles and anti-proliferative effects.

Conversely, it was of interest to understand how HDI treatment and cytokines secretion influence MyD88 expression and regulation, which was pursued via a set of experiments to study changes in MyD88 regulation, stability and acetylation status and provide an explanation to how changes in such status might modulate its

role a sensitivity determinant to HDI-treatment via investigating the interplay between MyD88 and a particular HDAC, namely HDAC6.

In summary, the outcomes of this project would pave the way towards further work to utilise MyD88 as a sensitivity determinant for HDI.

CHAPTER 2 MATERIALS AND METHODS.

2.1 Cell culture.

Cells were cultured at 37^{o}C in a humidified 5% CO_2 incubator. U2OS cells were maintained in DMEM supplemented with 10% foetal calf serum (FCS) and 1% penicillin/streptomycin (Invitrogen). The inducible U2OS-TET FLAG-MyD88 and FLAG-K132R-MyD88 cell line, both created previously by Dr Maria New in the La Thangue lab [272], were maintained in DMEM supplemented with 5% Tetracycline-negative FCS (PAA Laboratories), 100µg/ml G418 (Promega), 75µg/ml hygromycin B (Invitrogen), and 1% pen/strep. Ectopic Flag-protein expression was induced by the addition of doxycycline (Dox) (Sigma-Aldrich) at 1µg/ml according to manufacturer's instructions, with subsequent addition of Dox every 24 hours.

U2OS cell lines stably expressing both pCMV-FLAG-MyD88-WT and pCMV-FLAG-MyD88-L265P, both also previously created by Dr Maria New, were maintained in DMEM supplemented with 5% Tetracycline-negative FCS (PAA Laboratories), 100µg/ml G418 (Promega), and 1% pen/strep.

HDAC6 shRNA-knockdown U2OS cell lines were created in U2OS cells using HDAC6 or control shRNA lentiviral particles (Santa-Cruz), and stable clones were selected for puromycin resistance as per the manufacturer's protocol.

U2OS cells were from ATCC. Cells were regularly tested with a Lonza mycoplasma kit. Regular immunofluorescence microscopy with nuclear staining confirmed a negative mycoplasma result. Cells were passaged twice a week and used until 16 passages.

2.2 Plasmids, transfections and transductions.

For transient transfection; cells were transfected using GeneJuice (Merck) and harvested 24-72hour after transfection. USOS cells were transfected with siRNAs against MyD88 (Smart-Pool Dharmacon) and non-targeting control NT2 (Dharmacon) using oligofectamine (Invitrogen) to a final concentration of 50nM before harvesting.

The pCMV-FLAG-WT MyD88 mutant derivatives L265P and K132R were previously created by Dr Maria New using oligonucleotides designed in accordance with Stratagene's QuikChange Site-Directed Mutagenesis kit.

pcDNA-Flag-HDAC6 and ΔBUZ were obtained from Dr. Heidi Olzscha, which were previously given as a kind gift from T.P.Yao [124].

2.3 Drug Treatments.

Cells were treated with compounds SAHA (Sigma Aldrich), CXD101 (kind gift from Dr. Susan Fotheringham), tubastatin A (Chemietek), bortezomib (Millenium), at the indicated concentrations and for the indicated times before harvesting.

2.4 Antibodies.

The following antibodies were used; FLAG-M2 (Sigma), PARP (Cell Signalling), actin (Sigma), HDAC6 (Cell Signalling), phospho-Chk1 S317 (Millipore), Chk1 (Cell Signalling), cleaved caspase 3 (Cell Signalling), MyD88 (Cell Signalling for

immunoblotting and Abcam for immunohistochemistry), p53 (Santa-Cruz), IL-6 (Abcam) and acetylated lysine (Cell Signalling).

2.5 Fluorescence-activated cell sorting (FACS).

Trypsin-harvested cells were fixed in 70% ethanol/PBS overnight at 4^{o}C and incubated for 30 min with 1x RNAseA and 20ng/ml propidium iodide (Sigma). Samples were run on a FACScan Accuri flow cytometer (BD Bioscience) and the analysis was carried out using FACS Accuri C6 software.

2.6 Immunoblotting and Immunoprecipitation.

Seeded cells were washed with PBS, harvested by scraping or trypsinisation (in case of FACS) and pelleted by centrifugation for 5 minutes at 1000 rounds per minute (RPM) at 4°C, washed in PBS.

For immunoblotting, cells were lysed in 100uL RIPA buffer (Tris 50mM, NaCl 150mM, SDS 0.1 %, sodium deoxycholate 0.5 %, Triton X 100, NP40 1%) for 1 hour on ice. Extracts were centrifuged at 13200 RPM for 10 minutes to remove cell debri.

For immunoprecipitation, cells were lysed in TNN buffer (50mM Tris-HCl pH 7.3, 150mM NaCl, 5mM EDTA, 0.5% igepal (NP-40), 0.2mM Na_3VO_4, NaF 50mM and PIC) for 1 hour, and incubated overnight at 4^{o}C with the appropriate antibody, after which 30µl IgG beads were added and incubated for 2 hours at 4^{o}C. The beads were

washed four times with 1ml of TNN buffer, and boiled with SDS sample buffer containing 2-mercaptoethanol for 5 minutes.

Protein concentration was determined using Bradford reagent (Bio-Rad), and equal levels of protein loading were confirmed with Ponceau S staining before incubating with the antibody, and unless otherwise indicated, equal amounts of total protein were used. Total protein was resolved by denaturing sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis before electrotransfer to polyvinylidene fluoride (PVDF) membrane and probed with antibody overnight at 4°C. Membranes were washed three times for 10 minutes with phosphate buffered saline (PBST)/0.1% Tween 20). Membranes were incubated with corresponding secondary horseradish Peroxidase (HRP)-antibodies for 1hr30min at room temperature before washing three times with phosphate buffered saline with Tween 20 (PBST) as above and signal detection using enhancen chemi-luminescence (Perbio). Representative blots from at least three experiments are shown.

2.7 Immunofluorescence.

Cells were seeded onto coverslips, which were washed in PBS and fixed in 3.7% formaldehyde for 15 minutes, then permeabilised in PBS containing 0.5% Triton X-100 for 5 minutes and then blocked in 5% FCS/PBS for 30 minutes at room temperature before incubation with primary antibody overnight at 4°C. Coverslips were then washed three times in PBS 0.05% Tween-20 and incubated with corresponding secondary antibodies for 30 minutes at room temperature: Alexa Fluro 488 (green fluorescence) or Alexa Fluro 594 (red fluorescence). Finally, coverslips were washed in PBS 0.05% Tween-20 and mounted onto microscope

slides using Vectashield containing 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories). Protein expression and localisation was then visualized using a B202 light microscope (Olympus) and images were taken using OpenLab software (Improvision). All samples were processed simultaneously, with three images taken per sample, and representative sections are spanning the entire region of interest.

2.8 Colony formation assay (CFA).

U2OS-TET-Flag-MyD88 inducible cells were seeded at 1000 cells/well in 6-well plates. Flag-MyD88 was induced for 4 hours prior to 24 hour of drug treatment as indicated. At 9 days post drug-treatment, cells were washed twice in PBS, fixed in methanol for 10 minutes and stained with crystal violet solution for 30 minutes before extensive washing with water. Colonies were then automatically counted using GelCount colony counter (Oxford Optronix).

2.9 Cytokine level analysis.

The BD™ CBA Human Inflammatory Cytokines Kit was used to quantitatively measure IL-8, IL-1β, IL-6, IL-10, TNF, and IL-12p70 levels. This kit uses antibody-coated beads with unique fluorescence intensities to interact with a specific inflammatory cytokine. The unique fluorescence intensities of each of the six beads permit levels of several analytes to be simultaneously assessed and then measured using fluorescence detection by flow cytometry.

2.10 Immunohistochemistry pathology examination.

This protocol was optimised by Dr Semira Sheikh. Paraffin-embedded formalin-fixed tissue on slides were cleared of paraffin in histoclear for 5 minutes, and rehydrated through graded alcohol baths by incubation for 3 minutes in 100% IMS twice followed by 70% IMS. After a rinse in water, sections were heated at 95^{o}C for 20 minutes in 1mM EDTA pH9 for antigen retrieval. Slides were incubated in 0.5% hydrogen peroxide for 15 minutes, and then washed in PBS for 5 minutes before being blocked in serum for 20 minutes. Slides were incubated in the primary antibodies overnight at 4^{o}C, followed by incubation with corresponding anti-HRP secondary antibodies, ABC reagent (Vector Laboratories) and then substrate (DAB; Vector Laboratories). Slides were placed in hematoxylin (Sigma) for 60 seconds for nuclear counterstaining and then mounted on coverslips in AquaTex (Merck). Sections were examined under a light microscope.

CHAPTER 3 . MyD88 expression regulates cell sensitivity to HDI treatment.

3.1 Introduction.

HDIs were shown to exhibit good therapeutic indexes as antiproliferative agents in the treatment of some cancers. However, the lack of knowledge of how they act at the molecular level makes their use rather limited. An unbiased genome-wide loss-of-function screen has shown reduced

An unbiased genome-wide loss-of-function screen, where an shRNA library was library to silence a number of oncogenes involved in human cancer, helped identifying several potential sensitivity determinants to HDI treatment, after isolating colonies that have survived under conditions of 2μM SAHA treatment [227].

From the screen, 132 colonies were isolated, of which 17 survived under SAHA treatment conditions, relevant shRNA inserts were identified by PCR, and 12 genes were identified, including MyD88. As previously discussed, the screen identified MyD88, whereby cells where MyD88 was depleted survived under said SAHA treatment conditions [227].

As discussed in chapter 1, MyD88 interacts with a variety of TLRs, triggering the activation of a cascade of events that leads to promoting the release of several cytokines and subsequently moderating an inflammatory response [235]. MyD88 is known to interact all TLRs through its TIR domain, excluding TLR3, and upon ligand-simulation, its death domain interacts with IRAK's death domain. IRAK is then activated by phosphorylation leading to an interaction with TRAF6 and the activation of a cascade of signalling pathways, including NF-κβ pathway [235].

Moreover, MyD88 is known to play a role in cellular apoptosis and tumourigenesis across a number of tumours such as in hepatocellular carcinoma, where MyD88-deficient male mice were resistant to DEN-induced carcinogensis via an IL-6 dependant mechanism [246], and in skin carcinoma via activating EGFR–RAS–MAPK pathway in keratinocytes [244]. MyD88 was role in immuno-mediated antitumour activity has also been recently identified, whereas, together with CD40, in CART-cells, showing antitumorigenic effects in preclinical solid tumour models upon induction [250].

In addition, its frequent mutation in DLBCL, MyD88 L265P, particularly the activated B-cell-like (ABC) subtype, was found to be essential in promoting cell survival via constitutive activation of NF-κB that subsequently promotes cell survival [242 243].

Prior to the start of this project, previous work by Dr. Maria New has shown interplay between MyD88 levels and cells sensitivity to HDI treatment. The main focus of this chapter was to further confirm the previous findings by combining different ectopic overexpression experiments and provide further evidence via endogenous knowckdown approaches. The combination of both protocols aided at avoiding driving conclusions based solely on potential off-site effects, conformational changes, and negative or positive feedback loops induced by increasing a protein expression at high levels, which might have inhibited its own transcription or caused its own degradation.

The experiments conducted in this chapter have also been designed to explore that interplay between MyD88 and HDI-induced apoptosis in more details. via a combination of measuring apoptotic markers and cell-survival assays.

3.2 Initial study into the effects of different HDIs across different cell lines.

Although SAHA was the HDI of choice throughout the previous development of the shRNA loss-of-function screen, which made it reasonable to use it throughout the course of this project, yet it was important to screen its apoptotic effect with respect to other HDIs as well.

The percentages of apoptotic cells, those in sub-G1 phase, were measured by FACS in untreated U2OS cells and cells treated with single concentrations of 5μM of SAHA, CDX101 or tubstatin, for 0, 2, 4, 8, 24, 48 and 74 hours (Figure 3.1a). No significant effect was noticed across treatment with the three HDIs up until 8 hours of treatment. Noticeable apoptosis started to take place after 24 hours of treatment, with the maximum percentage of cells in sub-G1 phase occurring after 48 hours. Tubstatin was observed to be relatively the most potent compound with a maximum response of 22.56% apoptotic cells, compared to CXD101 (16.4%) and SAHA (12.35%). The response to the drugs started to decrease gradually thereafter, showing similar or slightly lower levels of cells in sub-G1 phase at 72 hours.

Additionally, cells were treated with a variety of SAHA concentrations to determine on the most optimal one for future experiments (Figure 3.1b). Cells treated with 10 and 20μM SAHA showed the highest efficacy, with not much noticeable difference in the percentage of sub-G1 cells between the two concentrations (14.5% and 16.1, respectively). Taking these results into account, as well as the previously reported clinical C_{max} range from 1μM to 9μM [273], it was therefore reasoned that SAHA is a

suitable HDI choice, with 10μM concentration and 24 hours treatment course to be used largely across future experiments.

It was also reasoned that using the same cell line used in the shRNA screen, U2OS, was the optimum system to work with throughout the project. However, it was of interest to see whether treating other cell lines, e.g. HeLa and A549, with the same concentration of SAHA would result in different apoptotic fates.

U2OS cell line, originally known as the 2T line, is a human bone osteosarcoma epithelial that was cultivated from the bone tissue of a fifteen-year-old human female suffering from osteosarcoma. Established in 1964, the original cells were taken from a moderately differentiated sarcoma of the tibia. U2OS are frequently used to screen different effects of cancer therapeutic agents, same as HeLa cell line, which is the oldest used human cell line [274] that was derived from cervical cancer cells taken on February 8, 1951 from Henrietta Lacks, a patient who died of her cancer on October 4, 1951 [275]. A549 cells are adenocarcinomic human alveolar basal epithelial cells that were first developed in 1972 by D. J. Giard, et al. through the removal and culturing of cancerous lung tissue of a 58-year-old caucasian male [276]. Cells representative of the three cells lines where treated with different SAHA concentrations (see figure 3.1c) for 24 hours and the changes in their sub-G1 populations was also measured by FACS. Marginal differences (< +/- 5%) between the numbers of cells going into sub-G1 phase were noticed across the three cells lines. Therefore, U2OS cell line was reasoned to be the experimental system of choice.

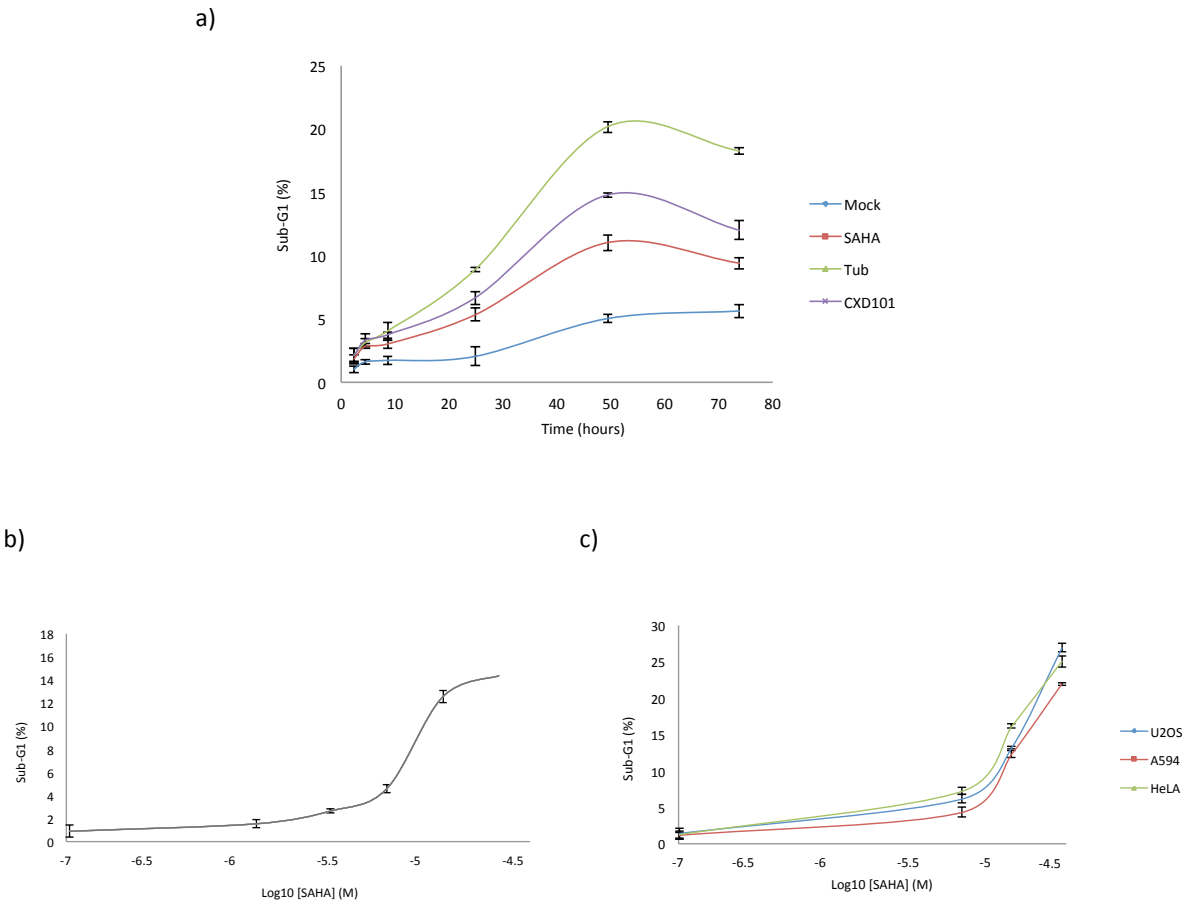


Figure 3.1 Initial analysis of the effects of different HDIs across different cell lines. (a) The percentage of cells in sub-G1 phase of the cell cycle after treatment of U2OS cells with the indicated drugs at 5µM for 0, 2, 4, 6, 24, 48 and 72 hours. (b) The percentage of cells in sub-G1 phase after treatment with increasing concentrations of SAHA for 24 hours. (c) The percentage of cells in sub-G1 phase in U2OS, A549 and HeLa cells after 24 hours treatment with 10µM SAHA. All data was normalized against vehicle control and is representative of three independent experiments. Error bars represent SEM.

3.3 MyD88 expression regulates cell sensitivity to HDAC inhibitor treatment.

One of the aims of this project was to investigate HDI treatment effects, apoptosis in particular, and how could changes into MyD88 statuses and levels possibly alter that. In order to determine the correlation between cellular MyD88 levels and apoptosis on HDI treatment, ectopic MyD88 levels were measured in a U2OS stable cell line expressing pCMV-Flag-MyD88 as well as in a conditionally regulated U2OS cell line (induction upon doxycycline treatment) stably expressing ectopic Flag-tagged MyD88 upon induction, in a Tet-on/Tet-off system, were used (developed by Dr Maria New).

One experimental method that was frequently used throughout the project to measure levels of apoptosis on HDI treatment is by observing Poly-ADP ribose polymerase (PARP) and caspase 3 cleavage levels during immunoblotting. Cleaved PARP is commonly used as a marker for apoptosis since its depletion plays a role in regulating NAD and ATP levels, which are thought to play a role in apoptosis [277]. Full-length PARP is a 116kDa protein and during apoptosis it is cleaved by caspase-3, and possibly other caspases, into a 89kDa fragment [278]. Caspase-3 is an executioner caspase that becomes activated during apoptosis, and contributes, when activated by cleavage, to the proteolytic cleavage of many key proteins [279].

Cells expressing pCMV-Flag-MyD88 were shown to exhibit higher sensitivity to apoptosis induced by SAHA in a dose-dependent manner with immunoblot results showing an increase in both PARP (cleaved) and caspase 3 (cleaved) in cells expressing Flag-MyD88 compared to cells lacking the same vector after 24 hours of SAHA treatment at the same concentrations (Figure 3.2a).

The same pattern was observed also when comparing uninduced cell (-DOX) to those where Flag-MyD88 was ectopically induced (+DOX), showing a significant increase in the levels of PARP (cleaved) and caspase 3 (cleaved) after 24 hours of 5 and 10μM SAHA treatment (Figure 3.2b).

It is worth nothing that variables including differences in SDS-gel batches, chemiluminescence reagent batches and developing films exposure times; these made it challenging to utilise cleaved PARP as a rather quantitative marker of apoptosis across different technical experimental repeats.

To further confirm these findings, another assay that was used is colony formation assay (CFA). CFA is an in vitro cell survival assay measuring the ability of a single cell to undergo "unlimited" divisions to grow into a colony [280]. Unlike measuring cleaved PARP levels, it does not give a direct measurement of cellular death, yet it presents an additive measurement of cellular proliferation. Cells were seeded in appropriate dilutions (1000 cells per a 34.8mm well, refer to section 2.8) where only a fraction of cells, that survived after a drug treatment, would form colonies that were later stained with a crystal violet dye and counted.

Changes into cellular proliferation and viability upon SAHA treatment with respect to MyD88-levels were measured by CFAs. Ectopic MyD88 overexpression (+ Dox in figure 3.2c) promoted an HDI-mediated inhibitory effect on cell growth as presented by the significant fall in the number of colonies formed in cultures treated with 5μM and 10μM SAHA treatments (for 24 hours). It was also noted that the number of colonies formed when cells were treated with 20μM SAHA was very small, irrelevant of MyD88 levels, which can be attributed to the unselective

cytotoxicity of the drug at high concentrations and for an extended period of time. Additionally, doxocyclin did not cause any changes in the number of colonies when it was used on its own, ruling out any potential additive anti-proliferative effects it might have caused.

These results suggest that MyD88 levels affect colony formation only during or after SAHA treatment, and that cells proliferation is more sensitive to SAHA treatment when MyD88 levels are high.

Moreover, experiments conducted by other members of the group has shown that MyD88 was able to senstise cells to a variety of other HDAC inhibitors, such as panobinostat, but not mechanistically unrelated agents (New et al, 2016. Supplementary Table S1) [272]. These results further confirmed that MyD88 regulates cell sensitivity to HDI treatment.

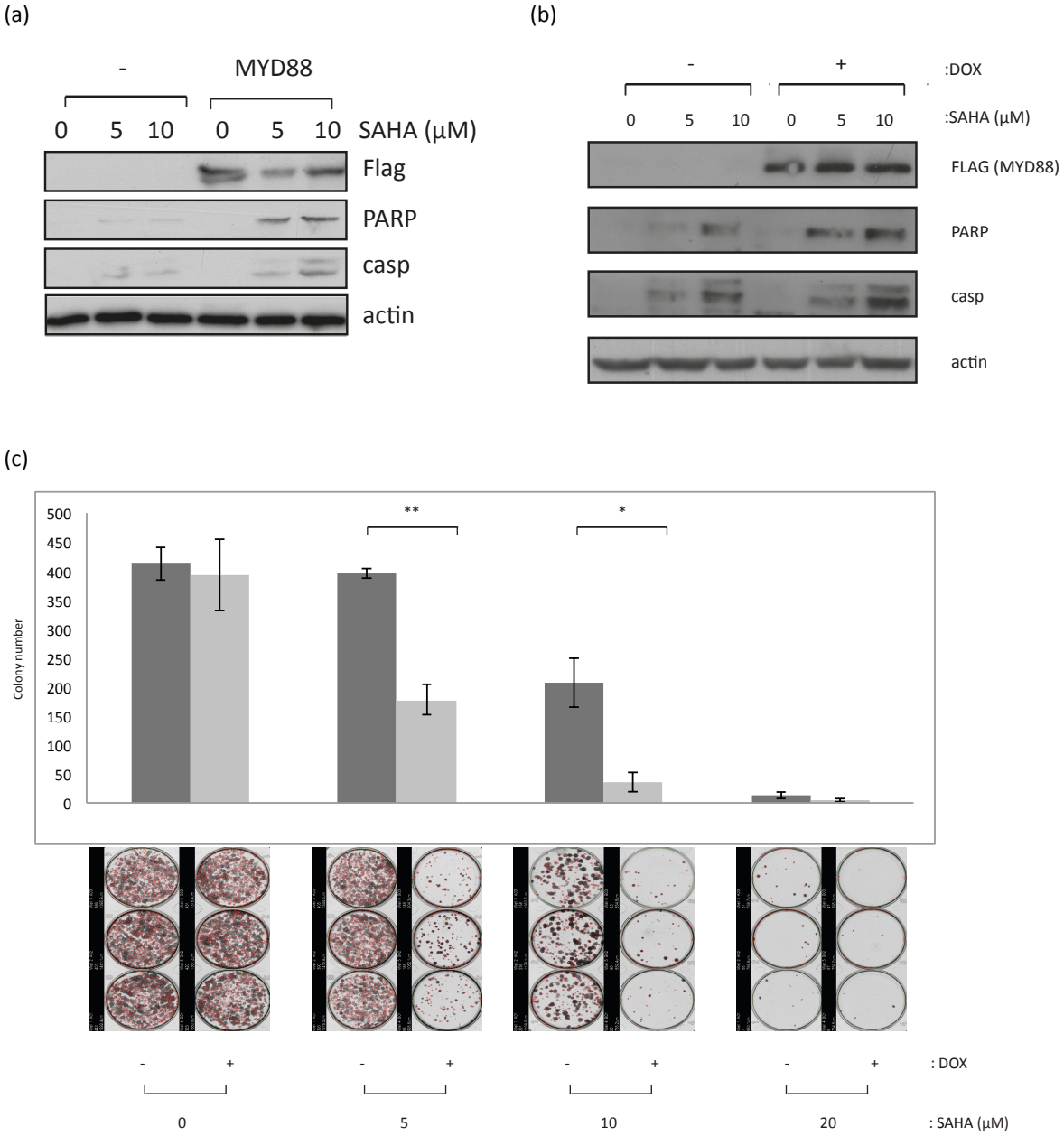


Figure 3.2 MyD88 sensitises cells to HDI-treatment. **(a)** Stable U2OS cell lines expressing an empty vector (-) or a pCMV-Flag-MyD88 one in G418-enriched media (MYD88) were treated with SAHA (5 or 10μM for 24 hours) and subsequently immunoblotted with anti-MYD88, anti-PARP (cleaved), anti-caspase-3 (cleaved), and anti-actin antibody. **(b)** Immunoblots of U2OS cells stably expressing Tet-On inducible FLAG-tagged MYD88 in the absence (-) or presence (+) of doxycycline treated as above. **(c)** Same cell line were grown in triplicate together with SAHA (5, 10 or 20μM) and, after 9 days, viable cell colonies was assessed by crystal violet staining (and quantitated). The untreated control (no SAHA) cells are shown for comparison. Graphical representation with example colony stains underneath (in triplicate). Statistical significance is indicated; * P≤0.05 and ** P≤ 0.01 and error bars represent SD. Shown examples are representatives of three independent experiments.

3.4 MyD88 knockdown reduced cells sensitivity to SAHA-associated apoptosis.

To further validate the previous findings, which showed that increasing ectopic MyD88 levels increases cells sensitivity to HDI treatment, it was essential to establish whether the opposite effect would occur as well when the endogenous MyD88 is depleted.

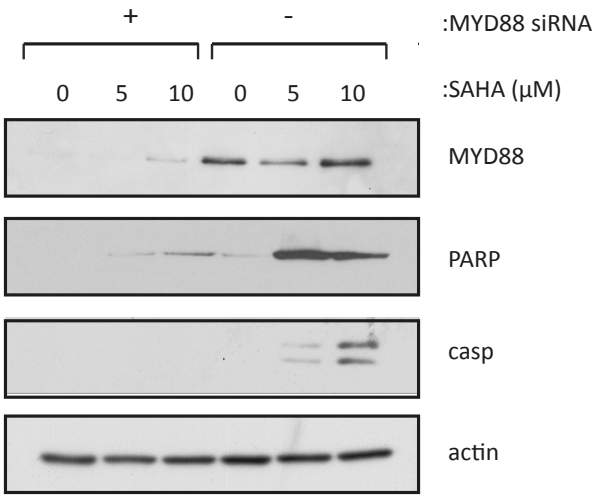
U2OS cells were transfected with MyD88 or non-targeting control (NT) siRNA (50nmol/L for 72 hours) in the presence or absence of SAHA and cell lysates were immunoblotted against anti-MyD88, anti-PARP (cleaved), anti-caspase-3 (cleaved), and anti-actin (loading control) antibodies. Cells with high levels of endogenous MyD88 levels (-MyD88 siRNA) displayed much greater sensitivity to apoptosis on HDI treatment compared to cells where MyD88 levels were reduced by siRNA (+MyD88 siRNA), as shown by PARP (cleaved) and caspase-3 (cleaved) levels (see Figure 3.3a).

Additionally, FACS measurement showed that MyD88 siRNA depletion also resulted in reducing cell population in sub-G1 phase after SAHA treatment. For example, a 10μM SAHA treatment caused lower levels of sub-G1 cells where MyD88 was knocked-down (4.6%), compared to those treated with the non-targeting control, where 11.4% of cells where in sub-G1 phase (see Figure 3.3b).

