Investigating the mechanisms by which histone deacetylase inhibitors induce apoptosis in cancer cells

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Submitted in total fulfilment of the requirement of the degree of
Doctor of Philosophy
April 2017

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The University of Melbourne

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I dedicate this thesis to my parents who have supported me through everything.
I hope you all are proud of what I have achieved.

Abstract

Cancer develops as a multi-step process through the accumulation of abnormal genetic alterations in tumour suppressor genes and oncogenes. Superimposed upon these genetic changes are changes in the epigenome which work together to induce the hallmarks of cancer. Histone deacetylase inhibitors (HDACi) are a class of epigenetic therapeutics approved for the treatment of cutaneous T-cell lymphoma (CTCL). These agents induce anti-tumour activity in a variety of ways, including inhibition of cell proliferation and induction of autophagy, differentiation and apoptosis. The mechanism by which HDACi induce apoptosis has been extensively investigated and shown to involve induction of a pro-apoptotic gene signature encompassing the up-regulation of pro-apoptotic genes such as BIM, BAX and BAK and the repression of anti-apoptotic genes such as BCL-2 and BCL-X_L. However, the specific molecular mechanisms by which this pro-apoptotic signature is induced have yet to be clearly identified.

Recent reports have demonstrated that HDACi-induced apoptosis is associated with upregulation of the AP-1 complex genes c-FOS, c-JUN and ATF3. The objective of this thesis was to determine whether induction of c-FOS, c-JUN or ATF3 is directly required for HDACi-induced apoptosis, and elucidate whether these changes in turn drive altered expression of the pro-apoptotic gene signature. The expression of c-FOS, c-JUN and ATF3 expression was found to be robustly and selectively induced upon HDACi treatment in HDACi-sensitive tumour cell lines. These effects transcended tumour type and included melanoma, colorectal, breast, lung, gastric and haematological cell lines.

Through systematic knockdown experiments, induction of ATF3 but not c-FOS or c-JUN was found to be a functional driver of HDACi-induced apoptosis. This was demonstrated in HDACi-sensitive lung, colorectal and gastric cancer cell lines. These results were further confirmed using $ATF3^{-/-}$ MEFs which were significantly less sensitive to HDACi-induced apoptosis compared to wild-type MEFs.

As HDACi treatment alters the expression of pro- and anti-apoptotic genes, we also determined the role of ATF3-induction in mediating these changes. Correlation of

HDACi-induced ATF3 expression with the altered expression of intrinsic apoptotic members across 15 different cancer cell lines, revealed an inverse correlation between the magnitude of ATF3 induction and the repression of expression of the pro-survival gene BCL-X_L. Furthermore, we demonstrated that ATF3 induction is directly required for HDACi-mediated repression of BCL-X_L.

A central role for repression of BCL-X_L in HDACi-induced apoptosis was demonstrated in knockdown studies whereby siRNA-mediated silencing of BCL-X_L was able to re-sensitise refractory cell lines to HDACi-induced apoptosis. Similarly, BH3 mimetics and BCL-X_L-specific inhibitors could also re-sensitise refractory cell lines to HDACi-induced apoptosis both *in vitro* and in xenograft models *in vivo.*

In addition to our finding that ATF3 induction is required for HDACI-induced apoptosis, it has previously been reported that proteasome inhibitor treatment can also induce ATF3 expression. Furthermore, the combination of HDACi and proteasome inhibitors has recently been approved for the treatment of multiple myeloma, although the mechanistic basis for this effect is unclear. We therefore postulated that additive or synergistic induction of ATF3 may underpin this effect. This thesis demonstrates that proteasome inhibitors robustly induce ATF3 in both colorectal cancer and multiple myeloma cell lines, and ATF3 induction is further enhanced by combination treatment with HDACi. We also demonstrate that these agents induce ATF3 through independent mechanisms, and that the combination treatment synergistically enhances apoptosis in these cell lines. Notably, knockdown of ATF3 attenuated the apoptotic response induced by the combination establishing ATF3 as a central component of the apoptotic response.

Collectively, these findings demonstrate that HDACi-induced apoptosis is driven by ATF3 induction and subsequent repression of BCL-X_L. We also demonstrate that combination treatment with a BCL-X_L inhibitor can overcome inherent resistance to HDACi. Additionally, we demonstrate combination treatment with HDACi and proteasome inhibitors synergistically enhances apoptosis through additive induction of ATF3. These studies provide novel insight into the basis for differential response of cell lines to single agent HDACi therapy, and identify avenues for enhancing the activity of HDACi through rationally developed drug combinations.

Declaration

This is to certify that:

(i) the thesis comprises only my original work towards the PhD, except when indicated in the Preface,

(ii) due acknowledgment has been made in the text to all other material used,

(iii) the thesis is fewer than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices.

Signed: _____ 2017

Janson Tse

Preface

In accordance with the regulations of The University of Melbourne, I acknowledge that some of the work presented in this thesis was undertaken collaboratively. Specifically:

(i) In **Chapter 3-Figure 3.9,** [mRNA and apoptotic response of HDACi in ATF3 WT and KO MEFs] was performed in collaboration with Dr. Anderly Chueh.

(ii) In **Chapter 4-Figure 4.1,** the data used in the correlation of HDACi-induced ATF3 expression and Bcl-xL and apoptosis was performed by Dr. Anderly Chueh. Statistical analysis and correlation was later performed by myself and Professor John M. Mariadason.

(iii) In **Chapter 4-Figure 4.5,** the hBCL-X_L cell line was generated by Dr. Erinna Lee.

(iv) In **Chapter 4, Figure 3.2 and 3.8**, the western blots were performed with the assistance of Mr. Paul Iaonnides.

(v) In **Chapter 4, Figure 4.17**, the animal study was assisted by Ms. Laura Jenkins and Mr. Ian Luk.

The remainder of this thesis comprises only my original work.

Publications

The work presented in this thesis has given rise to the following publications:

Anderly C. Chueh, **Janson W.T. Tse**, Lars Tögel, and John M. Mariadason (2015). Mechanisms of Histone Deacetylase Inhibitor-Regulated Gene Expression in Cancer Cells. *Antioxidants & Redox Signalling, 23 (1), 66-84.*

A.C Chueh***, J W.T Tse****, P. Ioannidis, L. Tögel, Bee S. Tan, I. Luk, L. Jenkins, M. Davalos-Salas, R. Nightingale, M R. Thompson, B G. Williams, D. Fairlie, M. Dickinson, A. S. Dhillon and J M. Mariadason (2016). ATF3-mediated repression of Bcl-xL drives HDAC inhibitor-induced apoptosis across tumour types. *Clinical Cancer Research, **DOI:** 10.1158/1078-0432.CCR-17-0466.*

* Denotes co-first authors

Acknowledgements

First and foremost, I would like to express my extensive gratitude towards my supervisor, Professor John M. Mariadason, for his support, patience, guidance and friendship throughout my PhD. I am very thankful for his encouragement and perseverance in passing his knowledge down to me and trying to make me the best research scientist I can be. Words cannot explain how thankful I am to have had you as a mentor over these years, not just in the lab but also in life. I would also like to extend my thanks to my co-supervisor, Dr. Anderly C Chueh, for his mentorship, ongoing support, comments and suggestions. Also to my other co-supervisor Dr. Amardeep S Dhillon, thank you for the intellectual support over the years and your blunt criticism.

I would also like to give thanks to the past Oncogenic Transcription Lab staff members: Sheren Al-Obaidi, Georgia A. Corner and Bee S. Tan. Thank you for all the techniques you have taught me along the way, your technical support and guidance have made me as into the scientist I am today. Special thanks to my fellow OTL crew members: Ian Luk, Laura Jenkins, Dr. Fiona Chionh, Dr. Jennifer Mooi, Dr. David Lau, Camilla Reehorst, Analia Lesmana, Mehmet Okray and Rui Wu, whom I have had the pleasure of working with throughout the years. Thank you for making my PhD a fun and memorable experience, and for helping me when times were tough to get through. I would also like to give thanks to Dr. Jennifer Huynh for all the late nights and for proof reading my thesis in such a timely manner.

I would also like to thank my dearest group of friends that have listened to me complain and whinge when science detests me. Also thanks to my boys from undergraduate school: Jason Nguyen, Nik Patsikatheodorou and Sam Khairallah, who have always given sound advice and solutions. Hope you all can join me soon in finishing you graduate studies!

Finally, and most importantly, I would like to thank my parents for their un-withering support. These 5 years would not have been possible without their love, support and strength. Thank you for always taking care of me when I was too busy to take care of myself.

Table of Contents

List of Figures

Chapter 1

Chapter 3

Chapter 4

Chapter 5

Chapter 6

List of Tables

Chapter 1

Chapter 2

Abbreviations

ACTB	Beta actin
ALL	Acute lymphoblastic leukemia
AP-1	Activating protein 1
APC	Adenomatous polyposis coli
APL	Acute promyelocytic leukemia
ATCC	American Type Culture Collection
ATF3	Activating transcription factor 3
BCL-6	B-cell lymphoma 6
BRF	Bio-Resources Facility
bZIP	Basic region-leucine zipper
CBHA	M-carboxycinnamic acid bis-hydroxamide
CBP	Creb-binding protein
CI	Confidence interval
c-FLIP	Cytoplasmic FLICE-like inhibitory protein
DISC	Death inducing signal complex
DLBCL	Diffuse large B-cell lymphoma
DMEM	Dulbecco’s Modified Eagles Medium
DPBS	Dulbecco's phosphate-buffered saline
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DUBs	De-ubiquitins
EKLF	Erythroid Kruppel-like factor
EMA	European Medicines Agency
EMBA	Diethyl bis (pentamethylene-N,N-dimethylcarboxamide) malonate
ERα	Estrogen receptor-alpha
FACS	Fluorescence activated cell sorting
FCS	Fetal Calf Serum
FDA	Food and Drug Administration
HATs	Histone acetyltransferases
HDAC	Histone deacetylase

HDACi	Histone deacetylase inhibitors
HMTs	Histone methyltransferases
KLF5	Kruppel-like factor 5
KO	Knockout
LB	Lysogeny broth
MAPK	Mitogen activated protein kinase
MEFs	Mouse embryonic fibroblasts
MLH1	MutL Homolog 1
MLH3	MutL Homolog 3
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
MSI	Microsatellite instability
MSS	Microsatellite stable
NaBu	Sodium butyrate
NF-κB	Nuclear Factor κB
PenStrep	Penicillin Streptomycin
PI	Propidium Iodide
PML	Promyelocytic leukemia
PTCL	Peripheral T-cell lymphoma
q-RT-PCR	Quantitative real time polymerase chain reaction
RARα	Retinoic acid receptor-alpha
RAREs	Retinoic acid responsive elements
Rb	Retinoblastoma protein
RNA	Ribonucleic acid
SAHA	Suberoylanilide hydroxamic acid
SB	Sample buffer
Sp1	Specificity protein 1
Sp3	Specificity protein 3
TCF	Ternary complex factors
TP53	Tumour Protein p53
TSA	Trichostatin A
VPA	Valproic acid
WT	Wild type

Chapter 1: Literature Review

1.1 Epigenetics and Cancer

Cancers develop in a step-wise process through the successive accumulation of genetic alterations in tumour suppressor genes and oncogenes, which can alter the control of cell proliferation, death and survival, angiogenesis, invasion and metastasis. In some cancers, this process is accelerated through the acquisition or inheritance of mutations in genes involved in deoxyribonucleic acid (DNA) repair such as MLH1 and MLH3. In parallel, tumour cells typically also undergo a number of epigenetic changes, which can be broadly defined as heritable changes in gene expression that do not alter the DNA sequence [1-3]. The three primary forms of epigenetic modifications associated with cancer development are CpG cytosine-5 methylation, histone modifications, and changes in expression of non-coding RNAs [1, 4, 5] and a key trait of epigenetics is that these modifications can be maintained and passed down through multiple cell divisions, yet can also be adapted to changing environmental cues.

A series of enzymes are involved in epigenetic regulation and can be broadly classified as initiators, readers, writers, erasers and re-modellers. Initiators include transcription factors and long non-coding RNAs which act to target epigenetic writers and erasers to specific regions in the genome. These enzymes create or remove epigenetic marks which include methylation of cytosine residues on DNA and the post-translational modification (typically acetylation or methylation) of lysine residues within DNA-bound histone proteins.

These epigenetic marks are subsequently recognised or “read” by epigenetic reader proteins, which translate the epigenetic marks to either increase or repress gene expression. Finally, chromatin re-modellers act on a broader scale to regulate gene expression by repositioning nucleosomes, while insulators form boundaries between different epigenetic domains [6, 7].

Cancer cells have long been known to undergo extensive epigenetic changes [4, 8]. In addition, in the last decade a number of mutations in epigenetic regulators have been discovered further underscoring the likely importance of alterations to the epigenome in cancer development and progression [9]. Thus, it is now clear that cancer is a disease that develops through both genetic and epigenetic mechanisms.

1.1.1 *Alterations to the epigenome*

In a typical human eukaryotic cell, approximately 1.8 meters of DNA needs to be packed into a nucleus which is only a few micrometres in diameter, thus requiring the DNA to be tightly compacted. The first step in compacting DNA involves the creation of the nucleosome, which consists of a histone octamer comprised of 2 subunits each of Histone H2A, Histone H2B, Histone H3 and Histone H4, around which 146 base-pairs of DNA is tightly wrapped (Figure 1.1) [10-12]. The DNA in between nucleosome units is termed 'linker DNA' and is further compressed and stabilised to form a 30 nM thick solenoid fibre by histone H1. Histone H1 wraps another 20 base pairs around the octamer to further stabilise it, while also functioning to bind linker DNA which allows the connection and subsequent compaction of the connected nucleosomes into the 30 nM thick solenoid fibres. The solenoid fibre is then further coiled into solenoid loops which are approximately 300 nM wide. Compaction of the solenoid loops ultimately forms the chromosomes [13, 14].

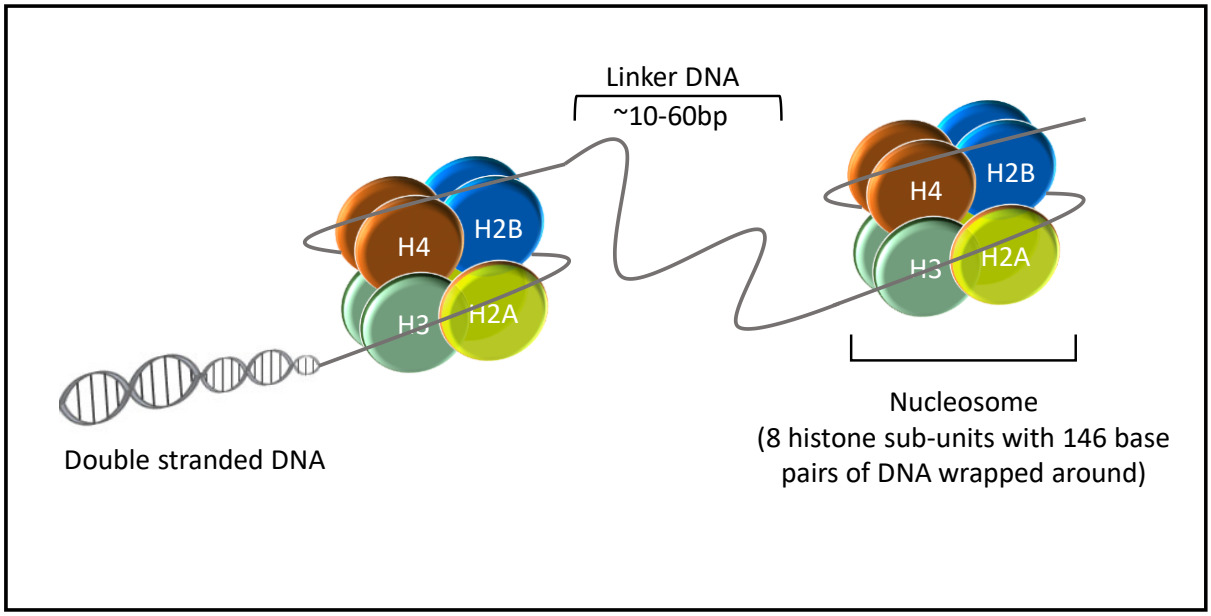

Figure 1.1: The nucleosome comprises of 146bp of DNA coiled around a histone octamer.

Epigenetic modifications regulate gene expression changes without altering the DNA sequence, and as such these modifications allow genetically identical cells to achieve diverse and stable phenotypes. These changes in gene expression are achieved by controlling the transcriptional activity of numerous parts of the genome through either

DNA methylation or chromatin remodelling. The primary mechanisms which induce epigenetic changes are DNA methylation and histone modifications [15, 16].

The initiation and progression of cancer has been established to be controlled by not only genetics, but also epigenetic events. As mentioned before, epigenetic alterations are not permanent and can potentially be reversible, unlike genetic alterations, which are almost impossible reverse. Therefore, the therapeutic targeting of factors which regulate epigenetic alterations emerged as a potential chemotherapy as well as a chemo-preventative measure to cancer. This thesis focuses on the mechanism of action of histone deacetylase inhibitors (HDACi), and as such the literature review will focus primarily on the HDAC class of epigenetic erasers and on histone modifications, specifically histone acetylation and de-acetylation.