Of relevant interest, the same results were obtained in a series of similar experiments, carried out by Dr. Semira Sheikh, in RIVA cells, an ABC-like DLBCL

cell line, where MyD88 knockdown was also shown to be responsible for depleting cellular sensitivity to HDI treatment (New et al, 2016, Figure 5c) [272].

(a)



(b)

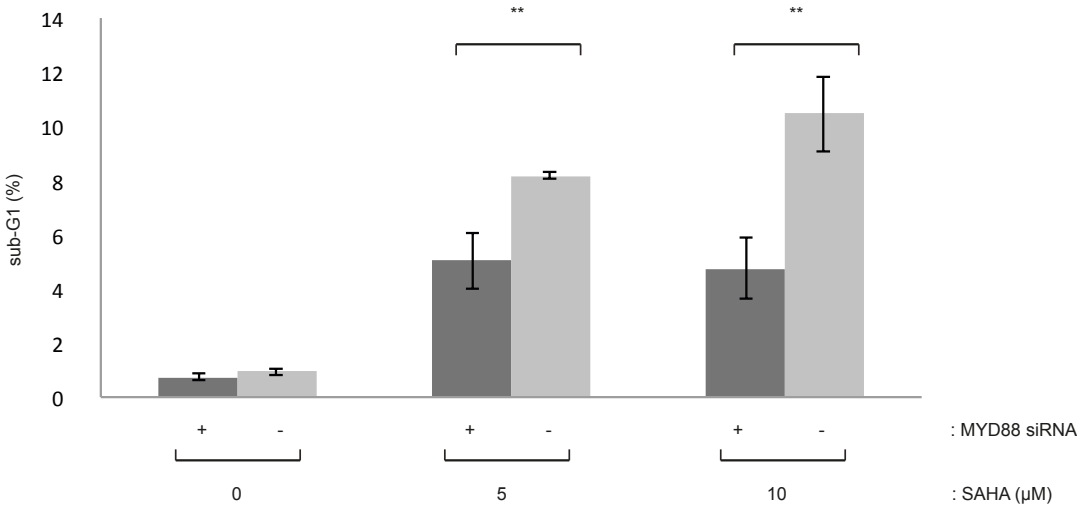


Figure 3.3 MyD88 knockdown reduces SAHA-induced apoptosis. **(a)** U2OS cells treated with MYD88 or control (NT) siRNA (50nM for 72hr) in the presence or absence of SAHA (5 or 10μM for 24hr) and subsequently immunoblotted with anti-MYD88, anti-PARP (cleaved), anti-caspase 3 (cleaved) and anti-actin antibody. **(b)** U2OS cells (in triplicate) from a) were analysed by flow cytometry. The sub-G1 phase (%) is shown. Statistical significance is indicated (**P≤0.01) and error bards represent SD. Shown examples are representatives of three independent experiments.

3.5 MyD88 changes cell cycle profiles.

Several components of the cell cycle machinery become functionally aberrant in cancer via dysregulation of a variety of genetic- and epigenetic-regulated proteins that governs the cell cycle progression from one phase to another. Tumour cells usually exhibit cell-cycle arrest profiles that occurs at the G2/M and/or G1/S interface(s), whereas regular checkpoints are compromised to allow tumour cells to progress into S phase and further proliferate. Therefore, a plethora of chemotherapeutic drugs have been developed to target such changes at the cell cycle level [281] [282], including HDIs that have shown to affect cells in S-phase through, for example, blocking the transcription of CTP synthase and thymidylate synthethase, two genes involved in DNA synthesis [104], or via disrupting the spindle assembly checkpoint and chromosomal passenger proteins as discussed in chapter 1 [165]. In addition, it was shown that different HDACs are essential for cellular progression into S-phase such as HDACs 1 and 2 role in maintaining S-phase chromatin and DNA replication fork progression [283] .

On the other hand, MyD88 has shown to play a role in regulating cell cycle progression as well. For example, overexpression of MyD88 resulted in p27 degradation; a tumour suppressor that plays a critical role in regulating progression through the G1-S phases of the cell cycle as previously discussed [239].

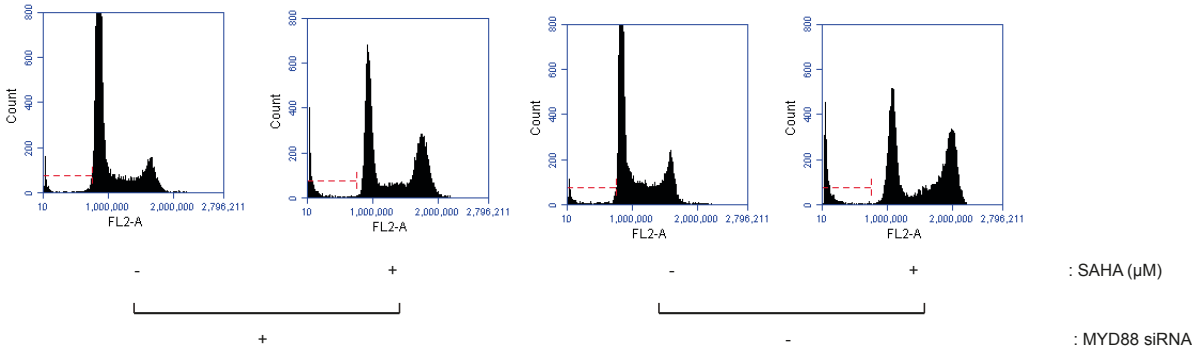
Such observations suggested that cells in S-phase may be particularly sensitive to HDI treatment, and therefore MyD88 might contribute to that role based on the previous results.

In order to test this hypothesis, FACS analysis of cellular profiles before and after SAHA treatment was conducted under high MyD88 levels (-MyD88 siRNA) as well as in cells with reduced MyD88 levels (+MyD88 siRNA) as shown in figure 3.4a. Neither changes into MyD88 levels alone, nor changes in MyD88 levels upon SAHA treatment, altered the percentage of cells undergoing S-phase (figure 3.4b).

On the contrary, the number of cells in S-phase decreased significantly before and after SAHA treatment, independent of MyD88 levels (Figure 3.4b). Although this result recapitulates previous findings where HDACs were shown to be required for S-phase progression and thus cells sensitivity to HDI treatment is partially dependant on the percentage of cells in S-phase, regardless of MyD88 levels, yet the result is not conclusive of whether cells in S-phase are more sensitive to SAHA than in other phases of the cell cycle, a property that can be further explored.

It was therefore concluded that MyD88 does not contribute to HDI-mediated changes into the S-phase on its own, and suggested that the MyD88-dependant increase into sub-G1 population upon SAHA treatment observed in previous experiments might indicate that it affected other phases of the cells cycle rather than the S-phase, which was further investigated later.

(a)



(b)

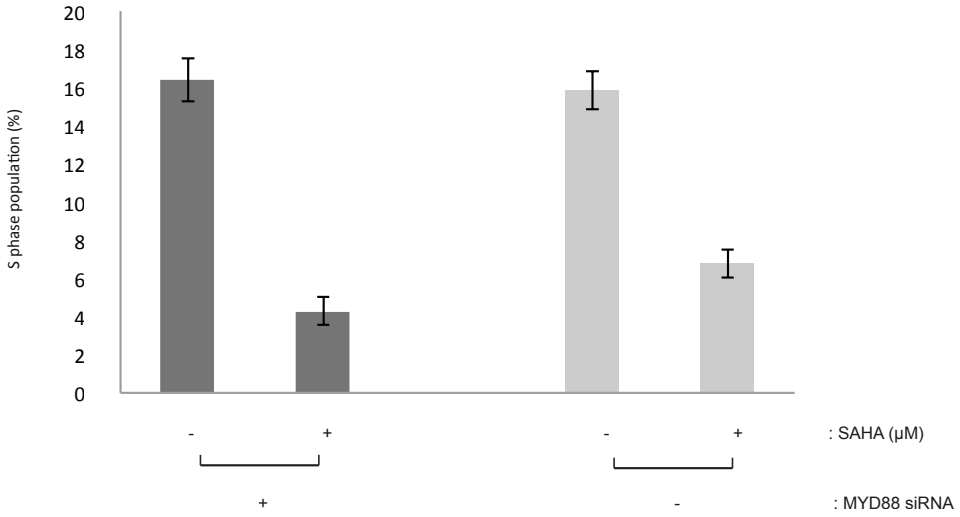


Figure 3.4 MyD88 has little effect on S-phase. **(a)** U2OS cells treated with MYD88 or control (NT) siRNA (50nM for 72hr) in the presence or absence of SAHA (10μM for 24hr). Cells were harvested and FACS analysed. The cell cycle profile of one of three triplicate plates is shown. **(b)** Quantification of the S-phase phase of the cell cycle from a). The mean reading derived from triplicate readings is shown, and the example shown is a representative of three independent experiments.

3.6 MyD88 cellular localisation.

In order to gain further insight on how MyD88 exhibits its role as a sensitivity determinant to HDI treatment, it was important to identify its cellular localisation and thereafter consider potential mechanistic interactions accordingly.

Using immunofluorescence, the localisation of MyD88 was shown to be mainly cytoplasmic in U2OS cells expressing pCMV-Flag-MyD88 (+ MyD88) as shown in Figure 3.5a. It was noted that MyD88 presented as small "speckle-like" condensed structures. One explanation could be that MyD88 exhibited condensed forms or aggregated structures in the cytoplasm, known as mydosomes, the formation of which depends on MyD88 dimerisation via cytoplasmic accumulation of sequestosome 1 (SQSTM1; also known as p62) and HDAC6, which are involved in accumulation of polyubiquitinated proteins.[284]. Mydosomes can recruit and activate IRAK 4, which in turn recruits and activates IRAK1 or 2. The MyD88:IRAK4:IRAK1/2 complex can then trigger NF-κB activation [285]. Such an explanation, along with over-ruling any artefacts-driven results, could have supported the previous conclusion via staining for endogenous MyD88, yet although several experimental attempts have been made, such a protocol proved to be challenging and the results were not conclusive.

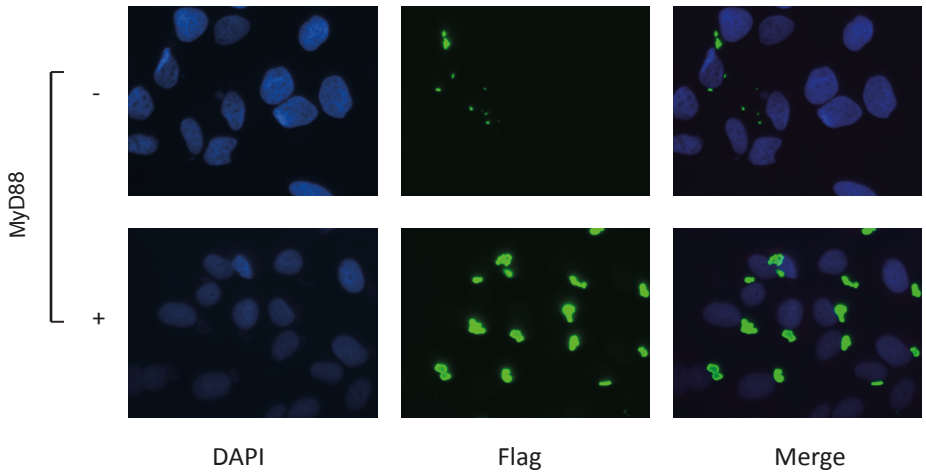
Moreover, as a potential biomarker, it was of further interest to look at the localisation of MyD88 expression in human tumour tissue by immunohistochemistry (IHC). Immunohistochemistry involves the use of specific antibodies to selectively image antigens in cellular tissues [286]. It is a widely used

technique both in basic research and clinical practice to provide better understanding of proteins and biomarkers localisation and distribution in a given sample. The technique usually involves two main steps: slide preparation and sample labelling.

IHC was performed in a small subset of biopsies from DLBCL patients with the antibody for MyD88, alongside the relevant IgG control (Figure 3.5b). IHC results have shown MyD88 to be expressed predominantly in the cytoplasm as well. Unfortunately, the limited availability of tissue samples for this experiment, which were mainly used to optimise the staining conditions, and the rather limited information provided about treatment received by each patient, made it difficult to draw conclusions out of this experiment about the impact of HDI treatment on MyD88 levels or localisation.

However, further IHC examination across a wider cohort of patients with different types of tumours was carried out by Dr. Semira Sheikh (New et al, 2016 supplementary Figure 3) [272]. A tissue microarray biopsies from patients with DLBCL were compared with biopsies taken from patients with prostate and colorectal cancer, and noncancerous tonsillar tissue biopsies; Waldenstrom macroglobulinemia. High levels of MyD88 were apparent in the DLBCL sections, but not prostate and colorectal cancer.

(a)



(b)

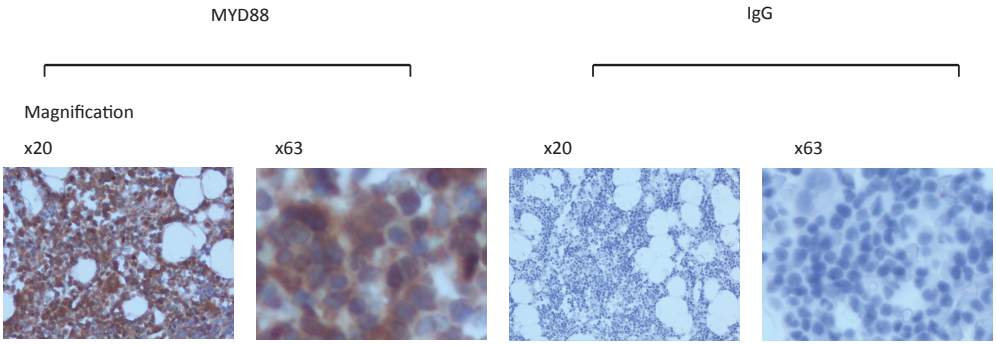


Figure 3.5 MyD88 localisation. **(a)** U2OS stable FLAG-MYD88 or pCMV (-) expressing cells were treated with SAHA (10µM) for 24 hr where immunoflourescence was carried out with ectopic Flag-MyD88 (green). Merged images with nuclear DAPI (blue) are shown. **(b)** MYD88 expression in TMA sections from a DLBCL patient stained for MYD88. IgG is was used as the negative control. All represented sections were stained under the same conditions, within the same experiment magnification is 20 and 63 times, and N=1.

3.7 Conclusion.

Overall, initial studies into the effects of different HDIs on different tumour cell lines survival have demonstrated that using U2OS cells as the biological system of choice and SAHA as a pan-HDI seemed to be reasonable. Although further investigations across an array of other cells lines utilising a wider variety of other HDIs should be considered, taking gene expression profiles of these cell lines after HDI treatment into account.

MyD88 was shown to be a valid sensitivity determinant to HDI treatment. Cells sensitivity to SAHA treatment was shown to be sensitive to both endogenous and ectopic MyD88 expression levels, where high levels of MyD88 senstised cells to SAHA and caused more apoptosis.

Although MyD88 levels contributed to changing the percentage of cells in sub-G1 phase upon SAHA treatment, yet little effect on the S-phase was observed that could be linked to MyD88 expression levels. However, and as expected, treatment with SAHA significantly reduced the number of cells in S-phase, regardless MyD88 expression levels.

Furthermore, and in agreement with the literature, MyD88 was shown to be expressed predominantly in the cytoplasm, in both U2OS cells [284] as well as in TMA sections from DLBCL patients [243].

CHAPTER 4 IL-6 and L265P-MyD88 sensitise cells to HDAC inhibition.

4.1 Introduction.

MyD88 role as a TLR adaptor protein underlies its function in mediating its effect on the inflammatory response via a cytokine-dependent mechanism as discussed in section 1.7 of the introduction.

PRPs signatures stimulate PRRs of immune cells in effected regions, which in result activate a number of pathways, inducing activation of MyD88-depedant TLR-pathway, common to all TLRs except TLR3, which in turn stimulate a range of signaling pathways including NF-κB, and enhance the transcription of the mRNA of pro-inflammatory cytokines such as IL-6 [256] [235].

IL-6 is an interleukin that consists of polypeptide cytokines with a four–α-helix structure and a molecular mass of 21 to 28 kDa. It is transiently produced in response to different stimuli, such as injuries and infections, and has pleiotropic effects on inflammation and immune response, acting as both a pro-inflammatory cytokine and an anti-inflammatory myokin. As a myokin, it is produced in muscles in response to muscles contraction, particularly during high-intensity exercising, exerting a number of anti-inflammatory functions in a fashion that is independent of TNF or NF-κB signalling [287].

On the other hand, its pro-inflammatory function is dependant on NF-κB signalling. Once released, Il-6 moderates a number of signalling pathways, including mediating STAT3, which as previously described has several effects on tumour cells and tumour microenvironment [264].

HDACs have important roles in modulating a variety of innate and adaptive immune response, particularly via IFN- and TLR-dependant pathways. IL-6 was shown to be both positively regulated by HDACs-mediated transcription [288], and in other studies the opposite effects were noticed; as the case in repressions of TLR-4 mediated chromatin modelling [289].

The clinical significance of IL-6 was first established in association with its role in autoimmune diseases [267], which has been further extended for cancer treatment due to its established role in modulating inflammation [268 269].

Several studies have shown an interplay between MyD88 and IL-6 in tumouregensis, with examples including blocking erlotinib-induced IL6 secretion upon MyD88 secretion; and therefore enhancing the antitumour activity of erlotinib in head and neck cancer cells [271].

On the other hand, MyD88 activating mutation, MyD88 L265P, that is highly present in DLBCL patients, 39%, and almost all WM patients (refer to section 1.7 of the introduction), was found to drive tumour growth and cytokine expression [242]. Intersetingly, gene expression profiling studies has shown high expression of IL-6 in WM samples [290]. This MyD88 recurring somatic mutation was proved to be associated with higher disease burden and progression; hence it can be considered as a potentially useful prognostic biomarker [252 291].

The focus of this chapter was to develop an understanding of how changes in cells response to HDI treatment as a function of MyD88 levels could affect the latter's role in cytokines regulation, and to further understand how changes into one or

more cytokine(s) level(s) might affect MyD88 role as a sensitivity determinant to HDI treatment. This chapter also investigated the changes in cells response when the mutant L265P was expressed, and how that subsequently could alter cytokines release levels.

4.2 Impact of MyD88 on cytokine expression upon HDI.

Prior to the initiation of this project, it was reasoned that as an inflammatory mediator, MyD88 effects on cytokines production might reflect upon its previously established role as a sensitivity determinant for HDI, and therefore further investigation to understand that role was carried out.

U2OS cells stably expressing Tet-On FLAG-tagged MyD88, non-induced (-) or induced (MYD), under doxycycline (DOX) treatment (1μg/ml; induction for 72 hours treatment) were harvested, nuclear fractions were extracted and immunoblotted with anti-FLAG (for ectopic MyD88), NF-κB p65 and actin antibodies as indicated (Figure 4.1a). It was observed that increasing levels of ectopic MyD88 caused activation of NF-κB, which recapitulates previous literature findings (refer to section 1.7).

It was then of interest to measure the extracellular levels of different cytokines regulated by NF-κB with respect to MyD88 levels as well as SAHA treatment conditions. A human inflammatory cytokine kit BD™ Cytometric Bead Array (CBA) kit was used in this investigation for that purpose to monitor levels of six inflammatory cytokines; namely IL-10, IL-8, IL-6, TNF-α, IL-12P70 and IL-1β (see Figure 4.1b). The kit provides a series of particles with distinct fluorescence

intensities to simultaneously detect and form complexes with multiple soluble analytes (cytokines) in the fluid (cell culture media), which then provides a fluorescent signal, proportional to the amount of cytokine, that can be measured using flow cytometry.

U2OS cell lines stably expressing Tet-On inducible FLAG-tagged MyD88 were seeded in triplicates and then treated with DMSO (control) and SAHA (10μM) for 24 hours in the presence and absence of doxycycline to induce ectopic MyD88 expression. Media was then collected from the cells cultures and levels of the 6 cytokines were measured by flow cytometery and subsequently plotted relative to the control-treated MyD88-uninduced cells (Figure 4.1b).

The relative levels of the six inflammatory cytokines measured are shown in Figure 4.1b. A media alone control was used to role out the effect of the potential presence of any inflammatory cytokines in the cell culture media fetal calf serum.

IL-12P70, IL-10, IL-1ß and TNF-α levels remained almost the same, and relatively similar to their levels in media alone, across all different MyD88 levels and SAHA treatment conditions (Figure 4.1b-iii, iv, v and vi respectively). Therefore, these cytokines were not investigated any further during the course of this project.

However, MyD88 induction and SAHA treatment changed IL-6 (see Figure 4.1b-i) and IL-8 (see Figure 4.1b-ii) levels significantly. IL-6 levels increased around twelve folds whereas IL-8 increased almost 8 folds, upon MyD88 induction, which is consistent with previous studies that have shown MyD88 to regulate the mRNA levels of IL-6 and IL-8 production and their activation by NF-κB [292 293].

Most interestingly, IL-6 levels were considerably reduced on SAHA treatment in MyD88-induced cells (see Figure 4.1b-i). Previous studies have also shown the effect of SAHA treatment on IL-6 expression and secretion levels, for example in osteoarthritis patients [294]. It is worth noting that levels of IL-6 levels were slightly higher in induced cells treated with SAHA treatment compared to uninduced ones under the same SAHA treatment conditions (Figure 4.1b-i).

On the other hand, IL-8 levels upon SAHA treatment were not as sensitive to MyD88 induction, with uninduced cells treated with SAHA showing 5-folds increase. Also relatively similar levels of IL-8 were seen in MyD88 induced cells media before and after SAHA treatment (Figure 4.1b-ii).

It was therefore of interest to further investigate the MyD88:IL-6:HDI interplay and how it could regulate cellular apoptosis associated with HDI treatment. Other members of the Cancer Biology group, University of Oxford, have also pursued further investigation on the MyD88-HDI-apoptosis-IL-8 relationship as recently published [272].

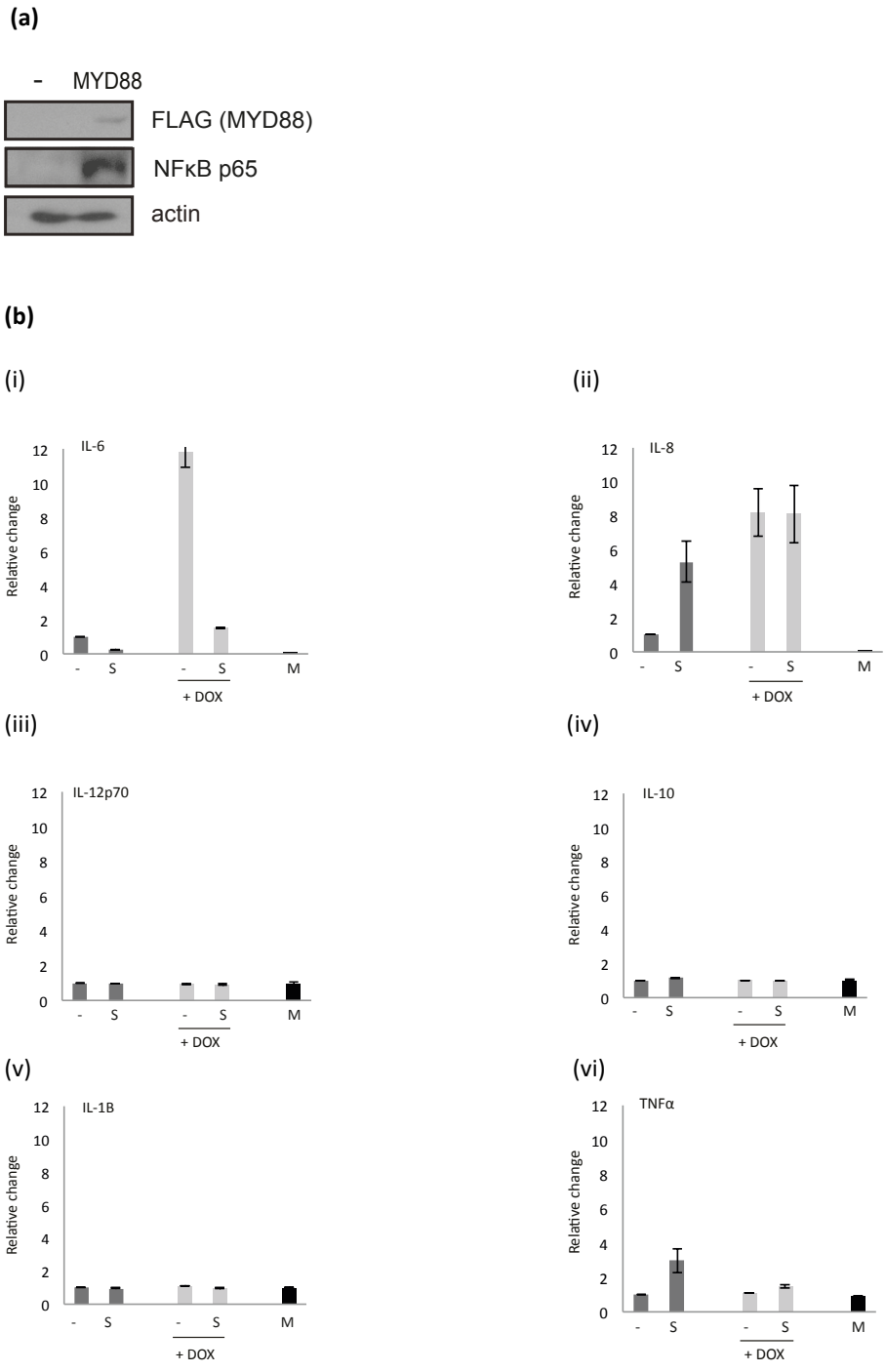


Figure 4.1 Impact of MyD88 on cytokine expression upon HDI. **(a)** U2OS cells stably expressing Tet-On inducible FLAG-tagged MyD88 under non-induced (-) or induced (MYD/+) doxycycline (DOX) treatment conditions (1µg/ml; induction for 72 hr treatment) were harvested, nuclear fractions were extracted and immunoblotted with anti-FLAG (for ectopic MyD88), NF-κB p65 and actin antibodies as indicated. **(b)** Cells from (a) were treated with SAHA (S; 10µM) for 24 hours, and relative levels of the indicated cytokines (IL6, IL8, TNFa, IL12p70, IL10, and IL1b) in the tissue culture media were measured using BD Cytometric Bead Array Human Inflammatory Cytokine measurement kit. The reading for preculture medium (M) is indicated. Mean values from triplicate readings of three independent experiments.

4.3 IL-6 neutralisation reduces MyD88-dependent HDI-mediated apoptosis.

Now that the relationship between MyD88 and IL-6 levels and its association with HDI treatment was established, the next step was to investigate whether IL-6 played a role in mediating MyD88 role as a sensitivity determinant to HDI treatment. An antibody that was previously used to effectively neutralise IL-6 in cell culture was used for the purpose of that experiment (advised by Dr Maria New) [295].

Apoptosis was abolished in U2OS cells stably expressing Tet-On inducible FLAG-tagged MyD88 and treated with SAHA as well as the IL-6 neutralising antibody as measured by PARP (cleaved) (Figure 4.2a), which suggested IL-6 role in moderating MyD88 role in the apoptoic response to SAHA treatment.

The same pattern was observed when different sub-G1 cells populations under SAHA treated conditions were compared by FACS (Figure 4.2b), whereby the percentage of apoptotic cells declined to 16.32% when IL-6 was neutralised in MyD88-induced cells, compared to cells where IL-6 was not neutralised (22.91%). No significant effect was seen though before and after IL-6 neutralistion in uninduced cells under the same SAHA treatment conditions.

In CFA, the number of USOS colonies formed by MyD88-induced cells were reduced significantly as expected upon SAHA treatment, yet the change in that number upon IL-6 neutralisation was quite moderate compared to the previous differences in apoptotic levels before and after IL-6 neutralisation (Figure 4.2c-i and

ii). Interestingly though, in un-induced cells, the number of colonies has declined significantly upon treatment with both IL-6 and SAHA.

One explanation for this could be that the interplay between MyD88 and HDI via an IL-6 dependent mechanism majorly influences apoptosis rather than cellular growth (Figure 4.2c-i and ii). Another explanation could be attributed to the treatment time (9 days), which made MyD88-cells more vulnerable to SAHA toxicity, where the differences before and after IL-6 neutralisation became insignificant. A third explanation could be attributed to IL-6 proliferative effects, whereas if inhibited, cellular progression would slow down, making it not possible to monitor changes as a function of cells sensitivity to HDI treatment.

It is worth mentioning that (work done by Dr Semira Sheikh) treating RIVA cells (ABD-DLBCL cells) with anti-TLR4 antibody has resulted in reducing SAHA-mediated apoptosis as presented by reduced levels of PARP (cleaved) as shown in Figure 5D at New et al., 2016 [272].

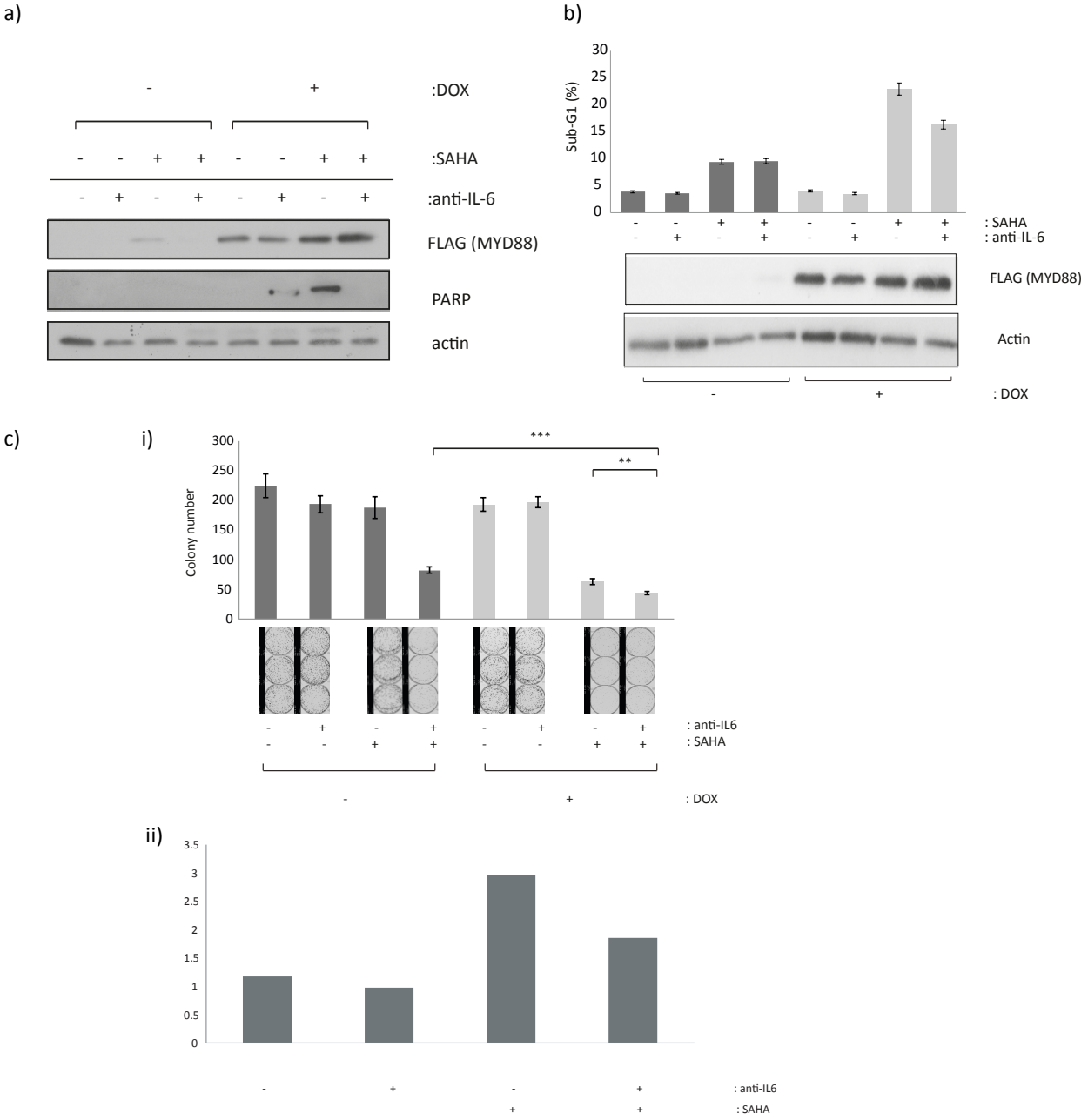


Figure 4.2 Interplay between IL-6 and MyD88 and its effect on HDI-mediated apoptosis. U2OS cells stably expressing Tet-On inducible FLAG-tagged MYD88 were treated with SAHA (10μM; +) and anti- IL6 neutralizing antibody (2 mg/mL) for 24 hours under noninduced (-) or induced (+) doxycycline (DOX) treatment conditions (1μg/ml; induction for 72 hours prior to treatment). **(a)** Cells were immunoblotted with anti-FLAG (for ectopic MYD88) or PARP (cleaved) and actin antibodies as indicated. Quantification indicated that cleaved PARP decreased by over 50% upon IL6 neutralization under conditions of MYD88 induction (+ DOX) and SAHA treatment. **(b)** U2OS cells stably expressing Tet-On inducible FLAG-tagged MYD88 were treated as in (a) and analyzed by flow cytometry for the level of sub-G1 phase cells (as a percentage of the total population). Immunoblot shows the level of MYD88 (anti-FLAG) and actin. **(c)** Same cells line were grown in triplicate together under the same treatment conditions and, after 9 days, viable cell colonies was assessed by crystal violet staining (and quantitated). The untreated control (no SAHA) cells are shown for comparison. Graphical representation with example colony stains underneath (in triplicate) (c-i) with a representation of relative change in colonies numbers after induction (c-ii). Statistical significance is indicated; *** P≤0.005 and ** P≤ 0.01 and error bars represent SD. Shown examples are representatives of three independent experiments.

4.4 Effect of IL-6 neutralisation on cell cycle progression.

To further understand how IL-6 altered cells response to HDI-mediated apoptosis in a MyD88-expression-depedant way; it was of interest to investigate its influence on cell cycle progression, a hypothesis that resonated with previous studies highlighting the role of IL-6 in promoting cellular growth [265] [296].

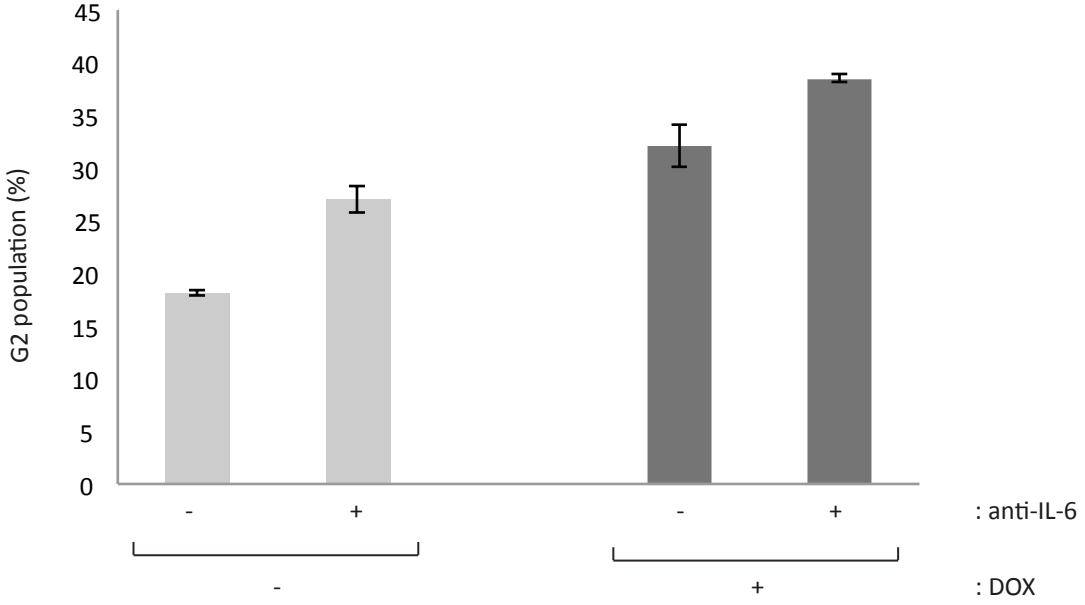
U2OS cells stably expressing Tet-On inducible FLAG-tagged MyD88 were treated with the IL-6 neutralising antibody under MyD88 uninduced (-DOX) and induced +DOX) conditions (Figure 4.3a) and cell cycle profiles were analysed by FACS. IL-6 neutralisation under conditions of MyD88 induction resulted in a higher percentage of cells in the G2 phase (40%) compared to both MyD88-induced cells where IL-6 was not neutralised (33%), as well as uninduced cells treated with IL-6 neutralising antibody (29%). These results suggested an interplay between cell cycle arrest in G2 and IL-6 neutralisation under conditions of MyD88 induction.

Because the cell-cycle profile of MyD88-induced cells treated with SAHA where IL-6 was neutralised was similar to that of an active G2 checkpoint, G2 check-point activation was studied. Protein lysates from cells under conditions of MyD88 induction, SAHA treatment and IL-6 neutralisation were immunoblotted to observe levels of Chk-1 phosphorylation on serine 317 u (Figure 4.3b). Chk1 is usually activated in response to replication blocks and certain forms of genotoxic stress whereby phosphorylation of serines 317 and 345 occur, which leads to cell cycle arrest during DNA damage [297]. Enhanced phosphorylation occurred at S317 upon IL-6 neutralisation (Figure 4.3b), consistent with a role of Chk1 in the G2

checkpoint and the increased cells population at G2 phase. An increase in phosphorylation at S317 also occurred in uninduced cells, irrespective of SAHA treatment conditions, although one would expect that, as a DNA damaging agent, SAHA treatment would have enhanced S317 phosphorylation. However, the effect in MyD88-induced cells was more apparent and dependent on SAHA treatment. These results suggest that an effect of IL-6 is to allow cell-cycle progression via a MyD88-mediated process and therefore to promote proliferation, whereby HDI treatment is more likely to target proliferating cells, which might therefore provide an explanation to the previously seen reduction of MyD88-dependant HDI-mediated apoptosis when IL-6 was neutralised.

It is worth noting though that IL-6 is not the only cytokine that MyD88 might mediate its cell cycle effects. Another previously mentioned example (chapter 1) is a study where it was found that flagellin, a TLR5 agonist, can induce cell cycle entry by overcoming p27-induced cell cycle arrest though a MyD88-dependent pathway as well [239].

a)



b)

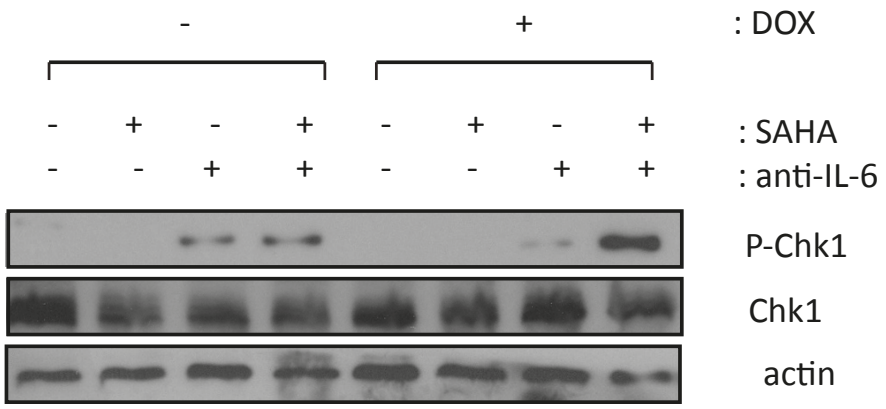


Figure 4.3 Effect of IL-6 neutralisation on cell cycle progression. **(a)** U2OS cells stably expressing inducible FLAG-tagged MYD88 were treated with IL-6 neutralising antibody (2µg/ml) for 24 hr under non-induced (-) or induced (+) doxycycline treatment conditions (1µg/ml; induction for 72 hr treatment). Cells were harvested and FACS analysed. The mean reading derived from triplicate readings is shown. **(b)** Cells from (a) were harvested and immunoblotted with anti-phospho-Chk1 (S317), Chk1 and actin antibodies as indicated. SAHA (10µM) treated cells were also included in the immunoblot analysis. Error bars represent SD. Shown examples are representatives of three independent experiments.

4.5 MyD88-L265P mutation sensitises cells to HDI.

As discussed in section 4.1, MyD88 is expressed at high levels in the hematopoietic lineage with L265P mutant being its most abundant isoform in a large number of ABC subtype of DLBCL and in almost all WM patients. Due to the significance of its clinical expression profile, L265P became an interesting research target recently, with several research groups screening its potential use as a biomarker across different types of cancers. Recently, a patient diagnosed with WM with secondary skin infiltration by clonal lymphoplasmacytic cells was also identified as MyD88-L265P positive [298]. L265P is believed to be an oncogenic gain-of-function mutation, which augments signalling through NF-κB. It was reasoned therefore that L265P might have an impact on MyD88 as a sensitiser to HDI treatment.

U2OS cells were transfected with an empty vector (-), FLAG-MyD88 (WT), or FLAG-L265P. Different concentrations of SAHA or DMSO were added, and 24 hours later cell lysates were immunoblotted. Cells transfected with the mutant-type MyD88-L265P showed higher levels of PARP (cleaved) and caspase 3 (cleaved) upon SAHA treatment compared to those expressing the wild type Flag-MyD88 (Figure 4.4a).

The relevant normalised quantification of caspase 3 (cleaved) shows approximately 20% increase of expression levels in cells transfected with the L265P plasmid treated with 10uM of SAHA compared to cells transfected with the WT MyD88 under the same SAHA treatment conditions (Figure 4.4b).

It is worth mentioning that reduced levels of FLAG-L265P in SAHA-treated cells were reproducibly observed in a noticeable number of similar experiments (as shown in Figure 4B at New et al., 2016 [272]); with the reasons for the reduction remained unclear. Yet, the enhanced levels of PARP (cleaved) and caspase 3 (cleaved) remained always enhanced compared to those of the wild type. That might be explained whereas the L265P mutant is transcribed into a MyD88-protein version of different stability compared to the wild type. Several protein rescue experiments were conducted, yet a conclusive result could not be drawn due to the continuously changing levels of L265P expression.

These results were further confirmed, by Dr Semira Sheikh, in two pathologically relevant ABC-like DLBCL cell lines, RIVA (MyD88 wild-type) and HBL-1 (heterozygous L265P-MyD88), whereby it was found that HBL-1 cells were more sensitive than RIVA cells to SAHA treatment [272]. Additionally, a very recent study has shown panobinostat to act synergistically with ibrutinib in diffuse large B cell lymphoma cells with MyD88 L265 mutations [299].

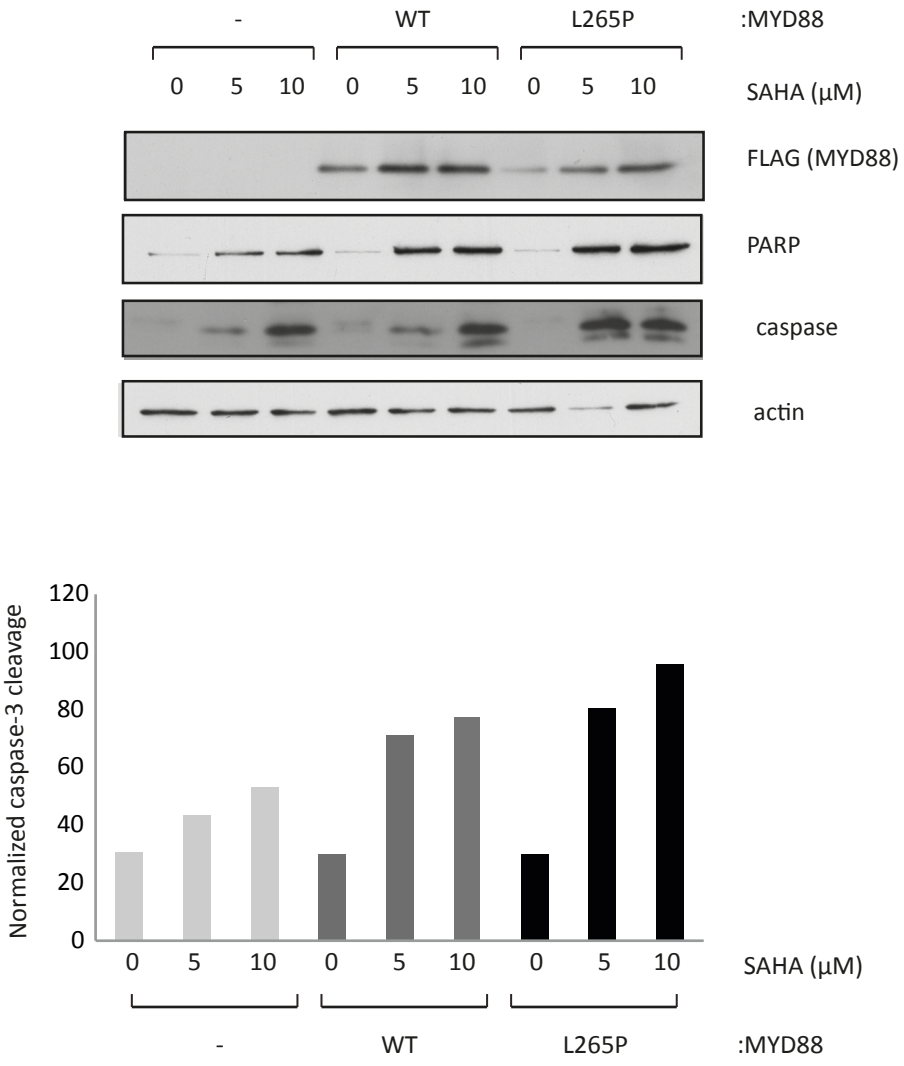


Figure 4.4 HDI effect on Wild type MyD88 vs L265P mutant. **(a)** U2OS cells were transfected with empty vector (-), FLAG-MYD88 (WT), or FLAG-L265P. SAHA (5, 10, or 20μM) or DMSO (0) was added, and 24 hours later, cells were immunoblotted with FLAG, caspase-3 (cleaved), or actin antibodies. **(b)** Graph represents the quantitated caspase-3 (cleaved) from (a).

4.6 L265P influence on cytokines levels.

The next step of investigating how L265P might act as a sensitiser to MyD88's role as a sensitivity determinant to HDI treatment is to study whether that effect is also influencing IL-6 expression levels and therefore might provide an explanation within the context of the previous results.

Using the BD™ Cytometric Bead Array (CBA), the relative levels of IL-6 in media were measured in cell cultures expressing pCMV-Flag-WT MyD88 wild type as well as derivative L265P (Figure 4.5), before and after SAHA treatment. L265P showed a 25-fold increase in IL-6 levels, compared to a 10-fold increase in case of the wild-type.

Most significantly, IL-6 levels in the mutant cells dropped considerably post-HDI treatment, exhibiting 2-times fold IL-6 expression level compared to the control, almost similar to that of the wild-type cells after treatment with SAHA as well. Collectively, these results suggest that the increased sensitivity of cells expressing L265P could reflect on the ability of IL-6 to promote proliferation, which confers with previous studies.

MyD88-L265P signalling was found to be constitutively active in both WM and DLBCL cells leading to increased levels of MyD88-L265P, IRAK and TRAF6 oligomerization and NF-κB activation. Also TAK1, a signalling protein, was found to be an essential mediator of MyD88-L265P-driven signalling, cellular proliferation and cytokine secretion in malignant B cells [254].

All together, these results provided promising evidence that MyD88 could be considered as a molecular candidate of potential interest to assess how the ABC subgroup in general, and the L265P subgroup in particular, responds to HDAC inhibitor-based therapeutics.

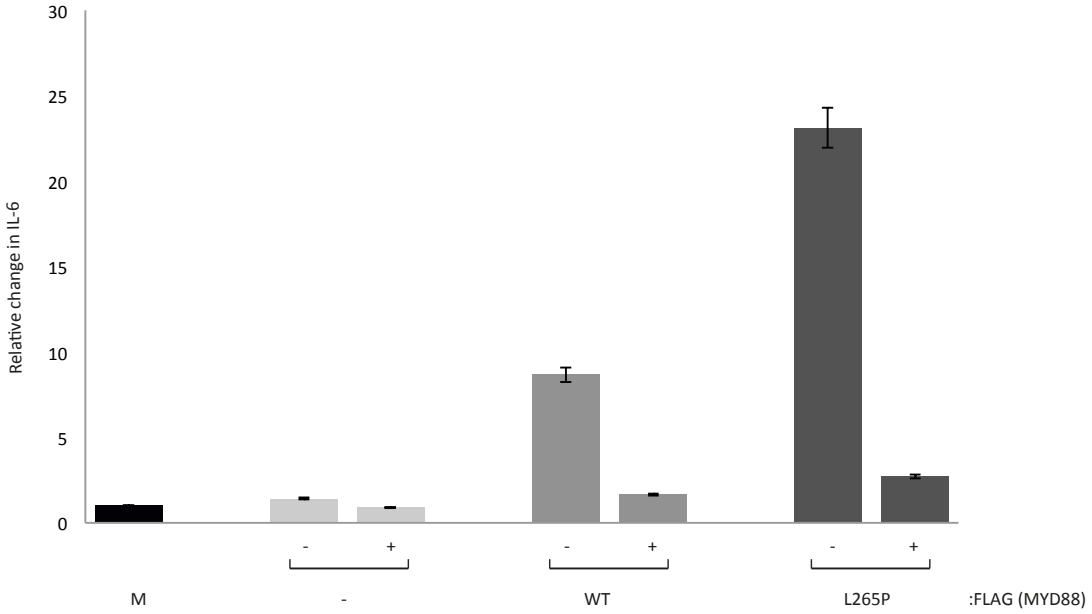


Figure 4.5 L265P influence on IL-6 expression. Relative levels of IL-6 in tissue culture medium (M) measured using BD Cytometric Bead Array Kit. Mean values from triplicate readings are shown in U2OS expressing WT or L265P MYD88 in the presence or absence of SAHA treatment (5μM). Error bars represent SD. Shown examples are representatives of triplicates of three independent experiments.

4.7 Conclusion.

Inducing ectopic MyD88 in FLAG-WT U2OS cells was coupled with an increase in the NF-κB p65 expression, which has subsequently resulted into the activation of several cytokines, including IL-6 and IL-8. Yet, only IL-6 levels changed upon SAHA treatment (Figure 4.1).

Further investigation showed that IL-6 neutralisation reduced cellular sensitivity to HDI treatment in cells rich with MyD88. That effect was more pronounced at the apoptotic level (Figure 4.2), which could be explained as a function of the autocrine action of IL6 in mediating cell sensitivity to HDAC inhibitors [300] [301].

It was also shown that IL-6 release as a consequence of MyD88-dependent signalling enabled cell cycle progression through the G2 phase check point which promoted faster cellular proliferation, making cells more vulnerable to HDI treatment, whereas IL-6 neutralisation resulted in cell cycle arrest at the G2-phase and the phosphorylation at S317, and therefore can explain the reduced overall cellular sensitivity to HDI treatment. These results recapitulates previous studies showing HDIs affecting mainly proliferative cells [302].

L265P, MyD88's gain-of-function mutation in ABC DLBCL and WM patients [243] [252], was shown to also sensitise cells to HDI-mediated apoptosis (Figure 4.4). That result coincided with an increase in the levels of IL-6 secretion in cells expressing L265P, which might therefore explain the enhanced sensitivity of L265P-expressing cells to SAHA treatment within the highlight of the previous findings (Figure 4.5).

CHAPTER 5 MyD88 acetylation.

5.1 Introduction.

After the identification of MyD88 as a potential sensitivity determinant to HDI in the shRNA loss-of-function screen, a set of different experiments were subsequently deployed to further explore whether manipulating MyD88 levels would alter cells response to HDI treatment. Cells expressing high levels of ectopic MyD88 have exhibited higher apoptotic profiles, and vice versa, those cells where endogenous MyD88 was depleted became less sensitive to SAHA treatment (Chapter 3).

As a TLR adaptor protein, it was reasoned that different MyD88 levels would trigger different cellular responses as a result of modulating NF-κB pathway activation and inflammatory cytokine release. It was shown that MyD88-assoicated IL-6 release prompted apoptosis upon HDI treatment (Chapter 4). However, these results did not provide an explanation to the molecular interaction between MyD88 and HDACs, and how could HDIs inhibition of deacetylation would inactivate MyD88.

As an HDI, SAHA has been previously shown to induce lysine acetylation in histone, for example in NB cells, where a quantitative proteomics study detected and quantified 28 histone lysine acetylation marks, the majority of which induced by SAHA [303].

Moreover, a recent study has shown that TLR4 gene transcription is epigenetically suppressed by treating Sprague–Dawley rats with kainic acid-induced seizures with SAHA [304].

As a result, it was hypothesised that the interplay between HDI and MyD88 may occur through promoting the latter's acetylation directly, or indirectly via the acetylation of associated proteins.

Until the initiation of the relevant background work by other members of the group and the publishing of this work, MyD88 acetylation has not been reported in the literature. Yet, directly prior to initiating this project, Dr Maria New, in collaboration with Benedikt Kessler's group, has been able to identify an acetylated lysine residue in MyD88 by tandem mass spectrometry analysis, whereby lysine (K) 132 was identified as a MyD88 acetylated residue [272]. Altogether, these observations formed the fundamentals to pursue further validation of MyD88 acetylation by SAHA, and to investigate how such an interaction would affect MyD88-HDI interplay and the subsequent effects on cellular apoptosis.

5.2 MyD88 is acetylated.

In order to validate the previous findings, cells stably expressing pCMV-Flag-WT MyD88 were treated with SAHA (to promote acetylation) for 16 hours and then immunoprecipitated with FLAG (MyD88) or nonspecific antibody (NS) prior to immunoblotting with MyD88 (FLAG) and acetyl-lysine antibody (Ac-K).

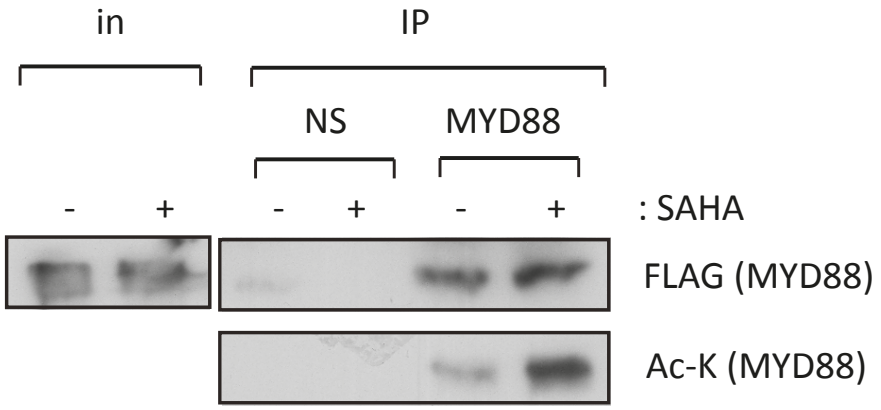
It was observed that a band appeared when the MyD88-immunoprecipitated protein lysates of the SAHA-treated-cells were probed with the Ac-K antibody, which overlapped with another band at the same molecular weight when the membrane was probed with FLAG-MyD88 (Figure 5.1a).

Conversely, a much weaker band was apparent at the same molecular weight in untreated cells upon probing with the Ac-K antibody (Figure 5.1a). Upon quantification, the relative change in the intensity of the acetylation band, normalised to the relevant control band, was shown to be almost twice the intensity SAHA-treated cells (Figure 5.1b).

In order to validate that this interaction is MyD88-specific, a non-specific IgG control of the same species as the MyD88 antibody was used, where a band at the same molecular weight was almost non-existent.

Furthermore, the endogenous MyD88 was also found to be acetylated in U2OS cells upon SAHA treatment (work conducted by Dr Maria New). These results collectively confirmed previous findings showing that MyD88 was acetylated.

a)



b)

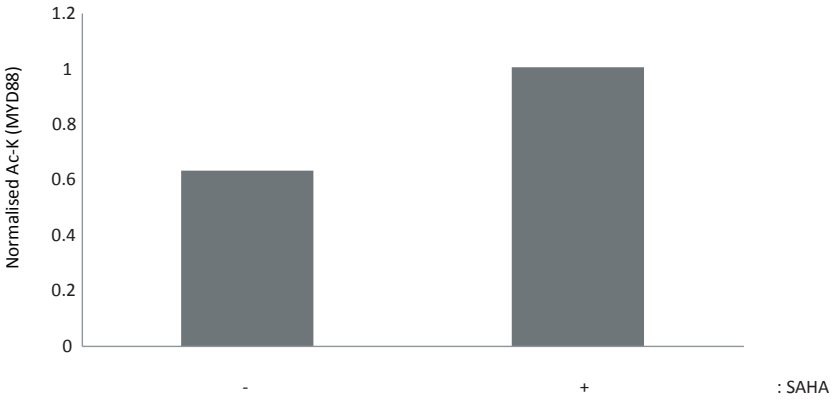


Figure 5.1 MyD88 is acetylated. **(a)** Cells stably expressing FLAG-MYD88 were treated with SAHA (+; 10µM) for 16 hours and immunoprecipitated with FLAG (M88) or nonspecific (NS) antibody prior to immunoblotting with MYD88 and acetyl-lysine antibody (Ac-K). Input (2%) level of MYD88 is shown. Shown example is representative of three independent experiments. **(b)** Graph represents the quantitated Ac-K (MYD88) from (a).