1.2 Histone modifications

The amino-terminal tails of histone proteins can undergo a range of post-translational modifications including methylation, acetylation, phosphorylation, ubiquitylation sumoylation and ADP ribosylation [17, 18]. These modifications are regulated by a series of enzymes including histone methyltransferases (HMTs) and demethylases [19], histone acetyltransferases (HATs) and histone deacetylases (HDACs) [20], kinases and phosphatases [21], ubiquitin ligases and de-ubiquitins (DUBs) [22, 23], small ubiquitin-related modifiers (SUMO) [24, 25] and poly-ADP ribose polymerases and ADP-ribosyltransferases [16, 26, 27].

1.2.1 Histone acetylation

Histone acetylation is regulated by the addition and removal of acetyl groups from lysine residues on histone tails, respectively. This is a key post-translational modification which significantly impacts on gene expression by determining whether chromatin exists in an open or closed conformation (Figure 1.2) [28]. In addition, these enzymes can also acetylate and deacetylate a number of non-histone substrates, including the transcription factors tumour protein p53 (TP53), nuclear factor κB (NF-κB), GATA transcription factors, erythroid kruppel-like factor (EKLF), E2F1, Kruppel-like factor 5 (KLF5) and B-cell lymphoma 6 (BCL-6). [29-31].

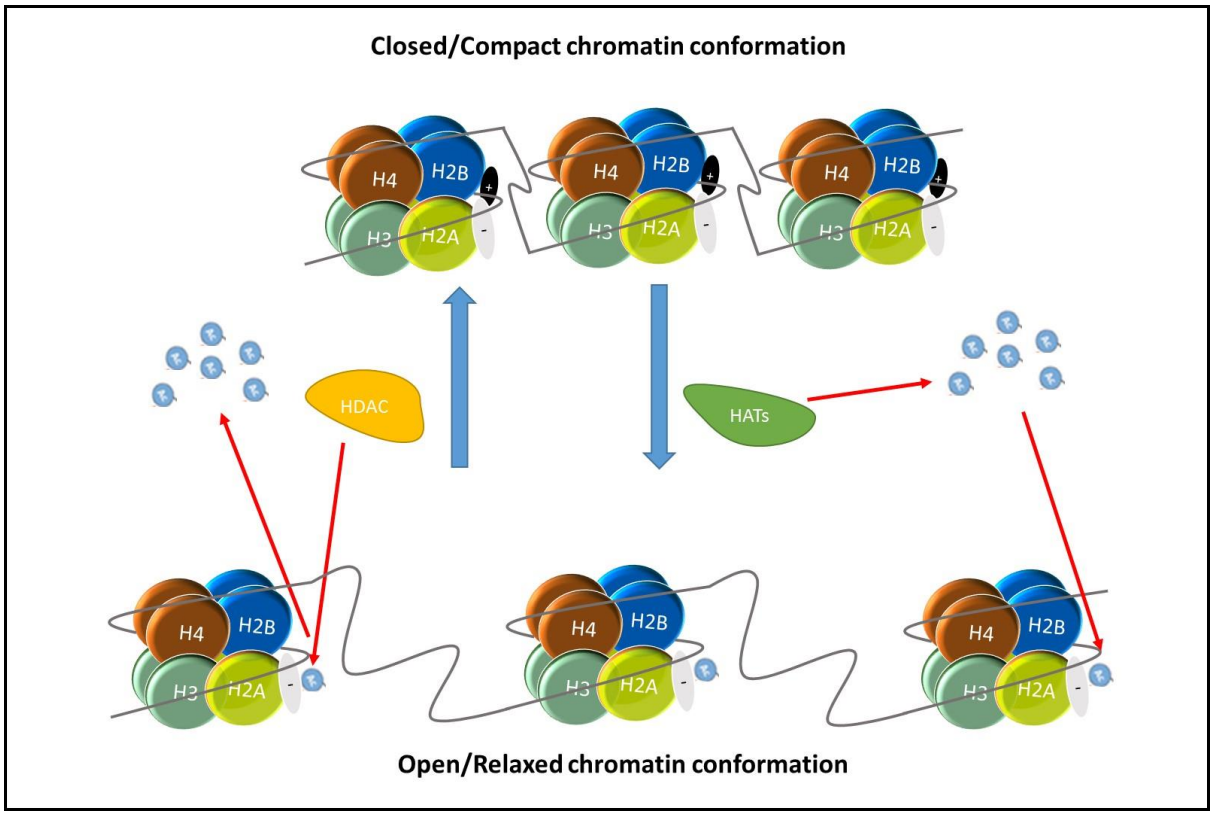

Figure 1.2: HDACs induce heterochromatin state by removing acetyl groups from histone tails, while HATs induce a euchromatin state by hyper-acetylating histones.

1.3 Histone Deacetylases

HDACs are enzymes that catalyse the de-acetylation of lysine residues in DNA bound core histone proteins [32, 33]. HDACs also catalyse the deacetylation of lysine residues in a number of non-histone proteins, including transcription factors and cytoplasmic proteins [34]. There are 18 mammalian HDAC family members, which can be classified into four main groups based on their homology to yeast proteins [35].

Class I HDACs (HDAC1, 2, 3 and 8) are approximately 50 kDa in size and are homologous to the yeast Rpd3, and show predominant nuclear localization. In comparison, Class II HDACs (HDAC4, 5, 7 and 9) have a high degree of homology to the yeast HDA-1, and are approximately 120-150 kDa in size. Class II HDACs localize to both the nucleus and cytoplasm, and their activity is dependent on nucleo-cytoplasmic shuttling. Class II HDACs have a large amino-terminal which upon

phosphorylation enhances their binding to the chaperone protein 14-3-3, which facilitates their shuttling out of the nucleus [36, 37]. The movement of Class II HDACs out of the nucleus tips the balance in favour of HATs which induce the hyperacetylation of histones or specific transcription factors [38-41]. Class II HDACs can be further separated in to 2 sub-groups, Class IIa (HDACs 4, 5, 7 and 9), and Class IIb (HDACs 6 and 10), based on the presence of two catalytic sites in HDAC 6 and 10. Structural studies into Class I and II HDACs have shown that they possess a zinc-containing catalytic binding domain which their activity is dependent on.

The Class III HDACs, which are also known as sirtuins, consist of 7 members (sirt1-7) and are homologous to the yeast Sir2 protein. Sirtuins have distinct sub-cellular localisation profiles, with sirt1, 6 and 7 localised in the nucleus, sirt 2 in the cytoplasm and sirt 3, 4 and 5 localised to the mitochondria. The Class III HDACs differ significantly from the Class I and II HDACs. Sirt1, 2, 3, and 5 have a NAD-dependent deacetylase domain while sirts, 4, 6 and 7, have an NAD^+ -dependent ADP ribosylation domain and deacetylase domain [42].

Finally, the Class IV HDAC, only consists of HDAC11, and is designated a Class on its own as it does not share sufficient homology with the other HDACs. However, it does have conserved residues in its catalytic centre that are shared by both Class I and II HDACs [10, 43-45].

Class I and II HDACs most typically exist within large multi-subunit complexes such as the Sin3, NuRD, CoREST and NCoR/SMRT complexes. The Sin3, NuRD and CoREST complexes contain HDACs 1 and 2 [46-49], while the NCoR/SMRT complex encompasses HDAC3, 4, 5, and 7 [50, 51]. These HDAC-containing complexes, along with HATs, are recruited to gene promoters through interactions with transcription factors and alter gene expression by regulating the acetylation of surrounding histones.

1.3.1 Expression profile and function of Class I and II Histone deacetylases

Class I HDACs are expressed in most tissues while Class II HDACs have more tissue-specific expression patterns [28, 52]. To investigate the biological function of

HDACs and their roles in specific tissue types, knockout mice have been generated for several of the Class I and II HDACs (Table 1). HDAC1-null mice are embryonic lethal dying in utero day 10.5, due to proliferation defects and growth retardation. MEFs isolated from these mice demonstrated an up-regulation of the cyclin-dependent kinase inhibitors p21 and p27 [53-55].

Comparatively, HDAC2-null mice are viable but significantly smaller in size at birth compared to WT mice. HDAC2-null mice develop cardiac defects, specifically displaying a thickened myocardial compact zone. From 2 months of age on-wards, HDAC2-null mice were found to be resistant to cardiac hypertrophy induced by hypertrophic stimuli [56]. Similar to HDAC1-null mice, HDAC3-null mice are also embryonically lethal, dying at embryonic day 9.5 due to defects in gastrulation [57-59]. Global deletion of HDAC8 in mice are viable however, they suffer perinatal lethality due to defects in the skull [60].