5.3 MyD88 acetylation enhances HDI cellular apoptosis.

In order to further determine which of the MyD88 10 lysine residues could be acetylated, an inducible cell line where MyD88 acetylation was mutated by site-directed mutagenesis was used against inducible Flag-WT MyD88. In the mutant cell line, Lysine 132 (K132), the MyD88 acetylated residue identified by previous mass spectrometry experiments, was replaced by arginine 132 (R132) to silence the MyD88 acetylation, where the mutant K132R could be induced upon doxycycline addition (provided by Dr Maria New).

FLAG-MyD88 WT and FLAG-K132R inducible U2OS cell lines were induced for 2 days with doxycycline (DOX). Cells were harvested and MyD88 and its lysine-deficient mutant immunoprecipitated with anti- FLAG antibody and immunoblotted with anti-FLAG or Ac-K antibody (Figure 5.2a).

No acetylation was observed when the ectopic MyD88-K132R protein was expressed in cells, while, on the contrary, the immunoprecipitated WT-MyD88 showed noticeable Ac-K signal (Figure 5.3a). This result confirmed that the lysine residue previously identified by mass spectrometry, K132, is acetylated.

Furthermore, it was then of interest to compare the properties of WT MyD88 to its K132R mutant. To monitor changes in cellular apoptosis, WT MyD88 cells were immunoplotted against those overexpressing the K132R mutant under the same SAHA no-/treatment conditions. As one would now expect, upon SAHA treatment, cells where WT MyD88 was induced showed higher levels of PARP (cleaved) compared to those where ectopic MyD88 was not induced, which confirmed the

previous results (chapter 3) where cellular apoptosis on SAHA treatment was dependent on MyD88 levels (Figure 5.2b). Overexpression of K132R further sensitised cells to SAHA treatment, resulting in higher PARP (cleaved) levels in K132R-MyD88 cells treated with SAHA, compared to WT-MyD88 cells treated with the same SAHA concentration (Figure 5.2b), whereby upon quantifying levels of normalized PARP (cleaved) expression K132R-overexpressing cells showed 5-folds compared to 3-folds in case of cells overexpressing WT-MyD88 (Figure 5.2c).

To further validate that K132R-MyD88 promotes more apoptosis compared to the WT, FACS analysis was used to measure changes in the number of cells in sub-G1 phase. 23.6% of the cells overexpressing K132R and treated with SAHA went into sub-G1 phase, compared to 15.24% in case of cells overexpressing WT and treated with the same SAHA concentration (Figure 5.2d). It was also noticed that the sub-G1 populations across the two isoforms prior to SAHA treatment were the same, suggesting that the subsequent changes in sub-G1 levels was a property associated with HDI treatment (Figure 5.2d).

Overall, these results suggested that MyD88 was thus a direct target for acetylation, and acetylation impacted on its biological properties, particularly those related to its role as a sensitivity determinant to HDI.

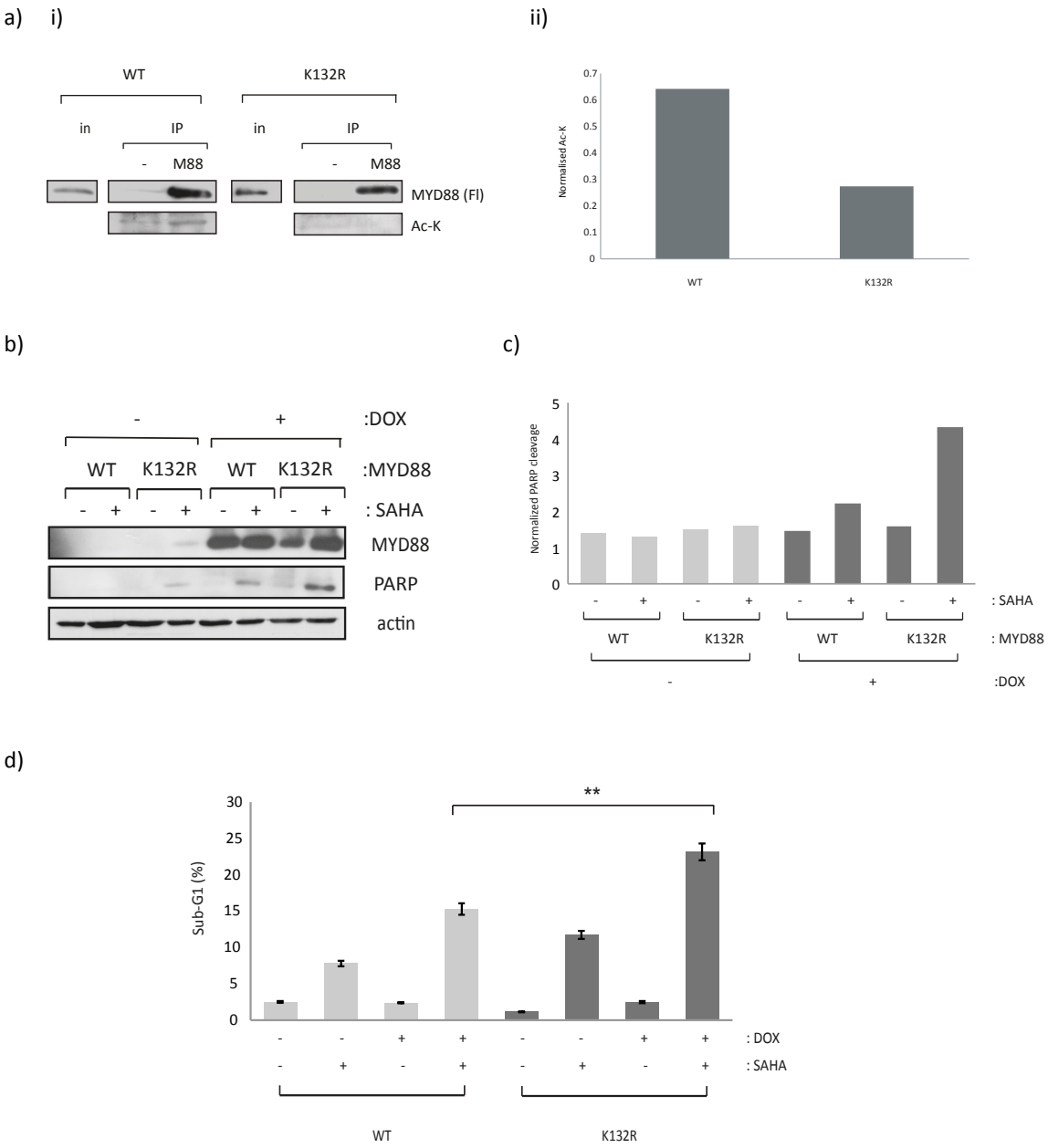


Figure 5.2 K132R mutant sensities cells to SAHA treatment. **(a) (i)** FLAG-MYD88 WT, FLAG-K132R, or control (-) inducible U2OS cell lines were induced for 2 days with doxycycline (DOX). Cells were harvested and MYD88 or its mutant immunoprecipitated with anti- FLAG antibody and immunoblotted with anti-FLAG or anti-acetyl-lysine antibody. Input is indicated (inset). (ii) Graph represents the quantitated Ac-K from (i) **(b)** Stable Tet-On inducible U2OS cell lines expressing WT or K132R FLAG-MYD88 were treated with SAHA (10μM) for 16 hours under noninduced (-) or induced (+) doxycycline (DOX) treatment conditions (1 mg/mL; induction for 72 hours prior to treatment). Cells were immunoblotted for MYD88 (anti-FLAG), PARP (cleaved), and actin antibody. **(c)** The graph represents quantitation of PARP cleavage from (b). **(d)** Level of sub-G1 in stable Tet-On inducible U2OS cell lines expressing WT or K132R FLAG-MYD88 U2OS under noninduced (-) or induced (+) doxycycline (DOX) treatment conditions by flow cytometry, upon SAHA treatment (10μM) for 16 hours, as described in (b). Statistical significance is indicated as ** P≤ 0.01. Shown examples are representatives of three independent experiments.

5.4 MyD88 acetylation alters its protein stability.

Further to previous findings, it was still unclear how MyD88 acetylation affects its properties. One assumption was that MyD88 acetylation would affect its stability. As previously discussed (chapter 1), acetylation can regulate protein stability in a refined mode and by several mechanisms [176]. The acetylation of lysine residues can block ubiquitination at the same residue, thereby preventing proteasomal degradation. An example is p53, which is tightly controlled by the Mdm2 E3 ligase-driven proteasomal degradation pathway. Acetylation abolishes complex formation between p53 and Mdm2, whereas an unacetylatable p53 mutant strongly interacts with Mdm2, resulting in p53 degradation [182].

Additionally, nonproteasomal degradation may also be facilitated by acetylation, as in the case of the acetylation of non-proteasomal protein HNF-6. It was seen that acetylation increases HNF-6 half-life, whereas a mutant incapable of being acetylated is degraded non-proteasomally [305].

Therefore, it was relevant to compare the protein stability levels of MyD88, which can be acetylated (WT), and MyD88 which cannot be acetylated (K132R). A time-course treatment of stable U2OS cells expressing Tet-On inducible WT or K132R FLAG-MyD88 with cycloheximide (100μM) was carried and the lysates were immunoblotted with FLAG, p53, and actin antibody (control).

Cycloheximide is commonly used to compare stability levels since it inhibits protein synthesis via acting specifically on the 60S subunit of ribosome and thus inhibiting translation elongation [306]. As seen in the immunoblot and the relevant quantitation graph (Figure 5.3), the mutant K132-MyD88 demonstrated a much higher half-life compared to the WT. To further confirm these findings, an alternative method to measure changes in protein turnover levels should be used, such as differential scanning fluorimetry (DSF) [307] or pulse-chase analysis [306].

This result might provide an explanation to how HDI-treatment affects MyD88 status and therefore its role in mediating apoptosis via a TLR-dependant mechanism, whereby the increase in the half-life of the hypoacetylated version (K132R) proposed that MyD88 acetylation, at least in part, promotes its signalling capacity. Although an explanation could not be made available, yet it is worth noting that since K132R is not acetylated, the change in its stability might be due to another set of molecular events, other that acetylation.

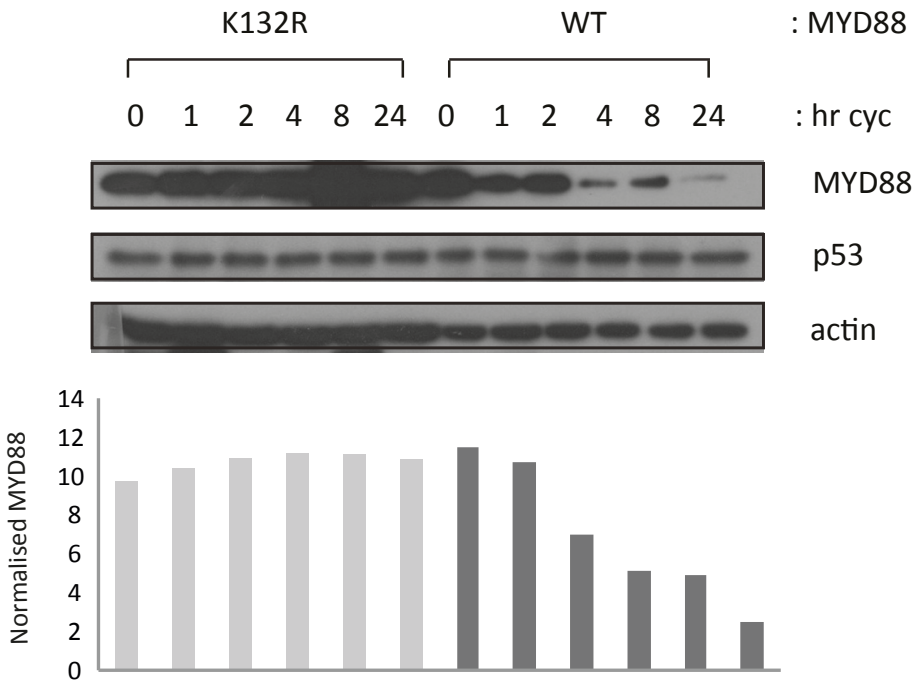


Figure 5.3 MyD88 acetylation affects its turnover. Stable U2OS cells expressing Tet-On inducible. WT or K132R FLAG-MYD88 were treated with cycloheximide (100μM) for the indicated time and immunoblotted with FLAG, p53, and actin antibody. Immunoblot quantitation of FLAG-MYD88 relative to actin performed using ImageJ software, with N=1.

5.5 Recognition of acetylated MyD88 by bromodomain proteins.

Further to establishing that MyD88 contribution into sensitising the cells to HDIs could be controlled by its K132 acetylation residues, and that silencing this site changed protein stability status, it was of interest to understand how that acetylation residue could be recognised, and whether that recognition and the subsequent acetylation would make MyD88 subject to structural changes that could be further translated into changes in its activity as an HDI-treatment sensitivity determinant. Interestingly, BRD-containing proteins were the only proteins known to date of pursuing this part of the project which could identify acetylated lysine resides on chromatins, where they could act as readers of this post-translational modification (see chapter 1). Therefore, a broad-spectrum bromodomain inhibitor, bromosporine, was used for that purpose.

The addition of bromosporine to U2OS cells stably overexpressing induced WT FLAG-MyD88 on its own at 3μM did not cause any significant cell death, however, it had significantly diminished cellular apoptosis caused by SAHA as measured by PARP (cleaved) and caspase 3 (cleaved) levels (Figure 5.4a). That effect was exclusive to cells expressing high levels of MyD88, since the same levels did not change after treating uninduced ones with both SAHA and bromosporine (Figure 5.5a).

It was then attempted to identify which particular bromodomain protein was important for recognition of MyD88, one that could potentially act as a reader for this acetylation site that recognises the acetylation residue and modulate the protein stability or the subsequent cellular outcome.

Previous work by Dr Maria New proposed BRWD3 as a potential BRD-containing reader, taking into account its cytoplasmic co-localisation with MyD88 as well its established role in JAK/STAT signalling [308].

TLR activation, where MyD88 is known to play a key role, causes release of interferon and pro-inflammatory cytokines, after which interferon activates the JAK/STAT signalling pathway [309]. Additionally, the L265P mutant promoted cell survival by spontaneously assembling a protein complex containing IRAK1 and IRAK4, leading to a downstream cascade of events that included JAK kinase activation of STAT3, and secretion of IL-6 [242].

To further validate that hypothesis, U2OS cells stably overexpressing WT FLAG-MyD88, induced and uninduced, were treated with SAHA for 24 hours, and the protein lysates were immunoprecipitated with FLAG antibody (for MyD88) as well as non specific IgG of the same strain (control), prior to blotting for endogenous BRWD3 and FLAG MyD88. An interaction between the ectopically overexpressed MyD88 and BRWD3 occurred (Figure 5.4b), while no signal was detected at the same molecular weight in case of probing for uninduced cell lysates and non-specific pull down, which conforms to previous results.

To further establish whether that interaction is acetylation-residue-dependant, the same protocol was followed, yet this time WT cells were compared against their K132R mutant. The interaction between the ectopically overexpressed MyD88 and BRWD3 disappeared in case of the mutant K132R (Figure 5.4c).

These results collectively established a clear interplay between MyD88 acetylation residue, K132, and BRWD3 as its potential reader, which might provide an explanation to how HDI-treatment influences MyD88 role in sensitising cells to HDI treatment via an acetylation-dependant mechanism that contributes to its role as a TLR adaptor protein.

It is worth noting that more recent studies have shown YEATS domains (named after Yaf9, ENL, AF9, Taf14 and Sas5) to also bind to histone lysine residues [310], in addition to Lys residues that are modified by crotonylation (a newly discovered acetylation-based PTM) [311], which therefore opens the door for further identification of more MyD88 lysine acetylation readers.

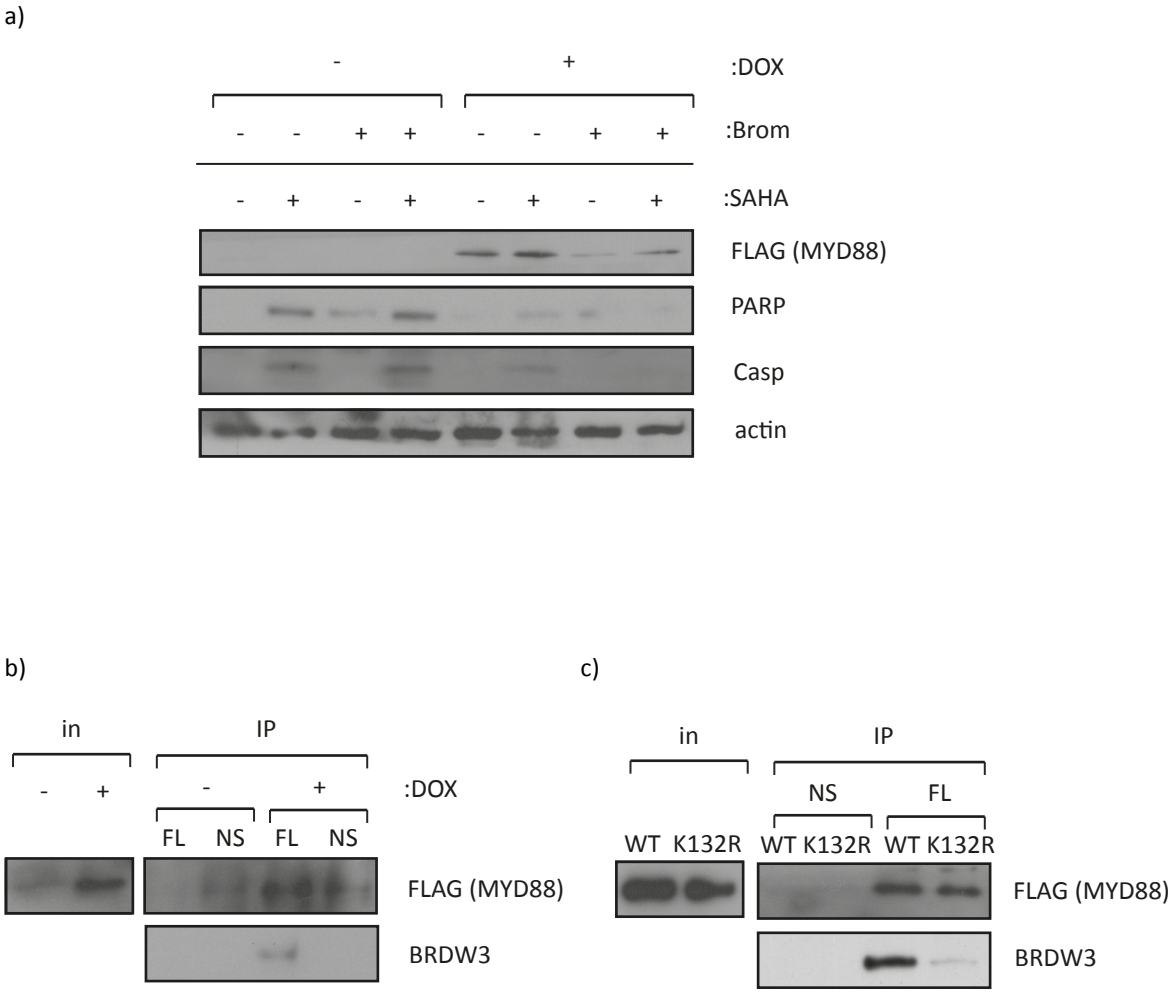


Figure 5.4 Acetylated MyD88 is recognised by bromodomain proteins. **(a)** Stable Tet-On inducible U2OS cell lines expressing WT were treated with SAHA (10µM) and/or bromosporine (3µM) for 24 hours under noninduced (-) or induced (+) doxycycline (DOX) treatment conditions (1 mg/mL; induction for 72 hours prior to treatment). Cells were immunoblotted for MyD88 (anti-FLAG), PARP (cleaved), caspase 3 (cleaved) and actin antibody. **(b)** FLAG-MyD88 WT and **(c)** FLAG-K132R, or control (-) inducible U2OS cell lines were induced for 2 days with doxycycline (DOX). Cells were harvested and MyD88 or its mutant immunoprecipitated with anti- FLAG (FL) or non-specific (NS) antibody and immunoblotted with anti-FLAG or BRWD3. Input is indicated (inset). Shown examples are representatives of three independent experiments.

5.6 Conclusion.

The changes in cellular cycle profiles under SAHA treatment conditions were shown to be dependant on MyD88 levels, suggesting an interplay between MyD88 and HDI. Previous work indicated endogenous MyD88 acetylation upon SAHA treatment, and further results confirmed the same results in ecoptic MyD88. To further explore that hypothesis, acetylation residue at location 132 was identified, by massspectrometry (Dr Maria new/Dr Benedikt Kessler). Additional investigation showed the K132 residue to be responsible for MyD88 acetylation, whereby its silencing yielded a more stable form of MyD88, K132R, that is not subject to acetylation.

Moreover, the hypoacetylated form of MyD88, K132R, promoted higher cell death under SAHA treatment conditions, compared to the WT-MyD88, as shown by both immunoblotting and FACS analysis. Respectively, K132R was shown to be more stable with longer half-life, a result that might provide an explanation on how MyD88 acetylation influenced its activity as a sensitivity determinant to HDI treatment.

Additionally, in a collaboration with Dr. Reuben M. Tooze's group, Leeds Institute of Molecular Medicine, University of Leeds, Leeds, United Kingdom, a bioinformatics analysis was performed to identify a gene-signature at the genome-wide level that could be associated with MyD88 expression levels across several cases of DLBCL. This GO (gene ontology) analysis showed that each ABC subgroup–specific cluster expressed a high content of lysine acetylation targets,

above the average lysine acetylation content of the top 500 genes. Therefore, it was concluded that in the ABC subgroup, MyD88 expression is associated with an enriched set of genes, which, in addition to their GO term, are related through their high level of lysine acetylation. For more details refer to New et al., 2016 [272].

MyD88 capacity to promote HDI-induced apoptosis was compromised by bromosporine treatment, where levels of PARP (cleaved) and caspase 3 (cleaved) under SAHA treatment conditions were reduced in cells overexpressing ectopic MyD88 when bromosporine was introduced. Also previous preliminary data suggesting MyD88 recognition by BRD-containing proteins, namely BRWD3, was further confirmed by mechanistic interactions between MyD88 and BRWD3, a property that was found to be exclusive to its WT when compared against its K132R mutant.

CHAPTER 6 MyD88 interplay with HDAC6.

6.1 Introduction.

MyD88 was identified as a sensitivity determinant to HDI in the loss-of-function screen, and its role in moderating cells response to SAHA treatment has been investigated (chapter 3). Its K132 acetylation residue was found important in regulating its activity, where if lost; its capacity to sensitse cells to HDI treatment changes (chapter 5). Therefore, it was of interest to identify which HDAC(s) might be responsible for these effects.

Although the reason has not been established at the time of this study, yet existent literature alongside previous results of this project indicated that MyD88 is thought to be present as aggregated structures or condensed forms, primarily in the cytoplasm (chapter 3), and therefore it was reasoned that an interplay is likely to occur with a co-localised HDAC(s). HDAC6 is the only HDAC that its known to be expressed predominantly in the cytoplasm.

HDAC6 belongs to class II of the HDAC family, and it contains an internal duplication of two catalytic domains that appear to function independently of each other (Chapter 1). Its roles in controlling protein stability and expression and cell fate was linked to its effect on different substrates, such as modulating metastasis via deacetylating α-tubulin and destabilising the microtubular network [121] [122] as well as modulating proteasomal degradation of misfolded proteins and the formation of polyubiquitinated aggregates via a p97/VCP-dependant pathway [123] [124].

HDAC6 also modulates a number of heat shock Hsp90-mediated regulatory processes via deacetylating Hsp90 and the subsequent recruitment of chaperone

complexes, which are involved in the control of the stability of a variety of downstream factors, and thus stimulating an acetylation-dependent protein degradation [125] [126].

Most relatively, previous studies have shown the suppressive effect of HDAC6 on MyD88 signalling, where HDAC6, together with SQSTM1, were required for the aggregation of the MyD88 via accumulation of polyubiquitinated proteins [312]. Additionally, knocking down SQSTM1 and HDAC6 showed an effect in promoting formation of the MyD88-TRAF6 complex and conversely decreased interaction of the ubiquitin-specific negative regulator CYLD with the complex [312].

Therefore, it was reasoned that an interaction between MyD88 and HDAC6 might take place, whereas if valid; it might impact its regulation of cellular response to HDI treatment.

6.2 HDAC6 moderates MyD88 expression.

It was shown that when MyD88 was acetylated; its stability became aberrant (chapter 5). Taking into account the cytoplasmic localisation of both HDAC6 and MyD88, it was of interest to further study the relationship between changes in MyD88 levels and those of HDAC6.

In order to investigate that; a cell line where HDAC6 was silenced has been made. HDAC6 shRNA (h) lentiviral particles that are transduction-ready and contain a target-specific construct that encodes a 19-25 nt (plus hairpin) shRNA designed to knock down HDAC6 gene expression were used to make a HDAC-deficient cell line. In parallel, control shRNA lentiviral particles containing a construct that encodes a scrambled sequence, which would not lead to the specific degradation of any known cellular mRNA, were used to make a control cell line.

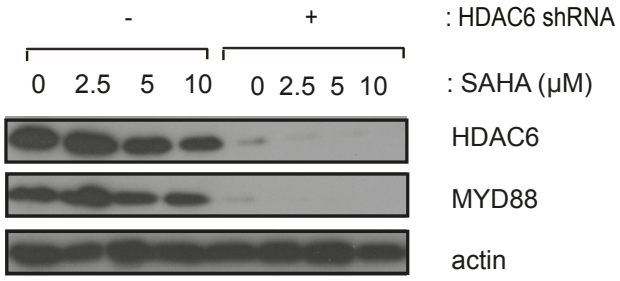
When HDAC6 was knocked down, cells capacity to express MyD88 proteins was lost (Figure 5.6a). It was therefore reasoned, taking previous effects of MyD88 acetylation status on its stability in account, that the absence of HDAC6 rendered MyD88 in the acetylated form and subsequently promoted its degradation.

Furthermore, MyD88 levels seemed to decrease when U2OS cells were treated with SAHA (Figure 5.6a), which might also be linked to decrease in HDAC6 levels. To further validate that this effect was HDAC6-dependant, both HDAC6-KD cells (+shRNA) and control cells (-shRNA) were treated with an HDAC6-specific inhibitor, namely tubstatin (tub) (discussed in more details in section 1.4). It was seen that the increase in tubstatin concentrations was coupled with a decrease in the

levels of MyD88 (Figure 6.1b) in control cells. Again, MyD88 was absent in cells treated with HDAC6 inhibitor (Figure 6.1b). Collectively, these results have suggested that HDAC6 plays a role in MyD88 expression.

These results were in accordance with other experiments run by other members of the group, whereby, for example, stable A549 HDAC6 shRNA (+) showed lower levels of apoptosis compared to control A549 HDAC6 shRNA (-) cells when both were treated with tubastatin [272].

a)



b)

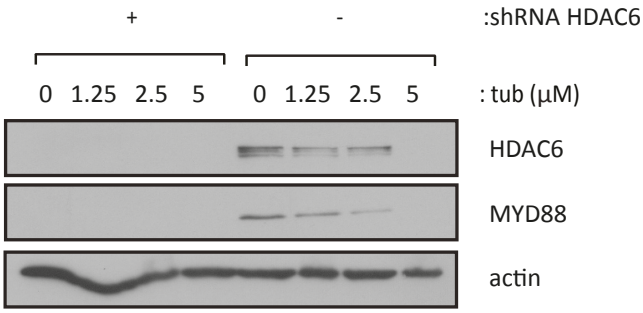


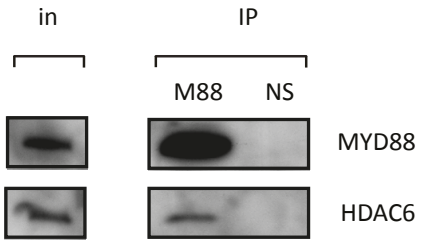
Figure 6.1 MyD88 levels are proportional to HDAC6 levels. Control (-) and HDAC6 knockdown (+; stable shRNA knockdown) U2OS cells were treated with SAHA **(a)** and tubstatin **(b)** at the indicated concentrations for 24 hours and analyzed by immunoblotting for HDAC6 and MYD88. Actin was the loading control. Shown examples are representatives of three independent experiments.

6.3 HDAC6 interacts with MyD88.

The next step of the investigation was to test if there was an interaction between MyD88 and HDAC6, and whether that interaction is dependant on MyD88 acetylation status. U2OS cells harvested and immunoprecipitated with anti-MyD88 antibody and immunoblotted with anti-MyD88 or anti-HDAC6 antibodies. An obvious interaction between HDAC6 and the endogenous MyD88 was observed upon immunoprecipitation (Figure 6.2a), which validates the previous hypothesis.

In order to validate that the interaction observed between MyD88 and HDAC6 is dependant on MyD88 acetylation, lysates from FLAG-MyD88 WT and its FLAG-K132R mutant were also immunoprecipitated and immunoblotted with anti-FLAG or anti-HDAC6 antibody. Only the WT MyD88 showed an interaction with HDAC6, with the mutant showing a much less degree of interaction (Figure 6.2b). Although that this result suggested that MyD88 K132 residue is vital for its interaction with HDAC6, yet the presence of an interaction signal with the K132R mutant, at rather low levels, indicates that either MyD88 harbours other acetylation residues that also interacts with HDAC6, in addition to its 132 acetylation site, or other regions of MyD88 might also interact with HDAC6. Nevertheless, these results confirmed that MyD88 is a substrate for HDAC6 acetylation.

a)



b)

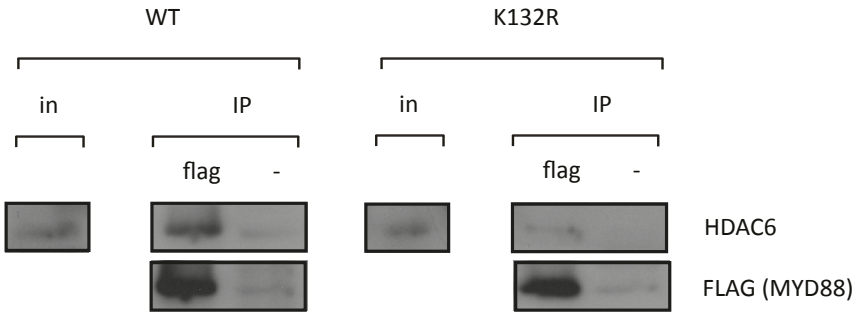


Figure 6.2 HDAC6 interacts with WT MyD88 but not with K132R. **(a)** U2OS cells harvested and immunoprecipitated with anti-MyD88 antibody and immunoblotted with anti-MyD88 or anti-HDAC6 antibody. Input is indicated **(b)** FLAG-MYD88 WT, FLAG-K132R, or control (-) inducible U2OS cell lines were induced for 2 days with doxycycline (DOX). Cells were harvested and MYD88 or its mutant immunoprecipitated with anti- FLAG antibody and immunoblotted with anti-FLAG or anti-HDAC6 antibody. Input is indicated (inset). Shown examples are representatives of three independent experiments.

6.4 HDAC6 promotes MyD88 protein stability.

Although transcriptional regulation at the gene levels rarely reflect on protein expression at the post-transcriptional level, mainly due to the regulation control at different levels, yet there have been instances where changes in mRNA levels strongly correlated with changes in protein levels with the assumption that regulation of gene expression of these genes could be tightly controlled at the levels upstream of translation. An example is a transcriptome study that showed the transcript and protein levels could correlate when a gene-specific RNA-to-protein (RTP) conversion factor independent of the tissue type was introduced, thus significantly enhancing the predictability of protein copy numbers from RNA levels [313]. Taking previous results showing a reduction of MyD88 protein levels upon HDAC6 depletion into account, it was of interest to further study whether that modulation happens at the post-transcriptional level only, or whether transcriptional events contribute to that interaction as well.

When the corresponding levels of MyD88 RNA were measured by RT-PCR, there were no differences at RNA levels of MyD88 between the control cells and HDAC6 KD cells. As a conclusion, it was assumed that the decrease in MyD88 protein levels upon changing HDAC6 levels was not a result of reduced transcription; instead it may be due to an effect on protein turnover subject to HDAC6-mediated stability (Figure 6.3a).

The next step was to investigate whether HDAC6 knockdown is responsible for reducing MyD88 protein stability and promoting its degradation. Protein levels are not solely determined by its synthesis rate, but also rates of its degradation. The

intracellular degradation of proteins usually occurs through two main pathways: the ubiquitin-proteasome pathway and lysosomal-mediated proteolysis degradation. In the lysosomal proteolysis pathway proteins are uptaken by lysosomes, whereby they are further digested by a number of enzymes, proteases included. The uptake of proteins by lysosomes is governed by a variety of pathways, such as autophagy and endocytosis [314].

The common pathway that is known to be the major pathway of selective protein degradation in eukaryotic cells is ubiquitin-proteasome pathway. Ubiquitin, a highly conserved 76-amino-acid polypeptide that is highly conserved in all eukaryotes, is attached to the amino group of the side chain of a lysine residue, whereas it functions as a marker that targets cytosolic and nuclear proteins for proteolysis. Additional ubiquitins are added subsequently forming a multiubiquitin chain, making polyubiquitinated proteins subject to recognition and degradation by a large, multisubunit protease complex; the proteasome.

Knowing that HDAC6 controls MyD88 expression at the post-transcriptional level, it was hypothesised that MyD88 down-regulation by HDAC6 neutralisation might involve the proteosomal degradation pathway. Bortezomib (Velcade) is an FDA-approved ubiquitin-proteasome pathway inhibitor that is widely used to study changes in protein degradation via the ubiquitin-proteasome pathway. It acts through inhibiting the chymotrypsin-like activity of the β5 subunit of the proteasome, which is associated with the rate-limiting step of proteolysis [315].

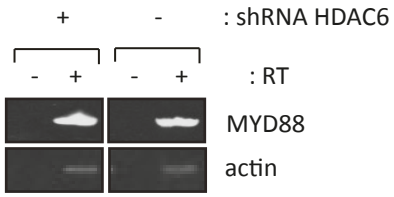
A time-course treatment of control (-shRNA) and HDAC6-KD cells (+shRNA) with brotezomib (100nM) was carried and the lysates were immunoblotted with MyD88,

HDAC6, and actin antibody. When HDAC6 KD cells, where MyD88 was previously shown to be absent, were treated with brotezomib, MyD88 started to occur clearly after 5 hours of the treatment (Figure 6.3b). This result suggested that the less stable version of MyD88, which is acetylated in absence of HDAC6, could be rescued from degradation by inhibiting the ubiquitin-proteasome pathway.

In order for this result to be more conclusive, other ubiquitin-proteasome inhibitors with different mechanisms of action should be further utilised. Examples include Mg-132, another commonly used proteosomal inhibitor, that also acts through binding to β-subunits of the proteasome, or an inhibitor of a different mode of action such as lactacystin, that becomes hydrolised in cells and covalently modifies the N-terminal threonine of subunit X in the mammalian 20S proteasome.

One potential explanation for this result is that HDAC6 capacity to deacetylate MyD88 renders the latter less vulnerable to proteosomal degradation and thus increasing its stability. Further work to explore other potential MyD88 degradation pathways that might be influenced by HDAC6, such as autophagy and lysosomal degradation, was under its way, yet conclusive results could not be obtained due to time constraints.

a)



b)

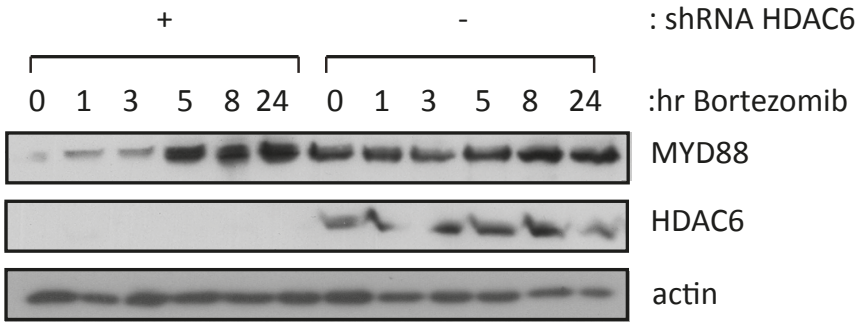


Figure 6.3 Proteasome inhibition rescues MyD88 in absence of HDAC6. **(a)** RT-PCR of MyD88 RNA in control (WT) and HDAC6 (KD) cells. Actin was used as control. **(b)** Control (-) and HDAC6 knockdown (+; stable shRNA knockdown) U2OS cells were treated with cycloheximide (100μM) for the indicated time and immunoblotted with MyD88, HDAC6, and actin antibody. Shown examples are representatives of three independent experiments.

6.5 Mechanism of MyD88 protein control by HDAC6.

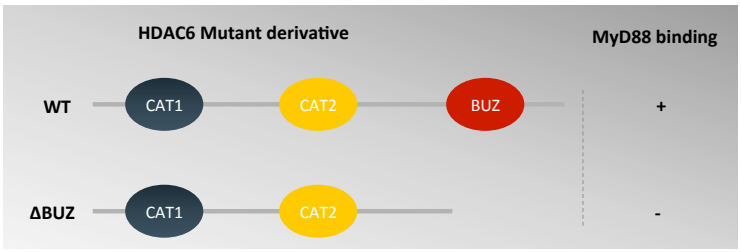
Previous results showing MyD88 as an HDAC6 substrate that promotes its stability via blocking ubiquitin-proteasome pathway led to further interest in deciphering the mechanistic interplay between MyD88 and HDAC6. Previous reports have indicated that HDAC6 is selectively involved in the polyubiquitin-positive, but not polyubiquitin-deficient, aggresomes [124]. The presence of an ubiquitin binding domain (the BUZ finger) previously suggested the possibility that the association of HDAC6 with aggresomes might be mediated by its binding to polyubiquitinated misfolded proteins [124]. It was also found that HDAC6 was associated with the dynein motor complex, a family of cytoskeletal motor proteins that move along microtubules in cells, and recruited polyubiquitinated proteins to dynein [124]. It was therefore reasoned that HDAC6-BUZ domain might be responsible for the protesomal-linked interaction between MyD88 and HDAC6 that was previously described.

U2OS HDAC6-KD cells (+shRNA) were transfected with HDAC6-FLAG WT and HDAC6-FLAG ΔBUZ (missing the BUZ domain) plasmids (Figure 6.4a), and lysates from harvested cells were immunoblotted against FLAG-HDAC6, endogenous MyD88 and actin (as a control) antibodies (Figure 6.4b). It was observed that cells transfected with the WT plasmid have successfully expressed endogenous MyD88, while MyD88 levels in cells transfected with the ΔBUZ plasmid were nearly undetectable. This result suggested that the BUZ domain might be responsible for HDAC6 interplay with MyD88, although such an interpretation could not be fully conclusive due to the rather weak ΔBUZ-Flag signal, which could have been antibody-dependant.

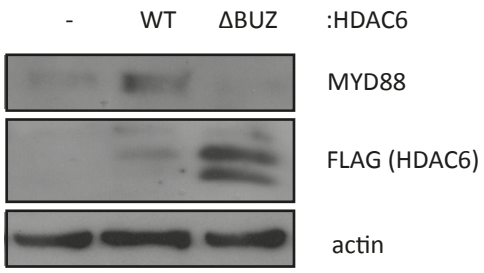
To further validate this hypothesis, lysates from HDAC-KD cells that were transfected with both plasmids, WT and ΔBUZ, were immunoprecipitated and immunoblotted against FLAG-HDAC6 and MyD88. An interaction between WT-HDAC6 and MyD88 took place. However, and despite its higher FLAG expression levels compared to WT, no interaction was detected between HDAC6 ΔBUZ and MyD88. It is worth noting that FLAG-HDAC6 bands were, albeit weak, detectable in the control lanes (-), which might suggest a certain degree of antibody lack of specificity or potential "leakage" in the cell line.

These results clearly suggested that HDAC6-BUZ domain plays a role in HDAC6 interaction with MyD88 and therefore that might explain, in part, how HDAC6 regulates MyD88 stability and the subsequent modulation of HDI-mediated cellular response via, at least partially, the ubiquitin-proteasome pathway. However, whether that interaction could be directly linked to the previously observed MyD88 acetylation-dependant stability and protein turnover needs to be further investigated in cells that express the K132R mutant version of MyD88.

a)



b)



c)

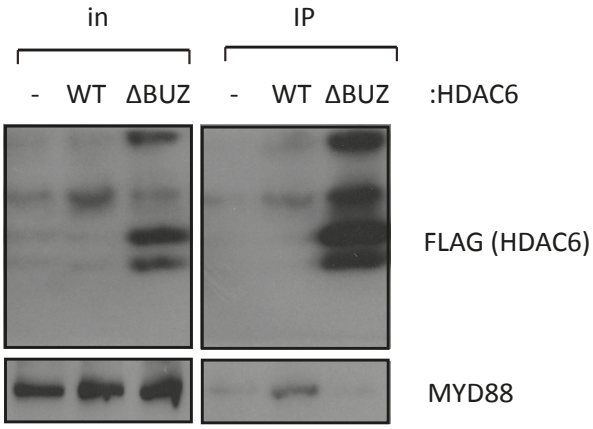


Figure 6.4 HDAC6 BUZ domain is required for HDAC6 interaction with MyD88. **(a)** A schematic showing HDAC6-WT and HDAC6-ΔBUZ plasmids domains composition. **(b)** U2OS HDAC6-KD (+shRNA) cells were transfected with empty vector (-), FLAG-HDAC6 (WT), or FLAG- ΔBUZ. Cells were immunoblotted with FLAG, MyD88, or actin antibodies. **(c)** Cells from (b) were immunoprecipitated with anti- FLAG antibody and immunoblotted with anti-FLAG or anti-MyD88 antibody. N=1.

6.6 Conclusion.

MyD88 stability, and thus its necessary presence to act as a TLR adaptor that modulates HDI-mediated apoptosis, was found to be largely in control by HDI-mediated acetylation, and it was reasoned that a co-localised HDAC, namely HDAC6, could play a role in mediating that control.

MyD88 was absent in cells where HDAC6 was depleted my means of shRNA knockdown. That result was further confirmed when cells were treated with a selective HDAC6 inhibitor, tubstatin.

To further explore that interplay, a set of mechanistic immunoprecepitation experiments confirmed an interaction between MyD88 and HDAC6. That interaction was absent in case of the K132R mutant, which confirms that the interaction is dependant on MyD88 acetylation capacity.

Interestingly, HDAC6 regulation of MyD88 was found to be restricted to changes at the post-transcriptional levels only, whereby proteosomal inhibition rescued MyD88 in HDAC6-deplated cells, suggesting that HDAC6 influence on MyD88 stability might be regulated via the ubiquitin-proteosome pathway.

That hypothesis was further validated when the interaction between MyD88 and HDAC6 was lost when the ubiquitination domain of the latter, its BUZ domain, was absent.

These results provided substantial about the interaction between MyD88 and HDAC6, which could provide a coherent explanation of how HDI-treatment influenced MyD88 levels and therefore its role in promoting a cascade of downstream effects, that eventually included apoptosis. However, further investigation of other degradation pathways, via Hsp90 for example, alongside studying any potential interactions with other HDACs should not be ruled out (discussed later, chapter 7).

CHAPTER 7 Discussion.

7.1 Conclusions.

7.1.1 MyD88 expression levels regulate sensitivity to HDIs.

The central scope of this project was to validate MyD88 as a potential sensitivity determinant to HDIs after its discovery at the genome-wide shRNA loss of function screen [227]. Functional analysis of MyD88 levels confirmed previous results and revealed its influence on cellular apoptosis on HDI treatment, where as inducing overexpression of ectopic MyD88 enhances cells response to SAHA treatment, and conversely silencing endogenous MyD88 reduces, yet does not completely abolish, their apoptosis. These results were an interpretation of the change into the levels of PARP (cleaved), caspase 3 (cleaved) and percentage of cells in sub-G1 phase as markers for apoptosis, and changes in cells capacity to form colonies in CFA as an indicator of cellular growth.

As a potential future biomarker that could be ultimately used in the clinic, MyD88 localisation was determined in both in vitro, by immunofluorescence, as well as in vivo samples, by IHC. MyD88 was found to be predominantly located cytoplasmically, although in some instances (experiments conducted by other group members) it could be seen in the nucleus. Due to limited access to patients' samples, it was hard to establish a conclusion on MyD88 localisation or any potential re-localisation upon HDI treatment. These results where in agreement with the literature, where MyD88 was shown to be expressed predominantly in the cytoplasm, in both U2OS cells [284] as well as in TMA sections from DLBCL patients [243].

Future experiments to further confirm MyD88 localisation should be conducted, such as cellular fractionation and immunoblotting the resultant fractions [316].

7.1.2 IL-6 sensitises cells to HDIs through a MyD88-dependent mechanism.

As a toll receptor protein modulator, MyD88 role in regulating inflammatory pathways and cytokines release was of an interest [258]. MyD88 was shown to influence NF-κB expression, whereas the overexpression of MyD88 coincided with high levels of NF-κB. It was reasoned therefore that this might have an affect upon the release of various cytokines. A cytokines screen was carried out, showing changes in both IL-6 and IL-8 levels in the tissue culture media to be dependent on changes in MyD88 levels. Furthermore, treating cells overexpressing MyD88 with SAHA coincided with a reduction in IL-6 secretion. In accordance, neutralising IL-6 in cells rich with MyD88 reduced HDI apoptotic effects on cells as validated by levels of PARP (cleaved) and the percentage of cells in sub-G1 phase. These results can be explained within the scope of HDACs roles in modulating innate and adaptive immune response, particularly via IFN- and TLR-dependant pathways, where IL-6 was shown to be both regulated by HDACs-mediated transcription repressions of TLR-4 mediated chromatin modelling [289]. Another explanation can be provided within the scope of interplay between MyD88 and IL-6, where previous studies have shown MyD88 and IL-6 interplay in tumouregensis, via, for example, blocking erlotinib-induced IL6 secretion upon MyD88 secretion [271].

However, neutralising extracellular IL-6 in cells overexpressing MyD88 did not cause major differences in cellular proliferation as shown in CFA experiments before and after SAHA treatment. That could be explained within the highlight of IL-6 function as a proliferative cytokine, whereas its depletion slowed down colonies growth unspecifically and therefore SAHA effects could not be pronounced.

Also it was noticed that IL-6 allowed cell-cycle progression via a MyD88-mediated process, whereby neutralising IL-6 stimulated more cells to go into the G2 phase as well as enhancing the phosphorylation of S317.

7.1.3 Somatic mutation in MYD88 influences HDAC inhibitor sensitivity.

L265P substitution is frequently observed in the ABC subtype of DLBCL and Waldenstrom macroglobulinemia as an oncogenic gain-of-function mutation, which augments signalling through NF-kB, and therefore it was of an interest for further investigation within the scope of this project [298]. Cells expressing L265P happened to be more sensitive to SAHA treatment when compared to cells expressing WT-MyD88 as observed in differences in PARP (cleaved) levels as well as sub-G1 cells populations. Moreover, L265P expression coincided with higher IL-6 secretion, which greatly dropped upon SAHA treatment. Such results support previous literature that suggests the potential use of L265P as a prognostic biomarker, where its expression was shown to be associated with higher disease burden and progression [252] [291]

Additionally, a collaborating group used a bioinformatics approach to determine at the genome-wide level whether a gene signature correlated with MyD88 expression by screening gene expression datasets derived from DLBCL clinical biopsies. When the 500 most correlated genes with MyD88 expression were clustered according to GO descriptors; each ABC subgroup–specific cluster had a high content of lysine acetylation targets. Thus, in the ABC subgroup, MyD88 expression was associated with an enriched set of genes, which, in addition to their GO term, are related through their high level of lysine acetylation [272].

7.1.4 MyD88 is acetylated.

Acetylation was shown to play a vital role in regulating several protein functions, by altering protein stability, localisation and interactions [317]. Many of these cellular proteins are relevant to cancer cell proliferation and tumourigenes, and targeting of such acetylation can be a viable cancer therapy approach [317].

Previous work has identified MyD88 to be acetylated at its K132 residue, a novel finding that was further investigated with respect to its role as a sensitivity determinant to HDI treatment. MyD88 was found to be acetylated upon immunoprecipitation and immunoblotting against an acetylated lysine antibody. When treated with SAHA, a mutant lacking the K132 acetyaltion residue, K132R, sensitised cells to higher levels of cellular apoptosis compared to the WT as seen by the increase in the levels of PARP (cleaved) levels as well as the number of cells in sub-G1 phase. Interestingly, K132R mutant was shown to be more stable, which might reflect on the effect of acetylation on MyD88 protein turnover. It is worth noting that since K132R is not acetylated, the change in its stability could not be exclusively attributed to change in acetylation status, with the likelihood of having a different unknown mechanism involved.

7.1.5 Acetylated MyD88 is recognised by a bromodomain reader.

A broad-spectrum bromodomain inhibitor, bromosporine, reduced cells response to HDI treatment, whereas only the acetylated version of MyD88 was recognised by the cytoplasmic domain reader BRWD3.

7.1.6 MyD88 is an HDAC6-substrate.

The established role of HDAC6 in MyD88 aggregate formation and its localisation in the cytoplasm made it a good candidate for studying how it could possibly control MyD88 status and the subsequent cellular fate on HDI treatment. Reduction in HDAC6 cellular levels by shRNA knockdown was shown to downregulate MyD88 levels and therefore reduced cells sensitivity to SAHA. That was shown to happen mainly due to an interaction between HDAC6 and the acetylatable form of MyD88, where the K132R mutant did not display an interaction with HDAC6.

One possible explanation could be made within the scope of MyD88-folding status, aggresome formation and HDAC6 activity. For example, one study has shown that cells treated with HDIs contained microaggregates, but these microaggregates were not transported to microtubule organising centers, and thus were never degraded [318]. Therefore, and despite that HDAC6 BUZ domain recognised the polyubiquitin on the MyD88 Mydosomes, which should lead to its degradation, yet another protein might protect MyD88 from aggregation, or also the formation of these micro-aggregates might fail to be transported to microtubule organising centers and therefore degradation does not take place.

That interplay was shown to happen at the post-transcriptional level only, whereas inhibiting the proteosomal degradation of the acetylated MyD88, in absence of HDAC6, promoted protein stability. It was also shown that the HDAC6 BUZ domain, which is mainly responsible for its ubiquitin binding activity, was vital in the interaction with MyD88, whereas if absent HDAC6 would lose its MyD88-interaction capacity.

Figure 7.1 is introducing a suggested working model of the study; illustrating the role of MyD88 as a potential sensitivity determinant to HDI-treatment via an IL-6-dependant mechanism, in a loop that is suggested to be dependant on HDAC6 influence on MyD88 acetylation status.

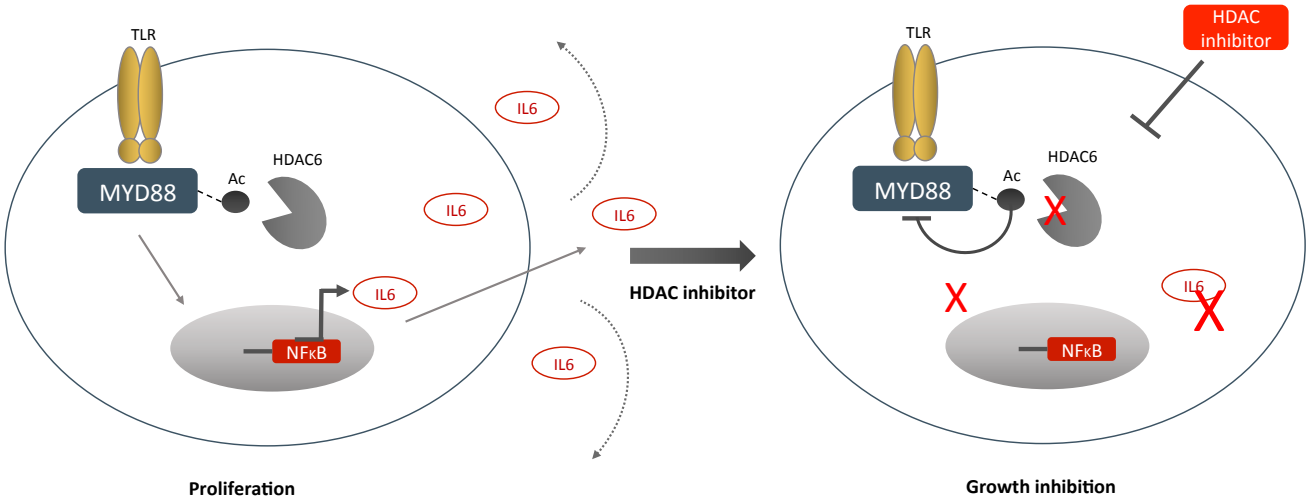


Figure 7.1 Model depicting the role of MyD88 as a sensitizer to HDAC inhibitor treatment. It is envisaged, through its role as an adaptor protein in TLR-dependent signaling, that in untreated lymphoma cells (left-hand diagram), MYD88 augments Nf-kB activity, which thereby increases the expression of target genes, including cytokine genes like IL-6. The autocrine action of IL-6 retains cells in the proliferative state and augments cell sensitivity to HDAC inhibitors. Acetylated MYD88 is deacetylated by HDAC6. Upon treating cells with HDAC inhibitor (right), increased acetylation of MyD88 occurs, resulting in reduced MyD88 and Nf-kB activity, lowering IL-6 expression and thereby prompting growth inhibition through reduced IL-6 levels. It is suggested that altered MYD88 acetylation contributes to the antiproliferative effects of HDAC inhibitor treatment by preventing the autocrine growth-promoting action of cytokines like IL-6. Adapted from New et al., 2016.

7.2 Project limitations.

7.2.1 Cell line system.

HDIs approved for clinical include Vorinostat for CTCL; romidepsin for CTCL and PTCL; belinostat for refractory PTCL and panobinostat for multiple myeloma. However, the genome-wide loss of function screen where MyD88 was identified as a potential sensitivity determinant to HDIs was conducted in U2OS cells [227], and therefore it was reasoned to use the same system to carry on this project.

Generally speaking, although a comparison of the different effects of SAHA treatment among other cell lines took place at the start of this project, yet it could not be conclusive and it would be interesting to validate the hypothesis in other cell lines, particularly those known to be more sensitive to HDI treatment. For example, RIVA and HBL cells were used, by Dr. Semira Sheikh, to show that levels of MyD88 dictates cells sensitivity to SAHA treatment [272]. However, all experimental cell lines are known to have mutations that makes them different compared to their actual genomic tissue profiles [319], that is in addition to their lack of evolution, complexity and heterogeneity capacity, as opposed in a tumour [320]

Also, and despite its convenience as an experimental tool, utilising overexpression systems might be disadvantageous. For example, the use of Tet-on MyD88 WT and mutant cells lines required doxycycline addition, which might affect cellular proliferation and metabolism. Additionally, whether ectopic proteins behave similar

to endogenous ones and whether their overexpression changes cellular translation and transcription remains questionable.

7.2.2 Measuring apoptosis.

One of the limitations of this project was the measurement of cell death only by immunoblotting for PARP (cleaved), caspase 3 (cleaved) and measuring sub-G1 phase population by FACS. Additional methods that could be used to validate this project's findings further include Annexin V staining to differentiate between apoptotic and necrotic cell death [321] and MTT assays to measure drug effect on cell metabolism and therefore viability [322].

Also although that apoptosis was the major focus of this project since the aim was to have a direct measurement of cellular sensitivity to HDI treatment, yet other cellular outcomes, such as autophagy, should be taken into account in future studies.

7.3 Future directions.

7.3.1 MyD88, a biomarker for HDI-treatment.

The results of this study paves the way for further investigation to establish MyD88 as a biomarker to stratify patients who are more likely to respond to HDI treatment. Several case studies have proposed L265P to be a valid potential biomarker for WM and ABC DLBL patients [323], yet the acetylation status of MyD88 and the

subsequent potential use of HDI as the treatment of choice are still to be thought after.

In order to achieve this, further experimentation to validate the molecular findings and link them to potential clinical applications should be considered in the future. Examples of validating the molecular findings include measuring changes in WM and ABC DLBCL cells sensitivity to SAHA treatment in co-relation with MyD88 levels, acetylation signature and its mutation status, and decide on the quantitative validity across a wide array of general population samples versus WM and ABC DLBCL samples across each of there three features. Once one (or more) of these molecular feature(s) prove(s) molecular viability, then the development of high sensitivity, cheap and standardised antibodies, optimised for clinical samples, should be achieved.

Following that development phase, the assay should be trialled in a comprehensive clinical trial to test its accuracy and reproducibility in the target group and understanding its limitations at end-points during the course of development, while conforming with the approval criteria, as those suggested, for example, by FDA's Center for Drug Evaluation and Research for defining the biomarker qualification process. as discussed in chapter 1 [216]. Once such trials are concluded, then the potential approval for MyD88 levels, acetylation or mutation status clinical use should be placed within the relevant context of use, level of evidence and said qualification criteria [216].