HDAC5 and 9 knockout mice, share similar phenotypes in which they are sensitive to cardiac stress signals and develop cardiac hypertrophy at older ages (6+ months). Furthermore, studies looking at the dual knockout of HDAC5 and 9 found that these mice were prone to embryonic lethality and early post-natal death due to an increased amount of cardiac abnormalities including ventricular septal defects and thinning of the myocardium walls [61, 62].

HDAC7 was shown to play a role in maintaining cardiac vascular integrity as HDAC7 knockout-mice were found to be embryonic lethal due to a failure in endothelial cell-cell adhesion, which subsequently results in dilation and rupturing of blood vessels. This phenotype has been shown to be driven by the increased expression of matrix metalloproteinase (MMP) 10, a secreted endoproteinase which is responsible for degrading the extracellular matrix. HDAC7 is required to repress MMP10 during critical stages angiogenesis and vascular re-modelling [63].

All the Class IIa HDACs, except for HDAC4, play a role in cardiac development. In contrast, HDAC4 knockout mice develop defects in bone formation, due to premature ossification of developing bones. HDAC4 has been shown to be crucial in the

regulation of chondrocyte hypertrophy which gives rise to this bone defect phenotype [64].

The Class IIb HDACs, HDAC6 and 10 are mostly cytoplasmic and are the only class of HDACs which display no phenotype when knocked out in mice. The only observation made in HDAC6 knockout mice was an increase in hyperacetylated tubulin in most tissues, but these mice were found to viable and fertile [65]. A HDAC10-null mice model has yet to be reported.

Table 1. Phenotype of histone de-acetylase knockout mice.

Class I HDACs				
	HDAC1	**HDAC2**	**HDAC3**	**HDAC8**
Localisation	Nucleus	Nucleus	Nucleus	Nucleus
Size (amino acids)	483	488	428	377
Knockout phenotype	Embryonically lethal	Cardiac defect	Embryonically lethal	Crano-facial defects
Class IIa HDACs				
	HDAC4	**HDAC5**	**HDAC7**	**HDAC9**
Localisation	Nucleus Cytoplasm	Nucleus Cytoplasm	Nucleus Cytoplasm	Nucleus Cytoplasm
Size (amino acids)	1,084	1,122	855	1,011
Knockout phenotype	Defects in chondrocyte differentiation	Cardiac defect	Maintenance of vascular integrity, increase in MMP10	Cardiac defect
Class IIb HDACs			**Class IV**	
	HDAC6	**HDAC10**	**HDAC11**	
Localisation	Mostly cytoplasm	Mostly cytoplasm	Nucleus Cytoplasm	
Size (amino acids)	1,215	669	347	
Knockout phenotype	None present	N/A	N/A	

1.4 HDACs as therapeutic targets in cancer

A role of HDACs in the development of cancer was initially demonstrated in acute promyelocytic leukemia (APL). A genetic characteristic of APL is the t(15;17) chromosomal translocation, which causes a fusion of the promyelocytic leukemia (PML) and retinoic acid receptor-alpha (RARα) genes. The resulting fusion protein

binds to retinoic acid responsive elements (RAREs) in target genes and recruits HDAC-containing co-repressor complexes with high affinity. This reduces the ability of RARα to bind retinoic acid, which normally serves to activate RARα and promote myeloid differentiation. Binding of the HDAC-corepressor complex therefore results in transcriptional repression of genes that normally regulate the differentiation and proliferation of myeloid cells [28, 66-68]. In addition, the expression of several HDACs have been found to be overexpressed in a range of tumours including Hodgkin's lymphoma, acute lymphoblastic leukemia, prostate, ovarian, bladder and colorectal cancer. Collectively, these findings have led to the investigation of HDACs as potential therapeutic targets in cancer.

1.5 HDAC mutations in cancer

In general, the large cancer sequencing studies have failed to identify mutations in any of the HDACs in high frequency. There has been some exception however, with Ropero *et al* identifying truncating mutations within a mononucleotide repeat region in the HDAC2 gene which resulted in loss of HDAC2 expression and activity in a subset of colorectal cancer cell lines with microsatellite instability. The authors went onto to demonstrate that these mutant cell lines were non-responsive to HDAC inhibitor therapy when compared to wild type cell lines. Inhibition of HDAC activity by Trichostatin A (TSA) treatment of RKO and Co115 cells displayed no decrease in proliferation or G2/M arrest, which were observed HDAC2 wild-type line, HCT116 [69]. However, the functional importance of this HDAC2 truncating mutation has been debated due to contrasting reports on the frequency of these mutations. Specifically, Ree *et al* reported that the RKO cells cultured in their laboratory expressed HDAC2, as did RKO cells obtained from the American Type Culture Collection (ATCC). They went onto demonstrate that regardless of HDAC2 expression, HDAC inhibitor treatment induced histone hyper-acetylation in all the investigated cell lines (RKO-ATCC, RKO-Ropero, Co115 and HCT116) and that radiotoxicity was enhanced by TSA treatment equally in both the RKO-Ropero and RKO-ATCC lines [70]. Hence, the authors concluded that the loss of HDAC2 was not a major determinant of HDAC inhibitor response.

The difference in HDAC2 mutation status between different RKO cell clones is likely due to the microsatellite instability (MSI) status of this cell line. MSI colorectal tumours are driven by mutation or epigenetic inactivation of DNA mismatch repair genes, such as MLH1. This results in a high mutation rate within mono-, di- or trinucleotide repeat elements which are especially prone to replication error. Hence, it has been suggested that the different HDAC2 mutation status of different RKO cell lines may be a consequence of chance propagation of different clones in different laboratories. In support of this theory, Hanigan *et al* also noted that the HCT116 cell line cultured in their laboratory was heterozygous for the HDAC2 mutation, while a newly passaged HCT116 line was wild-type [71]. Collectively, the majority of these studies suggest that the HDAC2 mutation observed by Ropero *et al* is most likely a bystander mutation that occasionally manifests in MSI tumours due to the presence of a microsatellite sequence within the coding sequence of HDAC2.

Sjöblom *et al* also reported a low frequency of HDAC4 mutations in breast cancer specimens in one of the first whole exome sequencing studies undertaken [72]. This finding was also confirmed in the TCGA 2015 data set in which HDAC4 mutations were identified in 13 out of 816 invasive breast carcinoma specimens [73]. However, the functional and clinical relevance of this mutation is yet to be determined.

1.6 HDAC expression in cancers

1.6.1 Role of HDACs in haematological cancers

Altered expression and activity of several HDACs has been reported in a number if haematological cancers. HDAC1, 2 and 3 are overexpressed in Hodgkin's lymphoma, although notably, decreased HDAC1 expression is associated with a worse outcome [74]. In acute lymphoblastic leukemia (ALL), expression levels of HDACs 1, 2 and 8 were each elevated compared to normal cells [75]. Furthermore, the elevated expression of HDAC1 and 2 were significantly correlated with poorer outcome. HDAC1, 2 and 6 are also overexpressed in diffuse large B-cell lymphoma (DLBCL) and peripheral T-cell lymphoma (PTCL), when compared to normal lymphoid tissue [76]. However, the association with outcome differs depending on tumour type.

For example, while both DLBCL and PTCL patients had overexpression of HDAC6, the overexpression was associated with a favourable outcome in DLBCL and poorer outcome in PTCL [76].

Direct evidence for a role for specific HDACs in haematological cancer progression includes the observation that HDAC1 or HDAC2 knockdown during the leukemic phase induces differentiation and apoptosis of APL cells, resulting in in increased survival in mice [77]. Conversely, knockdown of HDACs 1 and 2 in APL haematopoietic progenitors derived from PML-RAR transgenic mice resulted in accelerated leukemia development [66]. These results suggest a 'time' dependent dual role for HDACs 1 and 2 in APL, whereby they act as tumour promoters in pre-leukemic cells and tumour suppressors in established leukemic cells. HDAC3 on the other hand, has been demonstrated to only play an oncogenic role in leukemia, with HDAC3 knockdown inducing differentiation and apoptosis and reducing leukemia development [78].