Moreover, correlating the findings of this study with existent as well as future clinical data that indicate different MyD88 expression and/or acetylation levels in

different subsets of patients can be significantly important in patients' stratification, and investigating whether the MyD88-mediated antioproliferative effects of HDIs is a universal feature, or rather limited to certain types of cancer. For example, a recent study has shown that high levels of MyD88 staining can be associated with advanced stage and poorer survival in high-grade serous ovarian carcinomas, compared to improved survival in low-grade serous ovarian carcinomas [324].

7.3.2 MyD88-HDAC6 interplay via ubiquitination and Hsp90.

The results of this project suggested that the acetylatable version of MyD88 was subject to protesomal degradation in absence of HDAC6. Several experimental attempts have been made to develop more in depth understanding of how MyD88 acetylation status might affect its ubiquitnation, potentially via HDAC6, for example his-ubiquitin pull down; yet results were not conclusive due to low-binding affinity of antibodies used at the time of running that experiment. Also, albeit HDAC6 and MyD88 cytoplasmic localisation, it can be of interest to see whether other HDACs would have the same effect on MyD88.

Another interesting set of experiments that have been under their way is to establish whether the interaction between HDAC6 and MyD88 involves Hsp90. Hsp90 has been identified as an HDAC6-substrate. Inactivation of HDAC6 leads to Hsp90 hyperacetylation, its dissociation from an essential cochaperone, p23, and a loss of chaperone activity. In HDAC6-deficient cells, Hsp90-dependent maturation of the glucocorticoid receptor (GR) is compromised, resulting in GR defective in ligand

binding, nuclear translocation, and transcriptional activation [126]. As Hsp90 is known to play a role in protein folding and stabilisation, it will be interesting to see whether its activation by HDAC6 will have an effect on MyD88 protein turnover rates.

7.3.3 HDAC6 in immuno-oncology.

Immuno-oncology, the use of the immune system to treat cancer, has been recently on the rise, with several novel therapeutics under developed, including the recently approved CAR-T cell therapy CTL019 for the treatment of pediatric, young adult r/r B-cell ALL.

HDAC6 was shown to influence T-cell activation and survival for cancer immunotherapy [325]. Another line of investigation that was initiated on as part of this project but could not be completed due to time limitation was to identify the potential role of HDAC6 acetylation on programmed death-ligand 1 (PD-L1) regulation. Programmed death-ligand 1 (PD-L1) is a 40kDa type 1 transmembrane protein that has been speculated to play a major role in suppressing the immune system and is currently the target of several immunotherapy approaches. Potential interaction routes that are suggested include Epigenetic changes in master stimuli, such as IFN-gamma, will affect the expression of the whole cluster of genes, suggesting that epigenetic regulation could also work as an indirect mechanism to control the antigen presentation through the modulation of these master stimuli, modulation of an upstream target, for example a human gene which encodes a

protein called the class II, major histocompatibility complex, transactivator (CIITA) or via direct regulation of the epigenetic signature at the PD-L1 promoter.

7.4 Project Summary.

Although a growing interest in HDAC biology has grown over the past few years after the introduction of successful HDIs to the clinical setting, yet our understanding of how these agents work at the molecular level is still at its genesis. MyD88 has been identified as a potential sensitivity determinant to HDI treatment in a genome-wide loss-of-function screen [227]. The screen identified genes that were likely to sensitise cells to HDI-induced apoptosis. Altering MyD88 ectopic and endogenous levels were shown to play a role in mediating cells response to HDI treatment.

As a TLR adaptor, MYD88 role on controlling cytokines secretion upon HDI treatment was further investigated, and it was found that increased levels of extracellular IL6 played an important role in modulating cells sensitivity to HDI treatment. Moreover, the neutralisation of extracellular IL6 reduced the apoptotic effects of HDIs and captured cells in G2 phase.

MYD88 somatic gain of function mutation that exists frequently in DLBCL and Waldenstrom macroglobulinemia was further investigated within the context of interest of this project, and the results of showed that L265P impacted cellular sensitivity to HDI-treatment. The increased sensitivity of cells expressing L265P could reflect upon the ability of IL-6 to promote proliferation, which was further

confirmed by the results showing high level production of IL-6 in case of overexpressing the mutant ectopically.

An acetylation site at residue K132 was identified. The K132R- mutant derivative exhibited increased protein stability and caused higher levels of IL-6 secretion and HDI-induced apoptosis, relative to wild-type MyD88, establishing a mechanistic connection between MyD88 acetylation and cytokine signalling. Furthermore, that interaction was further explained within the context of utilising BRWD3 as an acetylation reader that HDACs can use to identify MyD88.

That relation was further investigated within the context of the interplay between HDAC6 and MyD88, where the former was found to be a key upstream regulator of the latter, through an acetylation-dependant mechanism, whereby HDAC6 inhibitors could subsequently have an impact on MyD88 activity. The reduced level of MyD88 in HDAC6-depleted cells is consistent with the observed increase in half-life of the hypoacetylated K132R mutant, proposing that deacetylation by HDAC6 renders MyD88 capacity for signalling. Furthermore, it was shown that this interaction did not include changes at the post-transcriptional level, whereas inhibiting the HDAC6-mediated proteosomal degradation of the acetylated MyD88 promoted protein stability, a property that was found to be attributed to HDAC6 ubiquitination domain.

Collectively, the results of this study paves the way for further investigation on the use of HDIs in different cancer types, taking MyD88 levels and its acetylation status into account.

References.

1. Slamon D, Eiermann W, Robert N, et al. Adjuvant trastuzumab in HER2-positive breast cancer. *N. Engl. J. Med.* 2011;365(14):1273-1283. doi:10.1056/NEJMoa0910383.

2. Romond EH, Perez EA, Bryant J, et al. Trastuzumab plus Adjuvant Chemotherapy for Operable HER2-Positive Breast Cancer. *N. Engl. J. Med.* 2005;353(16):1673-1684. doi:10.1056/NEJMoa052122.

3. Deininger MW. Milestones and monitoring in patients with CML treated with imatinib. *Hematology Am. Soc. Hematol. Educ. Program* 2008:419-426. doi:10.1182/asheducation-2008.1.419.

4. Kelley RK, Ko AH. Erlotinib in the treatment of advanced pancreatic cancer. *Biologics* 2008;2(1):83-95.

5. Baselga J, Albanell J, Ruiz A, et al. Phase II and tumor pharmacodynamic study of gefitinib in patients with advanced breast cancer. *J. Clin. Oncol.* 2005;23(23):5323-33. doi:10.1200/JCO.2005.08.326.

6. Green MD, Francis PA, Gebski V, et al. Gefitinib treatment in hormone-resistant and hormone receptor-negative advanced breast cancer. *Ann. Oncol.* 2009;20(11):1813-1817. doi:10.1093/annonc/mdp202.

7. Sosman JA, Kim KB, Schuchter L, et al. Survival in BRAF V600–Mutant Advanced Melanoma Treated with Vemurafenib. *N. Engl. J. Med.* 2012;366(8):707-714. doi:10.1056/NEJMoa1112302.

8. Schuster SJ, Bishop MR, Tam C, et al. Global Pivotal Phase 2 Trial of the CD19-Targeted Therapy CTL019 in Adult Patients with Relapsed or Refractory (R/R) Diffuse Large B-cell Lymphoma (DLBCL) -- An Interim Analysis. *Haematologica* 2017;102:LB2604. doi:10.1002/hon.2437_6.

9. Yuan Y, Van Allen EM, Omberg L, et al. Assessing the clinical utility of cancer genomic and proteomic data across tumor types. *Nat. Biotechnol.* 2014;32(7):644-652. doi:10.1038/nbt.2940.

10. Lawrence MS, Stojanov P, Mermel CH, et al. Discovery and saturation analysis of cancer genes across 21 tumour types. *Nature* 2014;505(7484):495-501. doi:10.1038/nature12912.

11. van de Vijver MJ, He YD, van't Veer LJ, et al. A gene-expression signature as a predictor of survival in breast cancer. *N Engl J Med* 2002;347(25):1999-2009. doi:10.1056/NEJMoa021967\r347/25/1999 [pii].

12. Rosenwald A, Wright G, Chan WC, et al. The Use of Molecular Profiling to Predict Survival after Chemotherapy for Diffuse Large-B-Cell Lymphoma. *N. Engl. J. Med.* 2002;346(25):1937-1947. doi:10.1056/NEJMoa012914.

13. Potti A, Mukherjee S, Petersen R, et al. A genomic strategy to refine prognosis in early-stage non-small-cell lung cancer. *N Engl J Med* 2006;355(6):570-580. doi:10.1056/NEJMoa060467.

14. Vargas AJ, Harris CC. Biomarker development in the precision medicine era: lung cancer as a case study. *Nat. Rev. Cancer* 2016;16(8):525-537. doi:10.1038/nrc.2016.56.

15. Hanahan D, Weinberg RA. Hallmarks of cancer: The next generation. *Cell* 2011;144(5):646-674. doi:10.1016/j.cell.2011.02.013.

16. Kinzler KW, Vogelstein B. Cancer-susceptibility genes. Gatekeepers and caretakers. *Nature* 1997;386(6627):761, 763. doi:10.1038/386761a0.

17. Esteller M. Aberrant Epigenetic Landscape in Cancer : How Cellular Identity Goes Awry. *Dev. Cell,* 2010;19(5):698-711. doi:10.1016/j.devcel.2010.10.005.

18. Jones PA, Baylin SB. The Epigenomics of Cancer. *Cell* 2007;128(4):683-692. doi:10.1016/j.cell.2007.01.029.

19. Jones PA. Functions of DNA methylation: islands, start sites, gene bodies and beyond. *Nat. Rev. Genet.* 2012;13(7):484-492. doi:10.1038/nrg3230.

20. Kluger J. A brief history of: epigenetics. *Cshl* 2008;172:22. doi:10.1016/j.calphad.2007.10.001.

21. Henikoff S, Shilatifard A. Histone modification: Cause or cog? *Trends Genet.* 2011;27(10):389-396. doi:10.1016/j.tig.2011.06.006.

22. Heijmans BT, Tobi EW, Stein AD, et al. Persistent epigenetic differences associated with prenatal exposure to famine in humans. *Proc. Natl. Acad. Sci.* 2008;105(44):17046-17049. doi:10.1073/pnas.0806560105.

23. McGowan, PO; Sasaki, A; D'Alessio, AC; Dymov, S; Labonté, B; Szyf MEA. Epigenetic regulation of the glucocorticoid receptor in human brain associates with childhood abuse. *Nat Neurosci* 2010;12(3):342-348. doi:10.1038/nn.2270.Epigenetic.

24. Sadikovic B, Al-Romaih K, Squire J a, Zielenska M. Cause and consequences of genetic and epigenetic alterations in human cancer. *Curr. Genomics* 2008;9(6):394-408. doi:10.2174/138920208785699580.

25. Mikkelsen TS, Ku M, Jaffe DB, et al. Genome-wide maps of chromatin state in pluripotent and lineage-committed cells. *Nature* 2007;448(7153):553-560. doi:10.1038/nature06008.

26. Choi S-W, Friso S. Epigenetics: A New Bridge between Nutrition and Health. *Adv. Nutr. An Int. Rev. J.* 2010;1(1):8-16. doi:10.3945/an.110.1004.

27. Feinberg AP, Vogelstein B. Hypomethylation distinguishes genes of some human cancers from their normal counterparts. *Nature* 1983;301(5895):89-

92. doi:10.1038/301089a0.

28. Greger V, Passarge E, Höpping W, Messmer E, Horsthemke B. Epigenetic changes may contribute to the formation and spontaneous regression of retinoblastoma. *Hum. Genet.* 1989;83(2):155-158. doi:10.1007/BF00286709.

29. Sakai T, Toguchida J, Ohtani N, Yandell DW, Rapaport JM, Dryja TP. Allele-specific hypermethylation of the retinoblastoma tumor-suppressor gene. *Am. J. Hum. Genet.* 1991;48(5):880-8. doi:10.1007/BF02890404.

30. Saito Y, Jones P a. Epigenetic activation of tumor suppressor microRNAs in human cancer cells. *Cell Cycle* 2006;5(19):2220-2. doi:10.4161/cc.5.19.3340.

31. Zoghbi HY, Beaudet AL. Epigenetics and human disease. *Cold Spring Harb. Perspect. Biol.* 2016;8(2):1-28. doi:10.1101/cshperspect.a019497.

32. Bjornsson HT, Daniele Fallin M, Feinberg AP. An integrated epigenetic and genetic approach to common human disease. *Trends Genet.* 2004;20(8):350-358. doi:10.1016/j.tig.2004.06.009.

33. Richardson B. Primer: epigenetics of autoimmunity. *Nat. Clin. Pract. Rheumatol.* 2007;3(9):521-527. doi:10.1038/ncprheum0573.

34. Mehler MF. Epigenetics and the nervous system. *Ann. Neurol.* 2008;64(6):602-617. doi:10.1002/ana.21595.

35. Esteller M. Epigenetics in cancer. - main article. *N. Engl. J. Med.* 2008;358(11):1148-1159. doi:10.1056/NEJMra072067.

36. Feinberg AP, Tycko B. The history of cancer epigenetics. *Nat. Rev. Cancer* 2004;4(2):143-153. doi:10.1038/nrc1279.

37. Hatzimichael E, Crook T. Cancer epigenetics: new therapies and new challenges. *J. Drug Deliv.* 2013;2013:529312. doi:10.1155/2013/529312.

38. Ellis L, Atadja PW, Johnstone RW. Epigenetics in cancer: Targeting chromatin modifications. *Mol. Cancer Ther.* 2009;8(6):1409-1420. doi:10.1158/1535-7163.MCT-08-0860.

39. La Thangue NB, Kerr DJ. Predictive biomarkers: a paradigm shift towards personalized cancer medicine. *Nat. Rev. Clin. Oncol.* 2011;8(10):587-596. doi:10.1038/nrclinonc.2011.121.

40. Mummaneni P, Shord SS. Epigenetics and oncology. *Pharmacotherapy* 2014;34(5):495-505. doi:10.1002/phar.1408.

41. Inche AG, La Thangue NB. Keynote review: Chromatin control and cancer-drug discovery: Realizing the promise. *Drug Discov. Today* 2006;11(3-4):97-109. doi:10.1016/S1359-6446(05)03691-3.

42. Olins DE, Olins AL. Nucleosomes: The structural quantum in chromosomes. *Am. Sci.* 1978;66(6):704-711.

43. Thomas JO, Kornberg RD. Cleavable cross-links in the analysis of histone-histone associations. *FEBS Lett.* 1975;58(1-2):353-358. doi:10.1016/0014-5793(75)80296-1.

44. Keating ST, Plutzky J, El-Osta A. Epigenetic changes in diabetes and cardiovascular risk. *Circ. Res.* 2016;118(11):1706-1722. doi:10.1161/CIRCRESAHA.116.306819.

45. Arents G, Moudrianakis EN. The histone fold: a ubiquitous architectural motif utilized in DNA compaction and protein dimerization. *Proc. Natl. Acad. Sci. U. S. A.* 1995;92(24):11170-4. doi:10.1073/pnas.92.24.11170.

46. Bannister AJ, Kouzarides T. Regulation of chromatin by histone modifications. *Cell Res.* 2011;21(3):381-395. doi:10.1038/cr.2011.22.

47. Peterson CL, Laniel M-A. Histones and histone modifications. *Curr. Biol.* 2004;14:R546-R551. doi:10.1016/j.cub.2004.07.007.

48. Lao VV, Grady WM. Epigenetics and colorectal cancer. *Nat. Rev. Gastroenterol. Hepatol.* 2011;8(12):686-700. doi:10.1038/nrgastro.2011.173.

49. Herman JG, Baylin SB. Gene Silencing in Cancer in Association with Promoter Hypermethylation. *N. Engl. J. Med.* 2003;349(21):2042-2054. doi:10.1056/NEJMra023075.

50. Jones P a, Baylin SB. The fundamental role of epigenetic events in cancer. *Nat. Rev. Genet.* 2002;3(6):415-28. doi:10.1038/nrg816.

51. Esteller M, Silva JM, Dominguez G, et al. Promoter hypermethylation and BRCA1 inactivation in sporadic breast and ovarian tumors. *J. Natl. Cancer Inst.* 2000;92(7):564-569. doi:10.1093/jnci/92.7.564.

52. Herman JG, Latif F, Weng Y, et al. Silencing of the VHL tumor-suppressor gene by DNA methylation in renal carcinoma. *Proc. Natl. Acad. Sci.* 1994;91(21):9700-9704. doi:10.1073/pnas.91.21.9700.

53. Herman JG, Civin CI, Issa JPJ, Collector MI, Sharkis SJ, Baylin SB. Distinct patterns of inactivation of p15(INK4B) and p16(INK4A) characterize the major types of hematological malignancies. *Cancer Res.* 1997;57(5):837-841.

54. Tate PH, Bird AP. Effects of DNA methylation on DNA-binding proteins and gene expression. *Curr. Opin. Genet. Dev.* 1993;3(2):226-231. doi:10.1016/0959-437X(93)90027-M.

55. Campanero MR, Armstrong MI, Flemington EK. CpG methylation as a mechanism for the regulation of E2F activity. *Proc. Natl. Acad. Sci. U. S. A.* 2000;97(12):6481-6486. doi:10.1073/pnas.100340697.

56. Issa JPJ, Vertino PM, Wu J, et al. Increased cytosine DNA-methyltransferase activity during colon cancer progression. *J. Natl. Cancer Inst.* 1993;85(15):1235-1240. doi:10.1093/jnci/85.15.1235.

57. P.J. L, L.L. W, D.J. L, et al. Limited up-regulation of DNA methyltransferase in human colon cancer reflecting increased cell proliferation. *Proc. Natl. Acad. Sci. U. S. A.* 1996;93(19):10366-70. doi:10.1073/pnas.93.19.10366.

58. Robertson KD, Uzvolgyi E, Liang G, et al. The human DNA methyltransferases (DNMTs) 1, 3a and 3b: Coordinate mRNA expression in normal tissues and overexpression in tumors. *Nucleic Acids Res.* 1999;27(11):2291-2298. doi:10.1093/nar/27.11.2291.

59. Bird A. DNA methylation patterns and epigenetic memory. *Genes Dev* 2002;16(1):6-21. doi:10.1101/gad.947102.

60. Cheah MSC, Wallace CD, Hoffman RM. Hypomethylation of DNA in human cancer cells: A site-specific change in the c-myc oncogene. *J. Natl. Cancer Inst.* 1984;73(5):1057-1065. doi:10.1093/jnci/73.5.1057.

61. Bhave MR, Wilson MJ, Poirier LA. C-h-ras and c-k-ras gene hypomethylation in the livers and hepatomas of rats fed methyl-deficient, amino acid-defined diets. *Carcinogenesis* 1988;9(3):343-348. doi:10.1093/carcin/9.3.343.

62. Ji W, Hernandez R, Zhang XY, et al. DNA demethylation and pericentromeric rearrangements of chromosome 1. *Mutat. Res. - Fundam. Mol. Mech. Mutagen.* 1997;379(1):33-41. doi:10.1016/S0027-5107(97)00088-2.

63. Chen RZ, Pettersson U, Beard C, Jackson-Grusby L, Jaenisch R. DNA hypomethylation leads to elevated mutation rates. *Nature* 1998;395(6697):89-93. doi:10.1038/25779.

64. Pakneshan P, Xing RH, Rabbani SA. Methylation status of uPA promoter as a molecular mechanism regulating prostate cancer invasion and growth in vitro and in vivo. *FASEB J.* 2003;17(9):1081-8. doi:10.1096/fj.02-0973com.

65. Pakneshan P, Têtu B, Rabbani SA. Demethylation of urokinase promoter as a prognostic marker in patients with breast carcinoma. *Clin. Cancer Res.* 2004;10(9):3035-41. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15131040.

66. Ateeq B, Unterberger A, Szyf M, Rabbani SA. Pharmacological Inhibition of DNA Methylation Induces Proinvasive and Prometastatic Genes In Vitro and In Vivo. *Neoplasia* 2008;10(3):266-278. doi:10.1593/neo.07947.

67. Pulukuri SMK, Estes N, Patel J, Rao JS. Demethylation-linked activation of urokinase plasminogen activator is involved in progression of prostate cancer. *Cancer Res.* 2007;67(3):930-939. doi:10.1158/0008-5472.CAN-06-2892.

68. Ehrlich M, Woods CB, Yu MC, et al. Quantitative analysis of associations between DNA hypermethylation, hypomethylation, and DNMT RNA levels in ovarian tumors. *Oncogene* 2006;25(18):2636-45. doi:10.1038/sj.onc.1209145.

69. Yegnasubramanian S, Haffner MC, Zhang Y, et al. DNA hypomethylation arises later in prostate cancer progression than CpG island hypermethylation and contributes to metastatic tumor heterogeneity. *Cancer Res.* 2008;68(21):8954-8967. doi:10.1158/0008-5472.CAN-07-6088.

70. Tahiliani M, Koh KP, Shen Y, et al. Conversion of 5-Methylcytosine to 5-Hydroxymethylcytosine in Mammalian DNA by MLL Partner TET1. *Science (80-.).* 2009;324(5929):930-935. doi:10.1126/science.1170116.Conversion.

71. Globisch D, Münzel M, Müller M, et al. Tissue distribution of 5-hydroxymethylcytosine and search for active demethylation intermediates. *PLoS One* 2010;5(12):1-9. doi:10.1371/journal.pone.0015367.

72. Kriaucionis S, Heintz N. The nuclear DNA base , 5-hydroxymethylcytosine is present in brain and enriched in Purkinje neurons. 2009;324(5929):929-930.

73. Kinney SM, Chin HG, Vaisvila R, et al. Tissue-specific distribution and dynamic changes of 5-hydroxymethylcytosine in mammalian genomes. *J. Biol. Chem.* 2011;286(28):24685-24693. doi:10.1074/jbc.M110.217083.

74. Pellakuru LG, Toubaji A, Argani P. Global 5-hydroxymethylcytosine content is significantly reduced in tissue stem/progenitor cell compartments and in human cancers. 2011;2(8):627-637.

75. Nebbioso A, Carafa V, Benedetti R, Altucci L. Trials with "epigenetic" drugs: An update. *Mol. Oncol.* 2012;6(6):657-682. doi:10.1016/j.molonc.2012.09.004.

76. Mariadason JM, Corner GA, Augenlicht LH. Genetic reprogramming in pathways of colonic cell maturation induced by short chain fatty acids: Comparison with trichostatin A, sulindac, and curcumin and implications for chemoprevention of colon cancer. *Cancer Res.* 2000;60(16):4561-4572.

77. Norton VG, Imai BS, Yau P, Bradbury EM. Histone acetylation reduces nucleosome core particle linking number change. *Cell* 1989;57(3):449-457. doi:10.1016/0092-8674(89)90920-3.

78. Ropero S, Esteller M. The role of histone deacetylases (HDACs) in human cancer. *Mol. Oncol.* 2007;1(1):19-25. doi:10.1016/j.molonc.2007.01.001.

79. New M, Olzscha H, La Thangue NB. HDAC inhibitor-based therapies: Can we interpret the code? *Mol. Oncol.* 2012;6(6):637-656. doi:10.1016/j.molonc.2012.09.003.

80. Kim MK, Kang YJ, Kim DH, et al. A novel hydroxamic acid derivative, MHY218, induces apoptosis and cell cycle arrest through downregulation of NF-??B in HCT116 human colon cancer cells. *Int. J. Oncol.* 2014;44(1):256-264. doi:10.3892/ijo.2013.2163.

81. Konstantinopoulos PA, Wilson AJ, Saskowski J, Wass E, Khabele D. Suberoylanilide hydroxamic acid (SAHA) enhances olaparib activity by

targeting homologous recombination DNA repair in ovarian cancer. *Gynecol. Oncol.* 2014;133(3):599-606. doi:10.1016/j.ygyno.2014.03.007.

82. Pasqualucci L, Dominguez-Sola D, Chiarenza A, et al. Inactivating mutations of acetyltransferase genes in B-cell lymphoma. *Nature* 2011;471(7337):189-195. doi:10.1038/nature09730.

83. Muraoka M, Konishi M, Kikuchi-Yanoshita R, et al. p300 gene alterations in colorectal and gastric carcinomas. *Oncogene* 1996;12(7):1565-9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8622873.

84. Ojesina AI, Lichtenstein L, Freeman SS, et al. Landscape of genomic alterations in cervical carcinomas. *Nature* 2013;506(7488):371-375. doi:10.1038/nature12881.

85. Kishimoto M, Kohno T, Okudela K, et al. Mutations and deletions of the CBP gene in human lung cancer. *Clin. Cancer Res.* 2005;11(2 I):512-519.

86. Tang Y, Luo J, Zhang W, Gu W. Tip60-Dependent Acetylation of p53 Modulates the Decision between Cell-Cycle Arrest and Apoptosis. *Mol. Cell* 2006;24(6):827-839. doi:10.1016/j.molcel.2006.11.021.

87. Sykes SM, Mellert HS, Holbert MA, et al. Acetylation of the p53 DNA-Binding Domain Regulates Apoptosis Induction. *Mol. Cell* 2006;24(6):841-851. doi:10.1016/j.molcel.2006.11.026.

88. Squatrito M, Gorrini C, Amati B. Tip60 in DNA damage response and growth control: many tricks in one HAT. *Trends Cell Biol* 2006;16(9):433-442. doi:10.1016/j.tcb.2006.07.007.

89. Bartkova J, Hořejší Z, Koed K, et al. DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. *Nature* 2005;434(7035):864-870. doi:10.1038/nature03482.

90. McLaughlin F, La Thangue NB. Histone deacetylase inhibitors open new doors in cancer therapy. *Biochem Pharmacol* 2004;68(6):1139-1144. doi:10.1016/j.bcp.2004.05.034.

91. Rundlett SE, Carmen a a, Kobayashi R, Bavykin S, Turner BM, Grunstein M. HDA1 and RPD3 are members of distinct yeast histone deacetylase complexes that regulate silencing and transcription. *Proc. Natl. Acad. Sci. U. S. A.* 1996;93(25):14503-14508. doi:10.1073/pnas.93.25.14503.

92. Yuan Z, Peng L, Radhakrishnan R, Seto E. Histone deacetylase 9 (HDAC9) regulates the functions of the ATDC (TRIM29) protein. *J. Biol. Chem.* 2010;285(50):39329-39338. doi:10.1074/jbc.M110.179333.

93. Imai S, Armstrong CM, Kaeberlein M, Guarente L. Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. *Nature* 2000;403(6771):795-800. doi:10.1038/35001622.

94. Liu T, Liu PY, Marshall GM. The critical role of the class III histone

deacetylase SIRT1 in cancer. *Cancer Res.* 2009;69(5):1702-1705. doi:10.1158/0008-5472.CAN-08-3365.

95. Kim HJ, Bae SC. Histone deacetylase inhibitors: molecular mechanisms of action and clinical trials as anti-cancer drugs. *Am J Transl Res* 2011;3(2):166-179. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21416059%5Cnhttp://www.ncbi.nlm.nih.gov/pmc/articles/PMC3056563/pdf/ajtr0003-0166.pdf.

96. Witt O, Deubzer HE, Milde T, Oehme I. HDAC family: What are the cancer relevant targets? *Cancer Lett.* 2009;277(1):8-21. doi:10.1016/j.canlet.2008.08.016.

97. Wu G, Broniscer A, McEachron TA, et al. Somatic histone H3 alterations in pediatric diffuse intrinsic pontine gliomas and non-brainstem glioblastomas. *Nat. Genet.* 2012;44(3):251-253. doi:10.1038/ng.1102.

98. Schwartzentruber J, Korshunov A, Liu X-Y, et al. Driver mutations in histone H3.3 and chromatin remodelling genes in paediatric glioblastoma. *Nature* 2012;482(7384):226-231. doi:10.1038/nature10833.

99. Ropero S, Fraga MF, Ballestar E, et al. A truncating mutation of HDAC2 in human cancers confers resistance to histone deacetylase inhibition. *Nat. Genet.* 2006;38(5):566-569. doi:10.1038/ng1773.

100. RUIJTER AJM de, GENNIP AH van, CARON HN, KEMP S, KUILENBURG ABP van. Histone deacetylases (HDACs): characterization of the classical HDAC family. *Biochem. J.* 2003;370(3):737-749. doi:10.1042/bj20021321.

101. Khan O, La Thangue NB. Drug Insight: histone deacetylase inhibitor-based therapies for cutaneous T-cell lymphomas. *Nat. Clin. Pract. Oncol.* 2008;5(12):714-26. doi:10.1038/ncponc1238.

102. Sasakawa Y, Naoe Y, Sogo N, et al. Marker genes to predict sensitivity to FK228, a histone deacetylase inhibitor. *Biochem. Pharmacol.* 2005;69(4):603-616. doi:10.1016/j.bcp.2004.11.008.

103. Chambers AE, Banerjee S, Chaplin T, et al. Histone acetylation-mediated regulation of genes in leukaemic cells. *Eur. J. Cancer* 2003;39(8):1165-1175. doi:10.1016/S0959-8049(03)00072-8.

104. Glaser KB, Staver MJ, Waring JF, Stender J, Ulrich RG, Davidsen SK. Gene expression profiling of multiple histone deacetylase (HDAC) inhibitors: defining a common gene set produced by HDAC inhibition in T24 and MDA carcinoma cell lines. *Mol. Cancer Ther.* 2003;2(2):151-63. doi:VL - 2.

105. Yang XJ, Seto E. Collaborative spirit of histone deacetylases in regulating chromatin structure and gene expression. *Curr. Opin. Genet. Dev.* 2003;13(2):143-153. doi:10.1016/S0959-437X(03)00015-7.

106. Kao HY, Downes M, Ordentlich P, Evans RM. Isolation of a novel histone

deacetylase reveals that class I and class II deacetylases promote SMRT-mediated repression. *Genes Dev.* 2000;14(1):55-66. doi:10.1101/gad.14.1.55.

107. Li J, Lin Q, Wang W, Wade P, Wong J. Specific targeting and constitutive association of histone deacetylase complexes during transcriptional repression. *Genes Dev.* 2002;16(6):687-692. doi:10.1101/gad.962502.

108. Yao YL, Yang WM, Seto E. Regulation of transcription factor YY1 by acetylation and deacetylation. *Mol. Cell. Biol.* 2001;21(17):5979-91. doi:10.1128/MCB.21.17.5979.

109. Weichert W, Röske A, Niesporek S, et al. Class I histone deacetylase expression has independent prognostic impact in human colorectal cancer: Specific role of class I histone deacetylases in vitro and in vivo. *Clin. Cancer Res.* 2008;14(6):1669-1677. doi:10.1158/1078-0432.CCR-07-0990.

110. Bertos NR, Wang AH, Yang XJ. Class II histone deacetylases: structure, function, and regulation. *Biochem Cell Biol* 2001;79(3):243-252. doi:10.1139/bcb-79-3-243.

111. Yang WM, Tsai SC, Wen Y Der, Fejé G, Seto E. Functional domains of histone deacetylase-3. *J. Biol. Chem.* 2002;277(11):9447-9454. doi:10.1074/jbc.M105993200.

112. Fischle W, Dequiedt F, Fillion M, Hendzel MJ, Voelter W, Verdin E. Human HDAC7 Histone Deacetylase Activity is Associated with HDAC3 in Vivo. *J. Biol. Chem.* 2001;276(38):35826-35835. doi:10.1074/jbc.M104935200.

113. Buggy JJ, Sideris ML, Mak P, Lorimer DD, McIntosh B, Clark JM. Cloning and characterization of a novel human histone deacetylase, HDAC8. *Biochem. J.* 2000;350 Pt 1:199-205. doi:10.1016/S0014-5793(00)01813-5.

114. Yang X-J, Seto E. The Rpd3/Hda1 family of lysine deacetylases: from bacteria and yeast to mice and men. *Nat. Rev. Mol. Cell Biol.* 2008;9(3):206-218. doi:10.1038/nrm2346.

115. Hu E, Chen Z, Fredrickson T, et al. Cloning and characterization of a novel human class I histone deacetylase that functions as a transcription repressor. *J. Biol. Chem.* 2000;275(20):15254-64. doi:10.1074/jbc.M908988199.

116. Bolden JE, Peart MJ, Johnstone RW. Anticancer activities of histone deacetylase inhibitors. *Nat. Rev. Drug Discov.* 2006;5(9):769-784. doi:10.1038/nrd2133.

117. McKinsey TA, Chun Li Zhang, Olson EN. Control of muscle development by dueling HATs and HDACs. *Curr. Opin. Genet. Dev.* 2001;11(5):497-504. doi:10.1016/S0959-437X(00)00224-0.

118. Fischle W, Dequiedt F, Hendzel MJ, et al. Enzymatic activity associated with class II HDACs is dependent on a multiprotein complex containing HDAC3 and SMRT/N-CoR. *Mol. Cell* 2002;9(1):45-57. doi:10.1016/S1097-2765(01)00429-4.

119. Zhou X, Marks PA, Rifkind RA, Richon VM. Cloning and characterization of a histone deacetylase, HDAC9. *Proc. Natl. Acad. Sci. U. S. A.* 2001;98(19):10572-7. doi:10.1073/pnas.191375098.

120. Bertos NR, Gilquin B, Chan GKT, Yen TJ, Khochbin S, Yang XJ. Role of the tetradecapeptide repeat domain of human histone deacetylase 6 in cytoplasmic retention. *J. Biol. Chem.* 2004;279(46):48246-48254. doi:10.1074/jbc.M408583200.

121. Hubbert C, Guardiola A, Shao R, et al. HDAC6 is a microtubule-associated deacetylase. *Nature* 2002;417(6887):455-458. doi:10.1038/417455a.

122. Zhang Y, Li N, Caron C, et al. HDAC-6 interacts with and deacetylates tubulin and microtubules in vivo. *EMBO J.* 2003;22(5):1168-1179. doi:10.1093/emboj/cdg115.

123. Kwon S, Zhang Y, Matthias P. The deacetylase HDAC6 is a novel critical component of stress granules involved in the stress response. *Genes Dev.* 2007;21(24):3381-3394. doi:10.1101/gad.461107.

124. Kawaguchi Y, Kovacs JJ, McLaurin A, Vance JM, Ito A, Yao TP. The deacetylase HDAC6 regulates aggresome formation and cell viability in response to misfolded protein stress. *Cell* 2003;115(6):727-738. doi:10.1016/S0092-8674(03)00939-5.

125. Boyault C, Zhang Y, Fritah S, et al. HDAC6 controls major cell response pathways to cytotoxic accumulation of protein aggregates. *Genes Dev.* 2007;21(17):2172-2181. doi:10.1101/gad.436407.

126. Kovacs JJ, Murphy PJM, Gaillard S, et al. HDAC6 regulates Hsp90 acetylation and chaperone-dependent activation of glucocorticoid receptor. *Mol. Cell* 2005;18(5):601-607. doi:10.1016/j.molcel.2005.04.021.

127. Li Y, Zhang X, Polakiewicz RD, Yao T-P, Comb MJ. HDAC6 is required for epidermal growth factor-induced beta-catenin nuclear localization. *J. Biol. Chem.* 2008;283(19):12686-90. doi:10.1074/jbc.C700185200.

128. Zhang W, Kone BC. NF-kappaB inhibits transcription of the H+-K+-ATPase alpha(2)-subunit gene: role of histone deacetylases. *Am. J. Physiol. Ren. Physiol.* 2002;283(5):F904-11. doi:10.1152/ajprenal.00156.2002.

129. Hada M, Kwok RPS. Regulation of ku70-bax complex in cells. *J. Cell Death* 2014;7(1):11-13. doi:10.4137/JCD.S13695.

130. Guardiola AR, Yao T-P. Molecular cloning and characterization of a novel histone deacetylase HDAC10. *J. Biol. Chem.* 2002;277(5):3350-6. doi:10.1074/jbc.M109861200.

131. Tong JJ, Liu J, Bertos NR, Yang X-J. Identification of HDAC10, a novel class II human histone deacetylase containing a leucine-rich domain. *Nucleic Acids Res.* 2002;30(5):1114-1123. doi:10.1093/nar/30.5.1114.

132. Kao H-Y, Lee C-H, Komarov A, Han CC, Evans RM. Isolation and characterization of mammalian HDAC10, a novel histone deacetylase. *J. Biol. Chem.* 2002;277(1):187-193. doi:10.1074/jbc.M108931200.

133. Lucio-Eterovic AK, Cortez MA, Valera ET, et al. Differential expression of 12 histone deacetylase (HDAC) genes in astrocytomas and normal brain tissue: class II and IV are hypoexpressed in glioblastomas. *BMC Cancer* 2008;8(1):243. doi:10.1186/1471-2407-8-243.

134. Gao L, Cueto MA, Asselbergs F, Atadja P. Cloning and functional characterization of HDAC11, a novel member of the human histone deacetylase family. *J. Biol. Chem.* 2002;277(28):25748-55. doi:10.1074/jbc.M111871200.

135. Thole TM, Lodrini M, Fabian J, et al. Neuroblastoma cells depend on HDAC11 for mitotic cell cycle progression and survival. *Cell Death Dis.* 2017;8(3):e2635. doi:10.1038/cddis.2017.49.

136. Stimson L, Wood V, Khan O, Fotheringham S, La Thangue NB. HDAC inhibitor-based therapies and haematological malignancy. *Ann. Oncol.* 2009;20(8):1293-1302. doi:10.1093/annonc/mdn792.

137. Dinarello CA, Fossati G, Mascagni P. Histone deacetylase inhibitors for treating a spectrum of diseases not related to cancer. *Mol. Med.* 2011;17(5-6):333-52. doi:10.2119/molmed.2011.00116.

138. Mai A, Massa S, Rotili D, et al. Histone deacetylation in epigenetics: An attractive target for anticancer therapy. *Med. Res. Rev.* 2005;25(3):261-309. doi:10.1002/med.20024.

139. Finnin MS, Donigian JR, Cohen a, et al. Structures of a histone deacetylase homologue bound to the TSA and SAHA inhibitors. *Nature* 1999;401(6749):188-193. doi:10.1038/43710.

140. Mohammed TA, Holen KD, Jaskula-Sztul R, et al. A Pilot Phase II Study of Valproic Acid for Treatment of Low-Grade Neuroendocrine Carcinoma. *Oncologist* 2011;16(6):835-843. doi:10.1634/theoncologist.2011-0031.

141. Raffoux E, Cras A, Recher C, et al. Phase 2 clinical trial of 5-azacitidine, valproic acid, and all-trans retinoic acid in patients with high-risk acute myeloid leukemia or myelodysplastic syndrome. *Oncotarget* 2010;1(1):34-42. doi:100518 [pii].

142. Brodie SA, Brandes JC. Could valproic acid be an effective anticancer agent? The evidence so far. *Expert Rev. Anticancer Ther.* 2014;14(10):1097-1100. doi:10.1586/14737140.2014.940329.

143. Richon VM, Webb Y, Merger R, et al. Second generation hybrid polar compounds are potent inducers of transformed cell differentiation. *Proc. Natl. Acad. Sci.* 1996;93(12):5705-5708. doi:10.1073/pnas.93.12.5705.

144. Richon VM, Emiliani S, Verdin E, et al. A class of hybrid polar inducers of

transformed cell differentiation inhibits histone deacetylases. *Proc. Natl. Acad. Sci.* 1998;95(6):3003-3007. doi:10.1073/pnas.95.6.3003.

145. Minucci S, Pelicci PG. Histone deacetylase inhibitors and the promise of epigenetic (and more) treatments for cancer. *Nat. Rev. Cancer* 2006;6(1):38-51. doi:10.1038/nrc1779.

146. Dokmanovic M, Clarke C, Marks PA. Histone Deacetylase Inhibitors: Overview and Perspectives. *Mol. Cancer Res.* 2007;5(10):981-989. doi:10.1158/1541-7786.MCR-07-0324.

147. Johnstone RW, Licht JD. Histone deacetylase inhibitors in cancer therapy: Is transcription the primary target? *Cancer Cell* 2003;4(1):13-18. doi:10.1016/S1535-6108(03)00165-X.

148. Gui C-Y, Ngo L, Xu WS, Richon VM, Marks PA. Histone deacetylase (HDAC) inhibitor activation of p21WAF1 involves changes in promoter-associated proteins, including HDAC1. *Proc. Natl. Acad. Sci. U. S. A.* 2004;101(5):1241-6. doi:10.1073/pnas.0307708100.

149. Butler LM, Zhou X, Xu W-S, et al. The histone deacetylase inhibitor SAHA arrests cancer cell growth, up-regulates thioredoxin-binding protein-2, and down-regulates thioredoxin. *Proc. Natl. Acad. Sci. U. S. A.* 2002;99(18):11700-5. doi:10.1073/pnas.182372299.

150. Furumai R, Matsuyama A, Kobashi N, et al. FK228 (depsipeptide) as a natural prodrug that inhibits class I histone deacetylases. *Cancer Res.* 2002;62(17):4916-4921. doi:10.1046/j.1365-2443.1997.1260323.x.

151. Eckschlager T, Plch J, Stiborova M, Hrabeta J. Histone deacetylase inhibitors as anticancer drugs. *Int. J. Mol. Sci.* 2017;18(7). doi:10.3390/ijms18071414.

152. Marks PA, Rifkind RA, Richon VM, Breslow R, Miller T, Kelly WK. HISTONE DEACETYLASES AND CANCER: CAUSES AND THERAPIES. *Nat. Rev. Cancer* 2001;1(3):194-202. doi:10.1038/35106079.

153. Lavu S, Boss O, Elliott PJ, Lambert PD. Sirtuins — novel therapeutic targets to treat age-associated diseases. *Nat. Rev. Drug Discov.* 2008;7(10):841-853. doi:10.1038/nrd2665.

154. Stiborova M, Eckschlager T, Poljakova J, et al. The Synergistic Effects of DNA-Targeted Chemotherapeutics and Histone Deacetylase Inhibitors As Therapeutic Strategies for Cancer Treatment. *Curr. Med. Chem.* 2012;19(25):4218-4238. doi:10.2174/092986712802884286.

155. Namdar M, Perez G, Ngo L, Marks PA. Selective inhibition of histone deacetylase 6 (HDAC6) induces DNA damage and sensitizes transformed cells to anticancer agents. *Proc. Natl. Acad. Sci. U. S. A.* 2010;107(46):20003-8. doi:10.1073/pnas.1013754107.

156. Pei X-Y, Dai Y, Grant S. Synergistic Induction of Oxidative Injury and Apoptosis in Human Multiple Myeloma Cells by the Proteasome Inhibitor

Bortezomib and Histone Deacetylase Inhibitors. *Clin. Cancer Res.* 2004;10(11):3839-3852. doi:10.1158/1078-0432.CCR-03-0561.

157. Nawrocki ST, Carew JS, Pino MS, et al. Aggresome disruption: A novel strategy to enhance bortezomib-induced apoptosis in pancreatic cancer cells. *Cancer Res.* 2006;66(7):3773-3781. doi:10.1158/0008-5472.CAN-05-2961.

158. Groh T, Hrabeta J, Khalil MA, Doktorova H, Eckschlager T, Stiborova M. The synergistic effects of DNA-damaging drugs cisplatin and etoposide with a histone deacetylase inhibitor valproate in high-risk neuroblastoma cells. *Int. J. Oncol.* 2015;47(1):343-352. doi:10.3892/ijo.2015.2996.

159. Daud AI, Dawson J, Deconti RC, et al. Potentiation of a topoisomerase i inhibitor, karenitecin, by the histone deacetylase inhibitor valproic acid in melanoma: translational and phase l/ll clinical trial. *Clin. Cancer Res.* 2009;15(7):2479-2487. doi:10.1158/1078-0432.CCR-08-1931.

160. Kim IA, Kim IH, Kim HJ, Chie EK, Kim J-S. HDAC Inhibitor-Mediated Radiosensitization in Human Carcinoma Cells: A General Phenomenon? *Regul. Pap. J. Radiat. Res* 2010;51:257-263. doi:10.1269/jrr.09115.

161. Munshi A, Kurland JF, Nishikawa T, et al. Histone deacetylase inhibitors radiosensitize human melanoma cells by suppressing DNA repair activity. *Clin. Cancer Res.* 2005;11(13):4912-22. doi:10.1158/1078-0432.CCR-04-2088.

162. Zhang Y, Carr T, Dimtchev A, Zaer N, Dritschilo A, Jung M. Attenuated DNA damage repair by trichostatin A through BRCA1 suppression. *Radiat. Res.* 2007;168(1):115-24. doi:10.1667/RR0811.1.

163. Kim IA, Shin JH, Kim IH, et al. Histone deacetylase inhibitor-mediated radiosensitization of human cancer cells: Class differences and the potential influence of p53. *Clin. Cancer Res.* 2006;12(3 I):940-949. doi:10.1158/1078-0432.CCR-05-1230.

164. Weller M, Gorlia T, Cairncross JG, et al. Prolonged survival with valproic acid use in the EORTC/NCIC temozolomide trial for glioblastoma. *Neurology* 2011;77(12):1156-1164. doi:10.1212/WNL.0b013e31822f02e1.

165. Stevens FE, Beamish H, Warrener R, Gabrielli B. Histone deacetylase inhibitors induce mitotic slippage. *Oncogene* 2008;27(10):1345-1354. doi:10.1038/sj.onc.1210779.

166. Mann BS, Johnson JR, He K, et al. Vorinostat for treatment of cutaneous manifestations of advanced primary cutaneous T-cell lymphoma. *Clin. Cancer Res.* 2007;13(8):2318-2322. doi:10.1158/1078-0432.CCR-06-2672.

167. Coiffier B, Pro B, Prince HM, et al. Results from a pivotal, open-label, phase II study of romidepsin in relapsed or refractory peripheral T-cell lymphoma after prior systemic therapy. *J. Clin. Oncol.* 2012;30(6):631-636. doi:10.1200/JCO.2011.37.4223.

168. Poole RM. Belinostat: First global approval. *Drugs* 2014;74(13):1543-1554. doi:10.1007/s40265-014-0275-8.

169. Garnock-Jones KP. Cobimetinib: First Global Approval. *Drugs* 2015;75(15):1823-1830. doi:10.1007/s40265-015-0477-8.

170. Dai Y, Rahmani M, Dent P, Grant S. Blockade of Histone Deacetylase Inhibitor-Induced RelA / p65 Acetylation and NF- κ B Activation Potentiates Apoptosis in Leukemia Cells through a Process Mediated by Oxidative Damage , XIAP Downregulation , and c-Jun N-Terminal Kinase 1 Activation Blockad. *Mol. Cell. Biol.* 2005;25(13):5429-5444. doi:10.1128/MCB.25.13.5429.

171. Falkenberg KJ, Johnstone RW. Histone deacetylases and their inhibitors in cancer, neurological diseases and immune disorders. *Nat. Rev. Drug Discov.* 2014;13(9):673-691. doi:10.1038/nrd4360.

172. Khan O, La Thangue NB. HDAC inhibitors in cancer biology: emerging mechanisms and clinical applications. *Immunol Cell Biol* 2012;90(1):85-94. doi:10.1038/icb.2011.100.

173. Petruccelli L, Dupéré-Richer D, Pettersson F, Retrouvey H, Skoulikas S, Miller W. Vorinostat induces reactive oxygen species and dna damage in acute myeloid leukemia cells. *PLoS One* 2011;6(6):e20987. doi:10.1371/journal.pone.0020987.

174. Lee J-H, Choy ML, Ngo L, Foster SS, Marks P a. Histone deacetylase inhibitor induces DNA damage, which normal but not transformed cells can repair. *Proc. Natl. Acad. Sci. U. S. A.* 2010;107(33):14639-44. doi:10.1073/pnas.1008522107.

175. Hajji N, Wallenborg K, Vlachos P, Füllgrabe J, Hermanson O, Joseph B. Opposing effects of hMOF and SIRT1 on H4K16 acetylation and the sensitivity to the topoisomerase II inhibitor etoposide. *Oncogene* 2010;29(15):2192-2204. doi:10.1038/onc.2009.505.

176. Sadoul K, Boyault C, Pabion M, Khochbin S. Regulation of protein turnover by acetyltransferases and deacetylases. *Biochimie* 2008;90(2):306-312. doi:10.1016/j.biochi.2007.06.009.

177. Nimmanapalli R, Fuino L, Bali P, et al. Histone deacetylase inhibitor LAQ824 both lowers expression and promotes proteasomal degradation of Bcr-Abl and induces apoptosis of imatinib mesylate-sensitive or -refractory chronic myelogenous leukemia-blast crisis cells. *Cancer Res.* 2003;63(16):5126-5135.

178. Fandy TE, Srivastava RK. Trichostatin a sensitizes TRAIL-resistant myeloma cells by downregulation of the antiapoptotic Bcl-2 proteins. *Cancer Chemother. Pharmacol.* 2006;58(4):471-477. doi:10.1007/s00280-005-0184-3.

179. Xu W, Ngo L, Perez G, et al. Intrinsic apoptotic and thioredoxin pathways in

human prostate cancer cell response to histone deacetylase inhibitor. *Proc Natl Acad Sci USA* 2006;103(42):15540-15545. doi:10.1073/pnas.0607518103.

180. Ocker M, Schneider-Stock R. Histone deacetylase inhibitors: Signalling towards p21cip1/waf1. *Int. J. Biochem. Cell Biol.* 2007;39(7-8):1367-1374. doi:10.1016/j.biocel.2007.03.001.

181. Gius D, Cui H, Bradbury CM, et al. Distinct effects on gene expression of chemical and genetic manipulation of the cancer epigenome revealed by a multimodality approach. *Cancer Cell* 2004;6(4):361-371. doi:10.1016/j.ccr.2004.08.029.

182. Zhao Y, Lu S, Wu L, et al. Acetylation of p53 at Lysine 373 / 382 by the Histone Deacetylase Inhibitor Depsipeptide Induces Expression of p21 Waf1 / Cip1. *Mol. Cell. Biol.* 2006;26(7):2782-2790. doi:10.1128/MCB.26.7.2782.

183. Mühlethaler-Mottet A, Meier R, Flahaut M, et al. Complex molecular mechanisms cooperate to mediate histone deacetylase inhibitors anti-tumour activity in neuroblastoma cells. *Mol. Cancer* 2008;7:55. doi:10.1186/1476-4598-7-55.

184. Ellis L, Hammers H, Pili R. Targeting tumor angiogenesis with Histone Deacetylase Inhibitors. *Curr. Opin. Genet. Dev.* 2010;280(2):1-15. doi:10.1016/j.canlet.2008.11.012.Targeting.

185. Kroesen M, Gielen PR, Brok IC, Armandari I, Hoogerbrugge PM, Adema GJ. HDAC inhibitors and immunotherapy; a double edged sword? *Oncotarget* 2014;5(16):6558-6572. doi:10.18632/oncotarget.2289.

186. Armeanu S, Bitzer M, Lauer UM, et al. Natural Killer Cell − Mediated Lysis of Hepatoma Cells via Specific Induction of NKG2D Ligands by the Histone Deacetylase Inhibitor Sodium Valproate. *Cancer Res* 2005;65:6321-6329.

187. Vianello F, Papeta N, Chen T, et al. Murine B16 Melanomas Expressing High Levels of the Chemokine Stromal-Derived Factor-1/CXCL12 Induce Tumor-Specific T Cell Chemorepulsion and Escape from Immune Control. *J. Immunol.* 2006;176(5):2902-2914. doi:10.4049/jimmunol.176.5.2902.

188. Vishwakarma S, Iyer LR, Muley M, et al. Tubastatin, a selective histone deacetylase 6 inhibitor shows anti-inflammatory and anti-rheumatic effects. *Int. Immunopharmacol.* 2013;16(1):72-78. doi:10.1016/j.intimp.2013.03.016.

189. Leoni F, Fossati G, Lewis EC, et al. The Histone Deacetylase Inhibitor ITF2357 Reduces Production of Pro-Inflammatory Cytokines In Vitro and Systemic Inflammation In Vivo. *Mol. Med.* 2005;11(1-12):1-15. doi:10.2119/2006-00005.Dinarello.

190. Pérez-Salvia M, Esteller M. Bromodomain inhibitors and cancer therapy: From structures to applications. *Epigenetics* 2016;12(5):1-17. doi:10.1080/15592294.2016.1265710.

191. Yang Z, Yik JHN, Chen R, et al. Recruitment of P-TEFb for stimulation of transcriptional elongation by the bromodomain protein Brd4. *Mol. Cell* 2005;19(4):535-545. doi:10.1016/j.molcel.2005.06.029.

192. Delmore JE, Issa GC, Lemieux ME, et al. BET bromodomain inhibition as a therapeutic strategy to target c-Myc. *Cell* 2011;146(6):904-917. doi:10.1016/j.cell.2011.08.017.

193. Zou Z, Huang B, Wu X, et al. Brd4 maintains constitutively active NF-κB in cancer cells by binding to acetylated RelA. *Oncogene* 2014;33(18):2395-2404. doi:10.1038/onc.2013.179.

194. Filippakopoulos P, Knapp S. Targeting bromodomains: epigenetic readers of lysine acetylation. *Nat. Rev. Drug Discov.* 2014;13(5):337-356. doi:10.1038/nrd4286.

195. Yu X, Li Z, Shen J. BRD7: A novel tumor suppressor gene in different cancers. *Am. J. Transl. Res.* 2016;8(2):742-748.

196. Mujtaba S, He Y, Zeng L, et al. Structural Mechanism of the Bromodomain of the Coactivator CBP in p53 Transcriptional Activation. *Mol. Cell* 2004;13(2):251-263. doi:10.1016/S1097-2765(03)00528-8.

197. Schiltz RL, Mizzen CA, Vassilev A, Cook RG, Allis CD, Nakatani Y. Overlapping but distinct patterns of histone acetylation by the human coactivators p300 and PCAF within nucleosomal substrates. *J. Biol. Chem.* 1999;274(3):1189-1192. doi:10.1074/jbc.274.3.1189.

198. Filippakopoulos P, Qi J, Picaud S, et al. Selective inhibition of BET bromodomains. *Nature* 2010;468(7327):1067-1073. doi:10.1038/nature09504.

199. French CA, Ramirez CL, Kolmakova J, et al. BRD–NUT oncoproteins: a family of closely related nuclear proteins that block epithelial differentiation and maintain the growth of carcinoma cells. *Oncogene* 2008;27(15):2237-2242. doi:10.1038/sj.onc.1210852.

200. Berenguer-Daizé C, Astorgues-Xerri L, Odore E, et al. OTX015 (MK-8628), a novel BET inhibitor, displays in vitro and in vivo antitumor effects alone and in combination with conventional therapies in glioblastoma models. *Int. J. Cancer* 2016;139(9):2047-2055. doi:10.1002/ijc.30256.

201. Coudé M-M, Braun T, Berrou J, et al. BET inhibitor OTX015 targets BRD2 and BRD4 and decreases c-MYC in acute leukemia cells. *Oncotarget* 2015;6(19):17698-712. doi:10.18632/oncotarget.4131.

202. Boi M, Gaudio E, Bonetti P, et al. The BET bromodomain inhibitor OTX015 affects pathogenetic pathways in preclinical B-cell tumor models and synergizes with targeted drugs. *Clin. Cancer Res.* 2015;21(7):1628-1638. doi:10.1158/1078-0432.CCR-14-1561.

203. Dawson M a., Prinjha RK, Dittman A, et al. Inhibition of BET recruitment to

chromatin as an effective treatment for MLL-fusion leukaemia. Supp data. *Nature* 2011;478(7370):529-33. doi:10.1038/nature10509.

204. Atkinson AJ, Colburn WA, DeGruttola VG, et al. Biomarkers and surrogate endpoints: Preferred definitions and conceptual framework. *Clin. Pharmacol. Ther.* 2001;69(3):89-95. doi:10.1067/mcp.2001.113989.

205. Aronson JK. Biomarkers and surrogate endpoints. *Br. J. Clin. Pharmacol.* 2005;59(5):491-494. doi:10.1111/j.1365-2125.2005.02435.x.

206. Füzéry AK, Levin JJ, Chan MM, et al. Translation of proteomic biomarkers into FDA approved cancer diagnostics: issues and challenges. *Clin. Proteomics* 2013;10(1):13. doi:10.1186/1559-0275-10-13.

207. Helleday T, Eshtad S, Nik-Zainal S. Mechanisms underlying mutational signatures in human cancers. *Nat. Rev. Genet.* 2014;15(9):585-598. doi:10.1038/nrg3729.

208. Berman DM, Bosenberg MW, Orwant RL, et al. Investigative pathology: leading the post-genomic revolution. *Lab. Investig.* 2012;92(1):4-8. doi:10.1038/labinvest.2011.147.

209. O'Connor JPB, Jackson A, Asselin M-C, Buckley DL, Parker GJM, Jayson GC. Quantitative imaging biomarkers in the clinical development of targeted therapeutics: current and future perspectives. *Lancet Oncol.* 2008;9(8):766-776. doi:10.1016/S1470-2045(08)70196-7.

210. Frank R, Hargreaves R. Clinical biomarkers in drug discovery and development. *Nat. Rev. Drug Discov.* 2003;2(7):566-80. doi:10.1038/nrd1130.

211. August J. Market watch: emerging companion diagnostics for cancer drugs. *Nat. Rev. Drug Discov.* 2010;9(5):351. doi:10.1038/nrd3173.