1.6.2 Role of HDACs in breast cancer

Estrogen is an established mitogenic factor highly implicated in breast cancer progression mediating its effects through binding and activation of the estrogen receptor (ERα). A number of studies have suggested that HDAC mediated chromatin modification plays a critical role in ERα silencing in breast cancer cells [79, 80]. In regard to HDAC expression, a study analysing 162 cases of invasive carcinoma of the breast found that HDAC1 mRNA was expressed at significantly higher levels in tumours from patients over 50 years of age and in those tumours without axillary lymph node involvement that were HER2 negative and interestingly, ER□/PgR positive. Furthermore, patients with high HDAC1 expression levels were further found to have a better prognosis in terms of both disease-free and overall survival [80]. In a more recent study, Muller *et al* examined HDAC1, 2 and 3 expression in 238 primary breast cancer tissue samples found that most of the tumour samples displayed an intermediate or high expression of all 3 HDACs. Furthermore, HDAC2 and 3 expression was found to be significantly higher in less differentiated breast cancers and negatively correlated with hormone receptor status. HDAC2 was also found to be

significantly associated with tumours with HER2 overexpression and nodal metastasis [81]. In a study by Senese *et al* the knockdown of HDAC1 and HDAC2 in breast cancer cells was shown to reduce cell proliferation [82]. Furthermore, the knockout of HDAC2, but not HDAC1 increased sensitivity to tamoxifen and topoisomerase inhibitor-induced apoptosis in breast cancer cells [83, 84].

1.6.3 Role of HDACs in prostate cancer

HDAC1, 2 and 3 expression has been reported to be elevated in prostate tumour tissue compared to normal prostate epithelium and expression was positively associated with an increase in tumour cell proliferation. Further to this, high HDAC2 expression was identified as an independent prognostic marker for prostate cancer [85]. A separate study by Wang *et al* also investigated the expression of HDACs in malignant prostate tissues and various prostate cancer cell lines compared to benign human prostate, and identified increased expression of HDACs 1-5 in the malignant samples and cancer cell lines [86]. Functionally, the overexpression of HDAC1 in prostate cancer cells induced an increase in proliferation and promoted an undifferentiated phenotype [87].

1.6.4 Role of HDACs in ovarian and endometrial cancers

In ovarian cancer, the expression of the Class I HDACs 1, 2 and 3 are all overexpressed and associated with a poor prognosis. Similar findings were also observed in endometrial cancers [88, 89]. Studies into the functional role of Class I HDACs in ovarian cancer cells demonstrated that their knockdown suppressed cell growth, with HDAC3 knockdown inducing the most robust effect [89, 90].

Platinum-based chemotherapy is currently the standard treatment for ovarian cancers, however resistance to these therapeutics commonly occurs. A study by Stronach *et al* found that tumours which had developed resistance to platinum-based therapies, displayed increases in HDAC4 expression compared to the paired pre-treatment biopsy [91]. A direct role for HDAC4 in mediating resistance to platinum-based chemotherapy was subsequently established by siRNA-mediated knockdown of HDAC4, which re-sensitised cells to this treatment. These findings suggest a potential

role for HDAC4 inhibition as a strategy for overcoming resistance to platinum-based chemotherapy in ovarian cancer.

1.6.5 Role of HDACs in bladder cancer

A few studies have investigated the expression levels of HDACs in bladder cancer with a similar trend to that observed in other cancer types emerging whereby HDACs 1, 2 and 3 are frequently overexpressed [92, 93]. In the study by Poyet *et al* expression of HDAC1, 2 and 3 was investigated in 174 urothelial carcinoma patients using a tissue microarray, with HDAC1 found to be overexpressed in 40% of cases, HDAC2 overexpressed in 42% of cases, and HDAC3 overexpressed in 59% of cases. Furthermore, high expression of HDACs 1 and 2 were associated with higher tumour grades. In a separate study, Niegisch *et al* reported increased expression of HDACs 2 and 8 in bladder cancer cell lines, whereas the majority of the Class II HDACS (HDAC4, 5 and 7) were found to be down-regulated. However, in a set of 24 urothelial cancer tissue samples, only the expression of HDAC8 mRNA was found to be significantly increased when compared to benign bladder tissues [92].

1.6.6 Role of HDACs in colorectal cancer

Several studies have demonstrated that various HDACs, particularly the Class I HDACs, are overexpressed in colorectal tumours [94-98]. HDACs 1, 2, 3 and 8 have each been shown to be overexpressed at both the mRNA and protein level in colorectal tumours compared to normal colon tissue [94-99]. Weichert *et al* analysed 140 colorectal carcinomas for their expression of Class I HDACs and reported high expression of HDAC1 in 36.4% of tumours, overexpression of HDAC2 in 57.9% of tumours and overexpression of HDAC3 in 72.9% of tumours analysed. HDAC2 expression was also shown to be an independent prognostic indicator with 5-year survival rates approximately 20% less in patients with high HDAC2 expressing tumours [100]. The overexpression of HDACs 1 & 2 have been linked to the transcriptional activity of Sp1 and Sp3 which orchestrate the expression of these HDACs [101]. HDAC2 overexpression in colorectal tumours has also been demonstrated to be driven by the Wnt-β-catenin-Myc signalling pathway, a critical driver of colon tumourigenesis [97]. Consistent with this, in LacZ-HDAC2 knockout

mice attenuated formation of adenomas was observed when these mice were crossed with Apc^{Min} mice [102]. Numerous studies looking into the functional role of HDACs in colorectal cancer have found that the knockdown of HDACs 1, 2 and 3, reduces growth of numerous colorectal cancer cell lines [94, 103, 104].

1.6.7 Mechanisms by which HDACs regulate gene expression in cancers

Collectively, these studies demonstrate that HDACs are overexpressed in several tumour types. Their overexpression may contribute to tumour progression in several ways. First, HDACs catalyse the de-acetylation of specific lysine residues in DNA-bound core histone proteins. This confers a positive charge on the histone proteins, which enhances the histone protein's affinity for negatively charged DNA. This produces a more compact chromatin conformation, which in turn leads to reduced accessibility of the RNA polymerase to the core DNA, resulting in transcriptional repression (Figure 1.2) [10, 11, 44, 105, 106]. Consequently, HDAC overexpression could contribute to tumour progression through the transcriptional repression of tumour suppressor genes. Second, HDACs regulate gene expression is through the deacetylation of transcription factors themselves. These include p53 [29], E2F [107], specificity protein 1 (Sp1) and specificity protein 3 (Sp3) [106, 108], TCF [109], HMG-1[110], and c-Myb [111], many of which play important roles in tumorigenesis. Depending on the transcription factor, acetylation can either increase or decrease their DNA binding activity, and thus modulate their ability to regulate gene expression.

Finally, HDAC overexpression can contribute to tumourigenesis by inducing the hypo-acetylation of cytoplasmic proteins such as the Hsp90 protein [30, 112]. Hsp90 protein is a chaperone protein required for the proper folding and maintenance of several pro-growth and pro-survival signalling proteins, including Bcr-Abl, mutant FLT-3, c-Raf, and AKT in human leukemia cells [113]. The binding of Hsp90 to Bcr-Abl is dependent on ATP, which prevents the poly-ubiquitination and degradation of Bcr-Abl [113, 114]. The chaperone activity of Hsp90 is regulated by its acetylation status, which is in turn controlled by HDAC6. HDAC6 knockout in cells results in Hsp90 hyperacetylation and inhibits ATP binding. Therefore, HDAC6 is crucial for the binding of ATP to Hsp90 hence overexpression of HDAC6 could in turn contribute to tumourigenesis through

facilitating the HSP90-dependent stabilization of pro-survival factors such as Bcr-Abl [115].

1.7 Histone Deacetylase inhibitors

Given that HDACs are frequently overexpressed in a range of cancers and often associated with poorer outcome, and the evidence implicating a direct role for these enzymes in tumour cell proliferation and survival, HDACs have emerged as attractive targets for cancer therapy. Consequently, a number of HDAC inhibitors have been developed and undergone extensive pre-clinical and clinical evaluation. Several of these compounds have demonstrated robust anti-tumour effects in multiple cancer cell lines including induction of growth arrest, differentiation and apoptosis [10, 44, 116], and a number of HDACi are now approved for the treatment of CTCL and multiple myeloma [117-121]. More recently, there have also been reports of HDACi having anti-tumour activity in acute myelogenous leukemia, and myelodysplastic syndrome [122].

1.7.1 Development of HDACi

HDACi were initially developed based on the observation that the compound dimethyl sulfoxide (DMSO) could induce differentiation of murine virus induced erythroleukemia cells. Friend *et al,* discovered that continuous incubation of leukemia cells in media containing 2% DMSO for 5 days resulted in their differentiation into more normal-like cells [123]. However, DMSO itself is not a clinically viable compound due to the high concentration required (280 nM) to treat cancers *in vivo*. Hence, a number of chemical modifications were made to DMSO in order to develop more potent compounds. Using this approach, three new compounds were developed which were able to induce differentiation and apoptosis of cancer cells at significantly lower concentrations: diethyl bis (pentamethylene-N,N-dimethylcarboxamide) malonate (EMBA), m-carboxycinnamic acid bis-hydroxamide (CBHA) and suberoylanilide hydroxamic acid (SAHA). Of these three compounds, SAHA demonstrated the most potent activity with an IC^{50} of 2 µM [124]. A number of other studies also demonstrated that treatment of cancer cells with these DMSO-based compounds induced an accumulation of acetylated histones, which was then later demonstrated to be a consequence of HDAC inhibition [125].

1.7.2 Classes of HDACi

Modern HDACi can be classified into four main groups based on their chemical structure; hydroxamates, short-chain fatty acids, benzamides and cyclic peptides (Figure 1.3) [12, 43, 126]. These compounds selectively inhibit Class I, II and IV HDACs, with no effect on the structurally distinct Class III HDACs (sirtuins). Biochemical studies have shown that different HDACi have stronger affinities for some HDACs over others, and can be further categorised as either pan or class specific HDACi [127, 128]. For example, the hydroxamates SAHA and Trichostatin A (TSA) are pan-HDACi which inhibit Class I, II and IV HDACs. Depsipeptide more potently inhibits HDACs 1 and 2 compared to the Class II HDACs, HDAC4 and 6 [129]. Short-chain-fatty acid based HDACi such as valproic acid (VPA) and sodium butyrate (NaBu) are Class I and IIa specific HDACi, but are rarely used in cancer therapy due to the active metabolism via β-oxidation. The benzamide MS-275 (Entinostat) preferentially inhibits HDAC1, and is less efficient in inhibiting HDACs 3 and 8 [130]. A summary of HDAC inhibitor compounds is listed in Table 2.

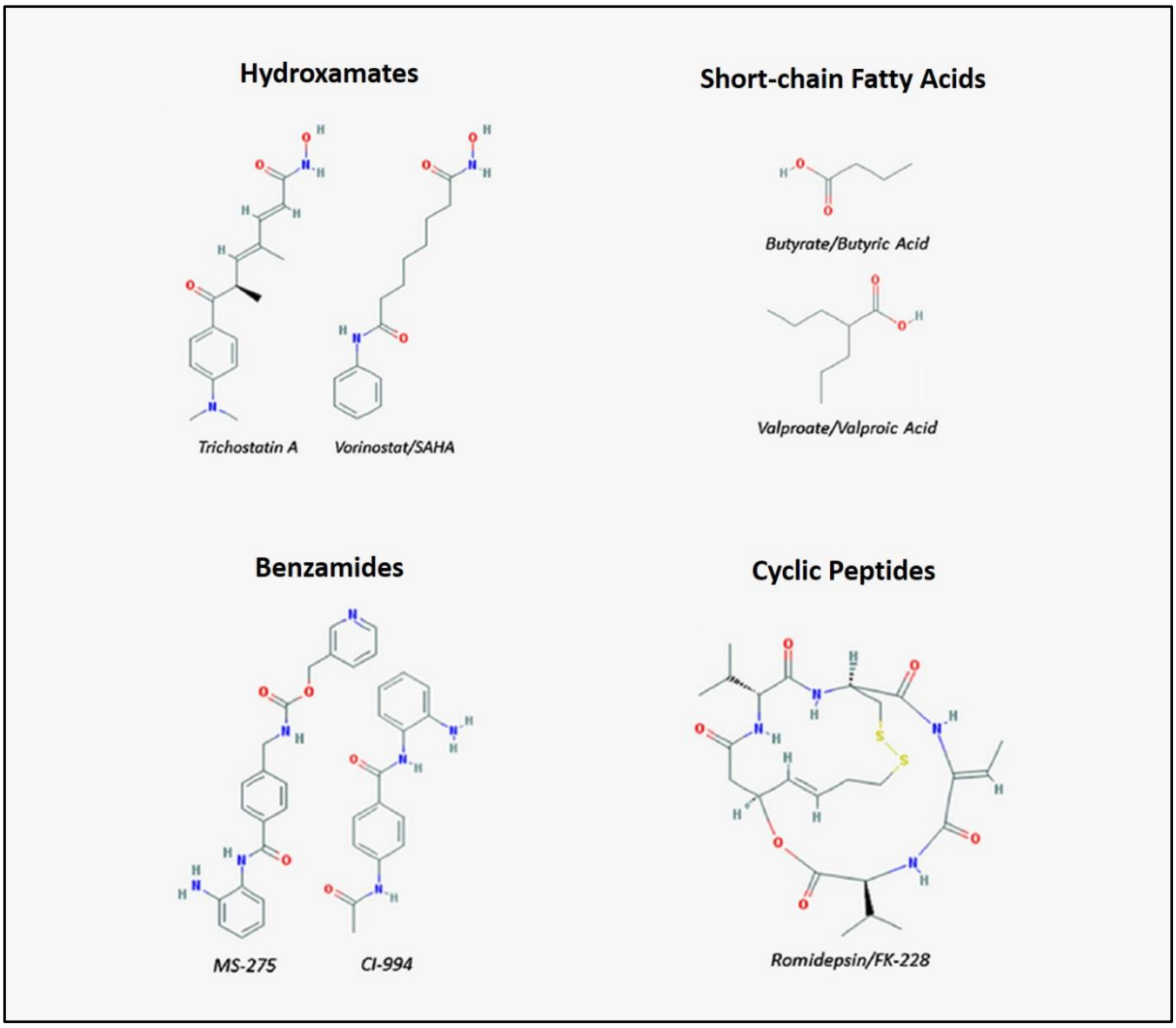

Figure 1.3: The four major chemical classes of HDAC inhibitors; hydroxamates, cyclic peptides, benzamides and short chain fatty acids.

Table 2. HDACi trialled as potential anti-cancer therapeutics and their specificity for different HDACs.

Class	Compound	HDAC specificity	Clinical trials
Short-chain Fatty Acids	Butyrate	Class I, IIa	Phase I, II
	Valproic acid (VPA)	Class I, IIa	Phase I, II
Hydroxamates	Trichostatin A (TSA)	Class I, II, IV	N/A
	Suberoylanilide hydroxamic acid (SAHA, Vorinostat)	Class I, II, IV	Phase I, II, III, Approved
	Belinostat (PXD101)	Class I, II	Phase I, II, Approved
	Panobinostat (LBH589)	Class I, II	Phase I, II, III, Approved
	Oxamflatin	N/A	N/A
	Scriptaid	N/A	N/A
	Tubacin	Class IIb	N/A
Benzamides	MS-275	HDACs 1, 2,3, 8	Phase I, II
	CI-994 (Tacedinaline)	N/A	N/A
Cyclic Peptides	Romidepsin (Istodax)/Depsipeptide	Class I, II	Phase I, II, Orphan approval
	Apicidin	HDACs 1 and 3	N/A

1.7.3 Clinical Activity of HDACi

HDACi are approved as single agents for the treatment of CTCL and peripheral T-cell lymphoma and in combination with the proteasome inhibitor Bortezomib for the treatment of multiple myeloma [117-121]. The efficacy of these drugs in solid tumours is more limited, although recent studies have demonstrated clinical activity in non-small cell lung cancer when used in combination with Azacitidine [131], and in ER-positive advanced breast cancer when used in combination with the aromatase inhibitor, Exemestane [132]. Furthermore, HDAC inhibitors have been shown to potently induce growth arrest and apoptosis in multiple solid tumour cell line models both *in vitro* and *in vivo* [43, 133-135], suggesting these agents may have activity in subsets of solid tumours.

Currently, there are four HDACi which are FDA approved for the treatment of various cancers: Vorinostat [117], Romidepsin [136], Belinostat [137] and Panobinostat [138]. The key trials which led to the approval of these drugs are described below.

- Suber-anilo-hydroxamic acid (SAHA/Vorinostat/Zolinza) is a pan-HDACi approved for the treatment of CTCL patients with progressive, persistent, and recurrent disease who have failed systemic therapy. Vorinostat inhibits the enzymatic activity of the Class I HDACs (HDAC1, 2, and 3), as well as the Class II HDAC, HDAC6. The key trial which led to the registration of this drug was a single-arm open-label phase II trial which enrolled 74 patients with stage IB or higher CTCL who had failed two prior systemic therapies. The primary efficacy endpoint was objective response assessed by the Severity-Weighted Assessment Tool. The trial demonstrated an objective response rate of 30% (95% confidence interval [CI], 19.7%-41.5%), with a median response duration of 168 days and the median time to tumour progression of 202 days. Another single centre study with 33 patients demonstrated similar results [117].