212. Sarker D, Workman P. Pharmacodynamic Biomarkers for Molecular Cancer Therapeutics. *Adv. Cancer Res.* 2006;96:213-268. doi:10.1016/S0065-230X(06)96008-4.

213. Zhou Q, Gallo JM. The pharmacokinetic/pharmacodynamic pipeline: translating anticancer drug pharmacology to the clinic. *AAPS J.* 2011;13(1):111-20. doi:10.1208/s12248-011-9253-1.

214. Raftery J. NICE and the challenge of cancer drugs. *BMJ* 2009;338(January):b67. doi:10.1136/bmj.b67.

215. Goossens N, Nakagawa S, Sun X, Hoshida Y. Cancer biomarker discovery and validation. *Transl. Cancer Res.* 2015;4(3):256-269. doi:10.3978/j.issn.2218-676X.2015.06.04.

216. Selleck MJ, Senthil M, Wall NR. Making Meaningful Clinical Use of Biomarkers. *Biomark. Insights* 2017;12:1-7. doi:10.1177/1177271917715236.

217. Shakir SAW, Layton D. Causal association in pharmacovigilance and pharmacoepidemiology: thoughts on the application of the Austin Bradford-Hill criteria. *Drug Saf.* 2002;25(6):467-71. doi:10.2165/00002018-200225060-00012.

218. Chan TA, Baylin SB. Epigenetic biomarkers. *Curr. Top. Microbiol. Immunol.* 2012;355:189-216. doi:10.1007/82-2011-165.

219. Esteller M, Corn PG, Baylin SB, Herman JG. A gene hypermethylation profile of human cancer. *Cancer Res.* 2001;61(8):3225-3229. doi:10.1126/science.270.5235.484.

220. Deiss LP, Feinstein E, Berissi H, Cohen O, Kimchi A. Identification of a novel serine/threonine kinase and a novel 15-kD protein as potential mediators of the γ interferon-induced cell death. *Genes Dev.* 1995;9(1):15-30. doi:10.1101/gad.9.1.15.

221. Iliopoulos D, Oikonomou P, Messinis I, Tsezou A. Correlation of promoter hypermethylation in hTERT, DAPK and MGMT genes with cervical oncogenesis progression. *Oncol. Rep.* 2009;22(1):199-204. doi:10.3892/or_00000425.

222. Jarmalaite S, Jankevicius F, Kurgonaite K, Suziedelis K, Mutanen P, Husgafvel-Pursiainen K. Promoter hypermethylation in tumour suppressor genes shows association with stage, grade and invasiveness of bladder cancer. *Oncology* 2008;75(3-4):145-151. doi:10.1159/000158665.

223. Song JZ, Stirzaker C, Harrison J, Melki JR, Clark SJ. Hypermethylation trigger of the glutathione-S-transferase gene (GSTP1) in prostate cancer cells. *Oncogene* 2002;21:1048-1061. doi:10.1038/sj/onc/.

224. Weichert W, Röske A, Gekeler V, et al. Histone deacetylases 1, 2 and 3 are highly expressed in prostate cancer and HDAC2 expression is associated with shorter PSA relapse time after radical prostatectomy. *Br. J. Cancer* 2008;98(3):604-610. doi:10.1038/sj.bjc.6604199.

225. Ferrari R, Pellegrini M, Horwitz GA, Xie W, Berk AJ, Kurdistani SK. Epigenetic reprogramming by adenovirus e1a. *Science* 2008;321(5892):1086-8. doi:10.1126/science.1155546.

226. Rasti M, Grand RJA, Mymryk JS, Gallimore PH, Turnell AS. Recruitment of CBP/p300, TATA-binding protein, and S8 to distinct regions at the N terminus of adenovirus E1A. *J. Virol.* 2005;79(9):5594-605. doi:10.1128/JVI.79.9.5594-5605.2005.

227. Fotheringham S, Epping MT, Stimson L, et al. Genome-wide Loss-of-Function Screen Reveals an Important Role for the Proteasome in HDAC Inhibitor-Induced Apoptosis. *Cancer Cell* 2009;15(1):57-66. doi:10.1016/j.ccr.2008.12.001.

228. Khan O, Fotheringham S, Wood V, et al. HR23B is a biomarker for tumor sensitivity to HDAC inhibitor-based therapy. *Proc. Natl. Acad. Sci.*

2010;107(14):6532-6537. doi:10.1073/pnas.0913912107.

229. New M, Olzscha H, Liu G, et al. A regulatory circuit that involves HR23B and HDAC6 governs the biological response to HDAC inhibitors. *Cell Death Differ.* 2013;20(10):1306-1316. doi:10.1038/cdd.2013.47.

230. Yeo W, Chung HC, Chan SL, et al. Epigenetic therapy using belinostat for patients with unresectable hepatocellular carcinoma: A multicenter phase I/II study with biomarker and pharmacokinetic analysis of tumors from patients in the mayo phase II consortium and the cancer therapeutics res. *J. Clin. Oncol.* 2012;30(27):3361-3367. doi:10.1200/JCO.2011.41.2395.

231. Takeda K, Akira S. TLR signaling pathways. *Semin. Immunol.* 2004;16(1):3-9. doi:10.1016/j.smim.2003.10.003.

232. Delneste Y, Beauvillain C, Jeannin P. Innate immunity: structure and function of TLRs. *Médecine Sci.* 2007;23(1):67-73. doi:10.1051/medsci/200723167.

233. O'Neill LAJ, Bowie AG. The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. *Nat. Rev. Immunol.* 2007;7(5):353-364. doi:10.1038/nri2079.

234. Ohnishi H, Tochio H, Kato Z, et al. Structural basis for the multiple interactions of the MyD88 TIR domain in TLR4 signaling. *Proc. Natl. Acad. Sci. U. S. A.* 2009;106(25):10260-5. doi:10.1073/pnas.0812956106.

235. Rakoff-Nahoum S, Medzhitov R. Toll-like receptors and cancer. *Nat. Rev. Cancer* 2009;9(1):57-63. doi:10.1038/nrc2541.

236. Salaun B, Romero P, Lebecque S. Toll-like receptor's two-edged sword: When immunity meets apoptosis. *Eur. J. Immunol.* 2007;37(12):3311-3318. doi:10.1002/eji.200737744.

237. Sun Y, Leaman DW. Ectopic expression of toll-like receptor-3 (TLR-3) overcomes the double-stranded RNA (dsRNA) signaling defects of P2.1 cells. *J. Interferon Cytokine Res.* 2004;24(6):350-61. doi:10.1089/107999004323142213.

238. Salaun B, Coste I, Rissoan M-C, Lebecque SJ, Renno T. TLR3 Can Directly Trigger Apoptosis in Human Cancer Cells. *J. Immunol.* 2006;176(8):4894-4901. doi:10.4049/jimmunol.176.8.4894.

239. Hasan UA, Trinchieri G, Vlach J. Toll-like receptor signaling stimulates cell cycle entry and progression in fibroblasts. *J. Biol. Chem.* 2005;280(21):20620-20627. doi:10.1074/jbc.M500877200.

240. Zambirinis CP, Levie E, Nguy S, et al. TLR9 ligation in pancreatic stellate cells promotes tumorigenesis. *J. Exp. Med.* 2015;212(12):2077-2094. doi:10.1084/jem.20142162.

241. Tye H, Kennedy CL, Najdovska M, et al. STAT3-Driven Upregulation of

TLR2 Promotes Gastric Tumorigenesis Independent of Tumor Inflammation. *Cancer Cell* 2012;22(4):466-478. doi:10.1016/j.ccr.2012.08.010.

242. Ngo VN, Young RM, Schmitz R, et al. Oncogenically active MYD88 mutations in human lymphoma. *Nature* 2011;470(7332):115-119. doi:10.1038/nature09671.

243. Choi JW, Kim Y, Lee JH, Kim YS. MYD88 expression and L265P mutation in diffuse large B-cell lymphoma. *Hum. Pathol.* 2013;44(7):1375-1381. doi:10.1016/j.humpath.2012.10.026.

244. Cataisson C, Salcedo R, Hakim S, et al. IL-1R–MyD88 signaling in keratinocyte transformation and carcinogenesis. *J. Exp. Med.* 2012;209(9):1689-1702. doi:10.1084/jem.20101355.

245. Mittal D, Saccheri F, Vénéreau E, Pusterla T, Bianchi ME, Rescigno M. TLR4-mediated skin carcinogenesis is dependent on immune and radioresistant cells. *EMBO J.* 2010;29(13):2242-2252. doi:10.1038/emboj.2010.94.

246. Naugler WE, Sakurai T, Kim S, et al. Gender Disparity in Liver Cancer Due to Sex Differences in MyD88-Dependent IL-6 Production. *Science (80-.).* 2007;317(5834):121-124. doi:10.1126/science.1140485.

247. Xiang F, Ni Z, Zhan Y, et al. Increased expression of MyD88 and association with paclitaxel resistance in breast cancer. *Tumor Biol.* 2016;37(5):6017-6025. doi:10.1007/s13277-015-4436-5.

248. Yang L, Inokuchi S, Roh YS, et al. Transforming growth factor-β signaling in hepatocytes promotes hepatic fibrosis and carcinogenesis in mice with hepatocyte-specific deletion of TAK1. *Gastroenterology* 2013;144(5). doi:10.1053/j.gastro.2013.01.056.

249. Coste I, Le Corf K, Kfoury A, et al. Dual function of MyD88 in RAS signaling and inflammation, leading to mouse and human cell transformation. *J. Clin. Invest.* 2010;120(10):3663-3667. doi:10.1172/JCI42771.

250. Mata M, Gerken C, Nguyen P, Krenciute G, Spencer DM, Gottschalk S. Inducible activation of MyD88 and CD40 in CAR T-cells results in controllable and potent antitumor activity in preclinical solid tumor models. *Cancer Discov.* 2017:CD-17-0263. doi:10.1158/2159-8290.CD-17-0263.

251. Ochi A, Nguyen AH, Bedrosian AS, et al. MyD88 inhibition amplifies dendritic cell capacity to promote pancreatic carcinogenesis via Th2 cells. *J. Exp. Med.* 2012;209(9):1671-1687. doi:10.1084/jem.20111706.

252. Treon SP, Xu L, Yang G, et al. MYD88 L265P Somatic Mutation in Waldenström's Macroglobulinemia. *N. Engl. J. Med.* 2012;367(9):826-833. doi:10.1056/NEJMoa1200710.

253. Gachard N, Parrens M, Soubeyran I, et al. IGHV gene features and MYD88 L265P mutation separate the three marginal zone lymphoma entities and

Waldenström macroglobulinemia/lymphoplasmacytic lymphomas. *Leukemia* 2013;27(1):183-189. doi:10.1038/leu.2012.257.

254. Ansell SM, Hodge LS, Secreto FJ, et al. Activation of TAK1 by MYD88 L265P drives malignant B-cell Growth in non-Hodgkin lymphoma. *Blood Cancer J.* 2014;4(2):e183. doi:10.1038/bcj.2014.4.

255. Yang G, Zhou Y, Liu X, et al. A mutation in MYD88 (L265P) supports the survival of lymphoplasmacytic cells by activation of Bruton tyrosine kinase in Waldenström macroglobulinemia. *Blood* 2013;122(7):1222-1232. doi:10.1182/blood-2012-12-475111.

256. Maslinska D, Laure-Kamionowska M, Maslinski S. Toll-like receptors as an innate immunity bridge to neuroinflammation in medulloblastoma. *Folia Neuropathol.* 2012;50(4):375-381. doi:10.5114/fn.2012.32367.

257. Dranoff G. Cytokines in cancer pathogenesis and cancer therapy. *Nat. Rev. Cancer* 2004;4(1):11-22. doi:10.1038/nrc1252.

258. Akira S, Takeda K. Toll-like receptor signalling. *Nat Rev Immunol* 2004;4(7):499-511. doi:10.1038/nri1391\rnri1391 [pii].

259. Moynagh PN. TLR signalling and activation of IRFs: Revisiting old friends from the NF-κB pathway. *Trends Immunol.* 2005;26(9):469-476. doi:10.1016/j.it.2005.06.009.

260. Medvedev AE, Kopydlowski KM, Vogel SN. Inhibition of Lipopolysaccharide-Induced Signal Transduction in Endotoxin-Tolerized Mouse Macrophages: Dysregulation of Cytokine, Chemokine, and Toll-Like Receptor 2 and 4 Gene Expression. *J. Immunol.* 2000;164(11):5564-5574. doi:10.4049/jimmunol.164.11.5564.

261. Schoenemeyer A, Barnes BJ, Mancl ME, et al. The interferon regulatory factor, IRF5, is a central mediator of toll-like receptor 7 signaling. *J. Biol. Chem.* 2005;280(17):17005-17012. doi:10.1074/jbc.M412584200.

262. Zambirinis CP, Miller G. Signaling via MYD88 in the pancreatic tumor microenvironment: A double-edged sword. *Oncoimmunology* 2013;2(1):e22567. doi:10.4161/onci.22567.

263. Chang J, Kunkel SL, Chang C-H. Negative regulation of MyD88-dependent signaling by IL-10 in dendritic cells. *Proc. Natl. Acad. Sci.* 2009;106(43):18327-18332. doi:10.1073/pnas.0905815106.

264. Grivennikov S, Karin E, Terzic J, et al. IL-6 and Stat3 Are Required for Survival of Intestinal Epithelial Cells and Development of Colitis-Associated Cancer. *Cancer Cell* 2009;15(2):103-113. doi:10.1016/j.ccr.2009.01.001.

265. Hodge DR, Hurt EM, Farrar WL. The role of IL-6 and STAT3 in inflammation and cancer. *Eur. J. Cancer* 2005;41(16):2502-2512. doi:10.1016/j.ejca.2005.08.016.

266. Moodley YP, Misso NLA, Scaffidi AK, et al. Inverse effects of interleukin-6 on apoptosis of fibroblasts from pulmonary fibrosis and normal lungs. *Am. J. Respir. Cell Mol. Biol.* 2003;29(4):490-498. doi:10.1165/rcmb.2002-0262OC.

267. Jones SA, Scheller J, Rose-John S. Therapeutic strategies for the clinical blockade of IL-6/gp130 signaling. *J. Clin. Invest.* 2011;121(9):3375-3383. doi:10.1172/JCI57158.

268. Waldner MJ, Foersch S, Neurath MF. Interleukin-6--a key regulator of colorectal cancer development. *Int. J. Biol. Sci.* 2012;8(9):1248-53. doi:10.7150/ijbs.4614.

269. Tsunenari T, Koishihara Y, Nakamura a, et al. New xenograft model of multiple myeloma and efficacy of a humanized antibody against human interleukin-6 receptor. *Blood* 1997;90(6):2437-2444.

270. Cheng Y, Du J, Han J, et al. Polymyxin B Attenuates LPS-Induced Death but Aggravates Radiation-Induced Death via TLR4-Myd88-IL-6 Pathway. *Cellular Physiology and Biochemistry*. 2017:1120-1126.

271. Koch AT, Love-Homan L, Espinosa-Cotton M, Stanam A, Simons AL. MyD88-dependent signaling decreases the antitumor efficacy of epidermal growth factor receptor inhibition in head and neck cancer cells. *Cancer Res.* 2015;75(8):1657-1667. doi:10.1158/0008-5472.CAN-14-2061.

272. New M, Sheikh S, Bekheet M, et al. TLR adaptor protein MYD88 mediates sensitivity to HDAC inhibitors via a cytokine-dependent mechanism. *Cancer Res.* 2016;76(23):6975-6987. doi:10.1158/0008-5472.CAN-16-0504.

273. Richardson PG, Mitsiades CS, Laubach JP, et al. Preclinical data and early clinical experience supporting the use of histone deacetylase inhibitors in multiple myeloma. *Leuk. Res.* 2013;37(7):829-837. doi:10.1016/j.leukres.2013.03.006.

274. Rahbari R, Sheahan T, Modes V, Collier P, Macfarlane C, Badge RM. A novel L1 retrotransposon marker for HeLa cell line identification. *Biotechniques* 2009;46(4):277-284. doi:10.2144/000113089.

275. Scherer WF, Syverton JT. Studies on the propagation in vitro of poliomyelitis viruses. III. The propagation of poliomyelitis viruses in tissue cultures devoid of nerve cells. *J. Exp. Med.* 1952;96:389-400. doi:10.1084/jem.96.4.389.

276. Giard DJ, Aaronson S a, Todaro GJ, et al. In vitro cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. *J. Natl. Cancer Inst.* 1973;51(5):1417-1423. doi:10.1093/jnci/51.5.1417.

277. Boulares a H, Yakovlev AG, Ivanova V, et al. Role of Poly (ADP-ribose) Polymerase (PARP) Cleavage in Apoptosis. *J. Biol. Chem.* 1999;274(33):22932-22940. doi:10.1074/jbc.274.33.22932.

278. Lazebnik YA, Kaufmann SH, Desnoyers S, Poirier GG, Earnshaw WC.

Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature* 1994;371(6495):346-347. doi:10.1038/371346a0.

279. Fernandes-Alnemri T, Litwack G, Alnemri ES. CPP32, a novel human apoptotic protein with homology to Caenorhabditis elegans cell death protein Ced-3 and mammalian interleukin-1??-converting enzyme. *J. Biol. Chem.* 1994;269(49):30761-30764.

280. Franken NAP, Rodermond HM, Stap J, Haveman J, van Bree C. Clonogenic assay of cells in vitro. *Nat. Protoc.* 2006;1(5):2315-2319. doi:10.1038/nprot.2006.339.

281. Dickson MA, Schwartz GK. Development of cell-cycle inhibitors for cancer therapy. *Curr. Oncol.* 2009;16(2):36-43. doi:10.1111/j.1582-4934.2009.00876.x.

282. Shah MA, Schwartz GK. Cell cycle-mediated drug resistance: An emerging concept in cancer therapy. *Clin. Cancer Res.* 2001;7(8):2168-2181. doi:10.1002/1097-0142(197501)35:1<98::aid-cncr2820350115>3.0.co.

283. Bhaskara S, Jacques V, Rusche JR, Olson EN, Cairns BR, Chandrasekharan MB. Histone deacetylases 1 and 2 maintain S-phase chromatin and DNA replication fork progression. *Epigenetics Chromatin* 2013;6(1):27. doi:10.1186/1756-8935-6-27.

284. Into T, Inomata M, Niida S, Murakami Y, Shibata KI. Regulation of MyD88 aggregation and the MyD88-dependent signaling pathway by sequestosome 1 and histone deacetylase 6. *J. Biol. Chem.* 2010;285(46):35759-35769. doi:10.1074/jbc.M110.126904.

285. Liu X, Hunter ZR, Xu L, et al. Targeting Myddosome Assembly in Waldenstrom Macroglobulinaemia. *Br. J. Haematol.* 2017;177(5):808-813. doi:10.1111/bjh.14103.

286. Matos LDLL De, Trufelli DC, Graciela M, et al. Biomarker Insights Immunohistochemistry as an Important Tool in Biomarkers Detection and Clinical Practice. *Biomark. Insights* 2010;5:9-20. doi:10.4137/BMI.S2185.

287. Febbraio MA, Pedersen BK. Contraction-induced myokine production and release: Is skeletal muscle an endocrine organ? *Exerc. Sport Sci. Rev.* 2005;33(3):114-119. doi:10.1097/00003677-200507000-00003.

288. Bode KA, Schroder K, Hume DA, et al. Histone deacetylase inhibitors decrease Toll-like receptor-mediated activation of proinflammatory gene expression by impairing transcription factor recruitment. *Immunology* 2007;122(4):596-606. doi:10.1111/j.1365-2567.2007.02678.x.

289. Foster SL, Hargreaves DC, Medzhitov R. Gene-specific control of inflammation by TLR-induced chromatin modifications. *Nature* 2007;447(7147):972-978. doi:10.1038/nature05836.

290. Chng WJ, Schop RF, Price-Troska T, et al. Gene-expression profiling of

Waldenström macroglobulinemia reveals a phenotype more similar to chronic lymphocytic leukemia than multiple myeloma. *Blood* 2006;108(8):2755-2763. doi:10.1182/blood-2006-02-005488.

291. Roumier C, Decambron A, Renneville A, et al. MYD88 L265P mutation in Waldenstrom macroglobulinemia. 2018;121(22):4504-4512. doi:10.1182/blood-2012-06-436329.There.

292. Cronin JG, Turner ML, Goetze L, Bryant CE, Sheldon IM. Toll-like receptor 4 and MYD88-dependent signaling mechanisms of the innate immune system are essential for the response to lipopolysaccharide by epithelial and stromal cells of the bovine endometrium. *Biol. Reprod.* 2012;86(2):51. doi:10.1095/biolreprod.111.092718.

293. Andreakos E, Sacre SM, Smith C, et al. Distinct pathways of LPS-induced NF-κB activation and cytokine production in human myeloid and nonmyeloid cells defined by selective utilization of MyD88 and Mal/TIRAP. *Blood* 2004;103(6):2229-2237. doi:10.1182/blood-2003-04-1356.

294. Makki MS, Haqqi TM. Histone deacetylase inhibitor vorinostat (SAHA, MK0683) perturb miR-9-MCPIP1 axis to block IL-1β-induced IL-6 expression in human OA chondrocytes. *Connect. Tissue Res.* 2017;58(1):64-75. doi:10.1080/03008207.2016.1211113.

295. Hubackova S, Krejcikova K, Bartek J, Hodny Z. Interleukin 6 signaling regulates promyelocytic leukemia protein gene expression in human normal and cancer cells. *J. Biol. Chem.* 2012;287(32):26702-14. doi:10.1074/jbc.M111.316869.

296. Chang Q, Daly L, Bromberg J. The IL-6 feed-forward loop: A driver of tumorigenesis. *Semin. Immunol.* 2014;26(1):48-53. doi:10.1016/j.smim.2014.01.007.

297. Christiano C, Chini S, Chen J. Human claspin is required for replication checkpoint control. *J. Biol. Chem.* 2003;278(32):30057-30062. doi:10.1074/jbc.M301136200.

298. Alegría-Landa V, Prieto-Torres L, Santonja C, et al. *MYD88* L265P mutation in cutaneous involvement by Waldenström macroglobulinemia. *J. Cutan. Pathol.* 2017;44(7):625-631. doi:10.1111/cup.12944.

299. Mondello P, Brea EJ, Stanchina E De, et al. Panobinostat acts synergistically with ibrutinib in diffuse large B cell lymphoma cells with MyD88 L265 mutations. *JCI Insight* 2017;2(6):1-14. doi:10.1172/jci.insight.90196.

300. Liu Q, Guntuku S, Cui XS, et al. Chk1 is an essential kinase that is regulated by Atr and required for the G2/M DNA damage checkpoint. *Genes Dev.* 2000;14(12):1448-1459. doi:10.1101/gad.14.12.1448.

301. Medema RH, Macůrek L. Checkpoint control and cancer. *Oncogene* 2012;31(21):2601-2613. doi:10.1038/onc.2011.451.

302. Reichert N, Choukrallah MA, Matthias P. Multiple roles of class i HDACs in proliferation, differentiation, and development. *Cell. Mol. Life Sci.* 2012;69(13):2173-2187. doi:10.1007/s00018-012-0921-9.

303. Xu G, Wang J, Wu Z, et al. SAHA regulates histone acetylation, butyrylation, and protein expression in neuroblastoma. *J. Proteome Res.* 2014;13(10):4211-4219. doi:10.1021/pr500497e.

304. Hu Q-P, Mao D-A. Histone deacetylase inhibitor SAHA attenuates post-seizure hippocampal microglia TLR4/MYD88 signaling and inhibits TLR4 gene expression via histone acetylation. *BMC Neurosci.* 2016;17(1):22. doi:10.1186/s12868-016-0264-9.

305. Sheng W, Yan H, Rausa III FM, Costa RH, Liao X. Structure of the hepatocyte nuclear factor 6α and its interaction with DNA. *J. Biol. Chem.* 2004;279(32). doi:10.1074/jbc.M403805200.

306. Zhou P. Determining protein half-lives. *Methods Mol. Biol.* 2004;284:67-77. doi:10.1385/1-59259-816-1:067.

307. Niesen FH, Berglund H, Vedadi M. The use of differential scanning fluorimetry to detect ligand interactions that promote protein stability. *Nat. Protoc.* 2007;2(9):2212-2221. doi:10.1038/nprot.2007.321.

308. Müller P, Kuttenkeuler D, Gesellchen V, Zeidler MP, Boutros M. Identification of JAK/STAT signalling components by genome-wide RNA interference. *Nature* 2005;436(7052):871-875. doi:10.1038/nature03869.

309. Paracha RZ, Ahmad J, Ali A, et al. Formal modelling of toll like receptor 4 and JAK/STAT signalling pathways: Insight into the roles of SOCS-1, interferon-b and proinflammatory cytokines in Sepsis. *PLoS One* 2014;9(9). doi:10.1371/journal.pone.0108466.

310. Andrews FH, Shanle EK, Strahl BD, Kutateladze TG. The essential role of acetyllysine binding by the YEATS domain in transcriptional regulation. *Transcription* 2016;7(1):14-20. doi:10.1080/21541264.2015.1125987.

311. Li Y, Sabari BR, Panchenko T, et al. Molecular Coupling of Histone Crotonylation and Active Transcription by AF9 YEATS Domain. *Mol. Cell* 2016;62(2):181-193. doi:10.1016/j.molcel.2016.03.028.

312. Into T, Inomata M, Niida S, Murakami Y, Shibata K. Regulation of MyD88 aggregation and the MyD88-dependent signaling pathway by sequestosome 1 and histone deacetylase 6. *J. Biol. Chem.* 2010;285(46):35759-69. doi:10.1074/jbc.M110.126904.

313. Edfors F, Danielsson F, Hallström BM, et al. Gene-specific correlation of RNA and protein levels in human cells and tissues. *Mol. Syst. Biol.* 2016;12(10):883. doi:10.15252/msb.20167144.

314. Cooper GM, Hausman RE. *The Cell: A Molecular Approach 2nd Edition*.; 2007.

315. Chen D, Frezza M, Schmitt S, Kanwar J, P Dou Q. Bortezomib as the first proteasome inhibitor anticancer drug: current status and future perspectives. *Curr. Cancer Drug Targets* 2011;11(3):239-253. doi:10.2174/156800911794519752.

316. Alberts A.; Lewis, J.; Raff, M.; Roberts, K.; Walter, P. B. J. Molecular biology of the cell (4th ed.): *Garl. Sci. New York* 2002;4:383. doi:10.1002/bmb.2003.494031049999.

317. Singh BN, Zhang G, Hwa YL, Li J, Dowdy SC, Jiang S-W. Nonhistone protein acetylation as cancer therapy targets. *Expert Rev. Anticancer Ther.* 2010;10(6):935-954. doi:10.1586/era.10.62.

318. Lisa J. Corcoran, Mitchison TJ, Liu Q. A Novel Action of Histone Deacetylase Inhibitors in a Protein Aggresome Disease Model. *Curr. Biol.* 2004;14:488-492. doi:10.1016/j.

319. Domcke S, Sinha R, Levine DA, Sander C, Schultz N. Evaluating cell lines as tumour models by comparison of genomic profiles. *Nat. Commun.* 2013;4. doi:10.1038/ncomms3126.

320. van Staveren WCG, Solis DY, Hebrant A, et al. Human cancer cell lines: Experimental models for cancer cells in situ? For cancer stem cells? *Biochim. Biophys. Acta* 2009;1795(2):92-103. doi:10.1016/j.bbcan.2008.12.004.

321. Crowley LC, Marfell BJ, Scott AP, Waterhouse NJ. Quantitation of apoptosis and necrosis by annexin V binding, propidium iodide uptake, and flow cytometry. *Cold Spring Harb. Protoc.* 2016;2016(11):953-957. doi:10.1101/pdb.prot087288.

322. Riss TL, Moravec RA, Niles AL, et al. Cell Viability Assays. *Assay Guid. Man. [Internet]* 2013;114(8):785-796. doi:10.1016/j.acthis.2012.01.006.

323. Lee J-H, Jeong H, Choi J-W, Oh H, Kim Y-S. Clinicopathologic significance of MYD88 L265P mutation in diffuse large B-cell lymphoma: a meta-analysis. *Sci. Rep.* 2017;7(1):1785. doi:10.1038/s41598-017-01998-5.

324. Civelek M, Lusis AJ, Genetics M, Angeles L. MyD88 and TLR4 Expression in Epithelial Ovarian Cancer. 2014;15(1):34-48. doi:10.1038/nrg3575.Systems.

325. Woods DM, Cheng F, Wang H, Sotomayor EM. Histone Deacetylase 6 (HDAC6) Influences T-Cell Activation and Survival: Implications For Cancer Immunotherapy. Laino AS, ed. *Blood* 2013;122(21):1050 LP-1050. Available at: http://www.bloodjournal.org/content/122/21/1050.abstract.