- Romidepsin (Istodax) is a cyclic peptide, Class I and II-specific HDACi for which it's activity has been linked to one of the sulfhydryl groups of the reduced form of the compound interacting with the zinc ions in the active site of the Class I and II HDAC enzymes, leading to potent inhibition of enzymatic activity [139]. The drug was initially fast tracked and granted orphan drug status by the FDA and European Medicines Agency (EMA) for monotherapy treatment of CTCL patients who had become refractory to systemic therapies in 2009 [117, 140, 141] and in the following years was further approved for the treatment of peripheral T-cell lymphomas [142]. The approval for CTCL was based on 2 multi-centre (US, Europe and Australia), single-arm trials in CTCL patients who had failed on at least one form of systemic therapy [141, 143]. The first study consisted of 96 patients who had received at least one prior systemic therapy, while the second consisted of 71 patients who had received a median of two prior systemic therapies. In both studies, patients could be treated until the end of the trial. The overall response rates obtained for both studies were very similar, 34% in study 1 and 35% in study 2. This was similarly observed for complete response rates where both trials achieved a complete response rate of 6%. Median response time was 15 months in study 1 and 11 months in study 2 [141, 144]. The approval for PTCL was approved based on a phase II study

for patients with relapsed or refractory disease, consisting of 131 patients. The study demonstrated an objective response rate of 25% including 15% with complete response, with median duration of response of 17 months. At median follow up time of 13.4 months, 89% of the patients had yet to experience disease progression. Long-term responses to Romidepsin were achieved in patients regardless of baseline characteristics, including subtype, heavy pre-treatment, response to prior therapy, or advanced disease [145].

- Belinostat (PXD101/Beleodaq) is a pan-HDACi FDA approved for the treatment of relapsed or refractory PTCL. Belinostat was granted approval based on the results of the phase II BELIEF (CLN-19) trial, which was a multi-centre, open label, non-randomised, single-arm study consisting of 129 PTCL patients who had progressed after receiving more than one systemic therapy. The overall response rate in the 120 evaluable patients was 25.8% (31 of 120), including 13 complete (10.8%) and 18 partial responses (15%). Median progression-free and overall survival were 1.6 and 7.9 months, respectively [146].

- Panobinostat (LBH-589/FARYDAK) is a pan-HDACi FDA and EMA approved for the treatment of multiple myeloma, when used in combination with Bortezomib and dexamethasone [121, 147] . Approval is only for multiple myeloma patients who have received at least two prior regimens, including Bortezomib and an immunomodulatory agent. Approval was initially based on the PANORAMA I trial, which was a randomised, multi-centre, phase III trial of Panobinostat (20mg orally) or placebo, combined with Bortezomib (1.3mg/m^2, IV) and Dexamethasone (20mg orally), in 193 patients. Patients enrolled in this trial were refractory multiple myeloma patients who had previously received at least two prior regimens, including Bortezomib and an immunomodulatory agent. The study demonstrated that the group who received Panobinostat plus Bortezomib gained significantly greater benefit than the control arm. Median PFS was 10.6 months in the Panobinostat-containing arm (Panobinostat + Bortezomib + Dexamethasone) versus 5.8 months in the control arm (placebo + Bortezomib + Dexamethasone) [HR 0.52 (95% CI: 0.36, 0.76)]. Overall response rates were 58.5% (95% CI:47.9, 68.6) in the Panobinostat arm and 41.4% (95% CI:31.6, 51.8) in the control arm [147]. The benefit of adding

Panobinostat to Bortezomib and Dexamethasone were further validated in the PANORAMA II trial which was a single-arm, open-label, multi-centre, phase 2 study of Panobinostat in combination with Bortezomib and Dexamethasone in 55 adult patients with Bortezomib refractory multiple myeloma. The overall response rate was 34.5% (1 near-complete response and 18 partial responses). A clinical benefit rate of 52.7% was achieved, and median exposure and progression-free survival were 4.6 months and 5.4 months, respectively. In patients who achieved a response, median time to response was 1.4 months, and median duration of response was 6.0 months [121].

1.8 Mechanism by which HDACi induce anti-tumour activity

1.8.1 Cell-cycle arrest

A key cellular effect induced by most HDACi is the induction of cell cycle arrest. This occurs primarily at the G1/S checkpoint, although induction of arrest at the G2/M phase also occurs when HDACi are used at higher concentrations [148-151]. Several mechanisms by which HDACi induce cell cycle arrest have been described. First, HDACi induce expression of CDKN1A/p21WAF1/CIP1, a negative regulator of G1/S transition. p21 induction by HDACi occurs in a p53-independent manner and inhibits CDK2 activity, which is required for the phosphorylation of the retinoblastoma protein. This results in reduced transcriptional activation of the E2F transcription factor which regulates the expression of several genes required for DNA replication, thus inducing a G0/G1 arrest [152-154]. Furthermore, HDACi treatment up-regulates several other CDK inhibitors such as $p15^{INK4B}$, $p19^{INK4D}$, and $p57^{KIP2}$ which inhibit cells from entering S phase [155].

Second, HDACi treatment directly mediates the repression of cell cycle progression genes including cyclin D1, cyclin E1,CDK2 and CDK4 [156-158], which are required for cell cycle progression. Specifically, the cyclin D1-CDK4/CDK6 complex is required for progression through the G1 checkpoint by inducing phosphorylation of the retinoblastoma protein and release of the E2F transcription factor. Similarly, cyclin E, which is synthesized during mid G1-phase and is jointly activated by E2F and Myc,

results in activation of CDK2 kinase which is required for G1/S transition [159]. Hence, the downregulation of these cyclins and CDK's have been postulated to contribute to HDACi-induced cell cycle arrest. Finally, HDACi can directly repress the transcription of CTP synthase and thymidylate synthase, which are required for DNA synthesis. Collective down-regulation of these key cell cycle regulators inhibits S-phase progression, and contributes to HDACi-induced G1/S arrest [160].

HDACi can also induce cell cycle arrest at the G2/M phase, which is independent of DNA damage [156, 161-163]. HDACi mediated G2/M arrest has been suggested to be mediated by the downregulation of cyclin A and cyclin B, which in turn prevent the formation of the cyclin A/CDK2 and cyclin B/CDK1 G2/M regulatory complexes [43, 156, 164-167]. In regards to cyclin A, it has been shown that HDACi treatment induces hyper-acetylation of cyclin A, causing it to be ubiquitinated and subsequently degraded [168, 169]. In comparison, the expression of cyclin B1 is regulated by the GADD45 family of cell cycle regulatory genes. HDACi induce expression of GADD45 which promotes G2/M arrest through inhibition of cyclin B1. HDAC-induction of GADD45 also causes the down-regulation of Aurora A and PLK1, additional factors involved in the transition of G2 to M phase, contributing to the induction of G2/M arrest [170-172].

1.8.2 Senescence

HDACi treatment of leukemia, colorectal and breast cancer cell lines has also been demonstrated to induce a senescence cell like morphology and increase expression of the senescence associated markers β-galactosidase (SAb-Gal), p21CIP1/WAF1 (Cdkn1n/p21) and p16 [173, 174]. Recent studies have found that polycomb repressor group proteins such Bmi1 [174-176] and EZH2 [177] are key mediators of HDACi-induced senescence. Specifically, BMI1 is a known repressor of the cell cycle inhibitors and senescence markers p16 and p19 [175]. In breast, tongue and leukemia cell lines, HDACi induced a strong repression of BMI1, which subsequently leads to the increased expression of p16 and p19, inducing senescence [176, 178, 179]. p16 (cyclin-dependent kinase inhibitor 2A) and p19 (cyclin-dependent kinase inhibitor 2D), are tumour suppressor proteins that have important roles in cell cycle regulation by prohibiting the progression of cells from the G1 phase to S phase. These two cell cycle inhibitors bind to CDK4 and CDK6, which inhibits the formation

of the cyclin D-CDK4/6 complex, and in turn inhibits CDK4/6-mediated phosphorylation of the retinoblastoma protein (Rb). This causes Rb to be inactivated and facilitates progression through the cell cycle progression [180-183]. HDACi also decrease expression of EZH2, a histone lysine methyltransferase, which catalyses the tri-methylation of H3K27, a repressive chromatin mark [184-186]. The depletion of EZH2 by HDACi in melanoma cells inhibits proliferation and induces a senescent phenotype. Studies looking into the depletion of EZH2 have demonstrated that p21/CDKN1A and p16 are induced upon knockdown of EZH2 in melanoma cells, which has been shown to be a crucial driver of the senescence phenotype observed [187-189].

1.8.3 Apoptosis

One of the consistent anti-tumour effects induced by HDACi is the induction of apoptosis. This effect has been demonstrated in multiple models including multiple cell lines derived from both haematological and solid tumours [10, 133, 153, 190, 191]. The efficacy of HDACi as a tumour-selective apoptotic drug has been demonstrated in several pre-clinical studies, where HDACi induce potent anti-tumour activity while causing minimal toxicities to the host [192-197]. However, the underlying mechanism as to why tumour cells are more sensitive to HDACi remains unclear. Bolden *et al* explored this by looking at the gene expression profiles induced by Vorinostat in matched human normal and transformed cells where transformation was induced through the sequential introduction of genes encoding the SV40 large T and small t antigens, hTERT and H-RAS^{G12V}. Gene-ontology analysis of the matched samples identified a tumour-cell-selective pro-apoptotic gene-expression signature that included altered expression of several members of the BCL2 family, such as BCL-X_L which was preferentially inhibited in transformed cells [198]. While numerous studies have demonstrated that HDACi-induced apoptosis typically occurs via activation of both the intrinsic and extrinsic apoptotic pathways, majority of evidence implicates activation of the intrinsic pathway as the primary mechanism.

1.8.3.1 Intrinsic (Mitochondrial) pathway

Numerous studies in various tumour types have demonstrated induction of apoptosis through activation of the intrinsic or mitochondrial apoptotic pathway in response to HDACi treatment [199-202]. Activation of this pathway is initiated predominantly at a transcriptional level, where several anti-apoptotic members of the BCL-2 family are repressed and conversely, a number of pro-apoptotic members are up-regulated following HDACi treatment [43, 163, 198, 203].

Anti-apoptotic members of the BCL-2 family including BCL-2, BCL-X_L and MCL-1, which function to protect mitochondrial integrity [204, 205], are repressed by HDACi treatment in multiple tumour types [198, 206-211]. BCL-X_L and MCL-1 preferentially bind to the BH3-only proteins BIM, PUMA, and NOXA but can also bind to BAK and BAX [204, 212, 213]. HDACi are thought to facilitate the initiation of apoptosis via the displacement model, whereby the repression of BCL-X_L and MCL-1 displaces their binding to BAX and BAK, subsequently allowing BAX and BAK to create pores in the mitochondrial membrane and initiate the release of cytochrome *c* [214-216]. The released cytochrome c binds to APAF-1 and initiates formation of the apoptosome. This activates the caspase cascade, starting with the activation by cleavage of the initiator caspase, caspase 9 [217-219]. The activation of caspase 9 in turn cleaves and activates the executioner caspases, 3 and 7, which subsequently drives apoptosis [218, 220-222]. The repression of pro-survival BCL-2 family members has been directly demonstrated to be important in HDACi-induced apoptosis in several ways. First, the overexpression of BCL-X_L, BCL-2 or MCL-1 attenuates HDACi-induced apoptosis in lymphomas, multiple myeloma, mesothelioma and head and neck squamous cell carcinoma [198, 210, 211, 223-230]. Furthermore, in an *in vivo* model of Eμ-myc-driven lymphoma the overexpression of BCL-2 or BCL-X_L protected lymphoma cells from HDACi-induced apoptosis [195, 231]

Conversely, HDACi treatment also induces the expression of several pro-apoptotic members of the BCL-2 family, which can be divided in 2 groups: multi-domain proteins which alter membrane permeability (BAX and BAK), and BH3-only proteins (BAD, BID, BIM, BMF, PUMA, and NOXA) which act as sensors of cellular stress leading to alterations in mitochondrial membrane remodelling and permeability [232-236]. First,

HDACi induce expression of BAX and BAK, which promote apoptosis via the cytochrome *c* apoptotic cascade [214-216]. Several studies have demonstrated the importance of Bax for the induction of apoptosis in numerous tumour cell types including prostate, glioma, breast, head and neck squamous cell carcinoma, and pancreatic cancer. Collectively, these studies have shown that overexpression of BAX can greatly enhance the apoptotic response of tumour cells to chemotherapeutic agents, or spontaneously induce apoptosis [237-241]. The transcriptional induction of BID, BIM, BAD, BMF, PUMA, and NOXA, by HDACi has been reported by several studies [43, 229, 231, 242, 243]. BID and BIM are also known as 'activator' BH3-only proteins and can directly interact with BAX and/or BAK to induce conformational changes that enhance the assembly of the BAX/BAK multimeric pores in the mitochondrial membrane [244, 245]. BAD, BMF, PUMA, and NOXA are considered as 'sensitiser' BH3-only proteins and facilitate the induction of apoptosis by binding to anti-apoptotic members of the BCL-2 family and displacing their binding to BAX and/or BAK [246-249]. The importance of BAX and BAK induction in HDACi-induced apoptosis has been demonstrated by the knockdown of BAX or BAK, in glioblastoma and pancreatic cells respectively, attenuating HDACi-induced apoptosis [250, 251]. This was similarly observed with the 'sensitiser' BH3-only pro-apoptotic proteins, where knockdown or overexpression of these members attenuated or increased HDACi-induced apoptosis, respectively. For example, knockdown of BIM in leukemia and myeloma cells significantly attenuated HDACi-induced apoptosis [252]. Similarly, the knockdown of NOXA in leukemia cells also attenuated HDACi-induced apoptosis [200].

1.8.3.2 Extrinsic (Death-receptors) pathway

While the majority of studies published to date implicate activation of the intrinsic apoptotic pathway in HDACi-induced apoptosis, studies in several tumour types including CTCL, multiple myeloma, peripheral T-cell lymphoma, non-small cell lung carcinoma, renal, pancreatic, breast and colorectal, have demonstrated activation of the extrinsic apoptotic pathway through up-regulation of TNF super family members (DR4, DR5, and Fas) and their related ligands (TRAIL, TRAIL-receptors and FasL) [253-258]. These proteins initiate the extrinsic apoptotic pathway by recruiting the

adaptor proteins, FADD and TRADD, as well as pro-caspases -8 and -10 [193, 259-262]. Specifically, in peripheral T-cell lymphomas and prostate cancer cells, HDACi-induced up-regulation of the TRAIL receptor, TRAIL-R2 and reduced the levels in cytoplasmic FLICE-like inhibitory protein (c-FLIP). This enabled the formation of the death inducing signal complex (DISC) and subsequent activation of caspase-8 leading to apoptosis [255, 263, 264]. Inhibition of HDACi-mediated up-regulation of these death receptors using siRNAs or their blockade using DR5-Fc and Fas-Fc chimeras, leads to the attenuation of HDACi-induced apoptosis demonstrating the importance of this pathway in HDACi-induced apoptosis [192, 193, 265]. Notably, the induction of these receptors and ligands has been shown to be tumour cell specific [192, 193, 266]. For example, Insinga *et al* demonstrated selective induction of TRAIL, DR5, FasL and Fas by HDACi in leukemic cells, but not in normal hematopoietic progenitor cells [192]. However, the functional importance of activating the extrinsic pathway in HDACi-induced apoptosis is cell type dependent. For example, a study in acute myeloid leukemia cells demonstrated that knockdown of TRAIL attenuated HDACi-induced apoptosis [192], whereas TRAIL inhibition in the Eμ-myc lymphoma model had no effects on HDACi-induced apoptosis [195].

1.8.4 Differentiation

Sodium-butyrate, a first generation HDACi, was initially explored as a potential anti-cancer therapeutic due its ability to induce differentiation in erythroleukemia cells [267]. The ability of a number of HDACi to induce differentiation has subsequently been demonstrated in several other haematological cancer cell lines as well as in solid tumour cell lines, including colorectal, gastric and breast cancer cells [267-275]. Specifically, VPA effectively drives differentiation of PML/RAR fusion harbouring AML cells *in vitro* [276]. AML sub-class 3, better known as acute promyelocytic leukemia (APL), is a haematological cancer characterised by a chromosomal translocation t(15;17), that causes a fusion of the PML and RAR genes. The PML-RARα fusion protein binds to RAREs, and recruits HDAC-corepressor complexes with high affinity. This effectively competes with the ability of retinoic acid to bind to the fusion protein, resulting in the repression of target genes involved in myeloid differentiation. HDACi treatment is able to reverse this process and enables re-expression of these genes, promoting myeloid differentiation [28, 66-68]. Notably though, differentiation therapy

with ATRA, which restores the binding of retinoic acid, has made APL an essentially curable disease [277, 278]. However, having demonstrated that HDACi also induce differentiation of APL, this provided a strong molecular rationale for the combination of HDACi and ATRA [279]. The addition of HDACi can also re-sensitise ATRA-resistant APL cell lines to ATRA [280], and pilot studies in a small number of patients have shown that combining the HDACi VPA with ATRA has shown promising clinical activity [281-283], with one study demonstrating mono-myelocytic differentiation in patients with stable disease [282].

HDACi also induce differentiation in NUT midline carcinoma (NMC) cells. NMC is driven by an oncogenic fusion (BRD4-NUT) between BRD4 and NUT genes which prevents squamous cell differentiation [284]. The mechanism by which this occurs is postulated to be through the BRD4-NUT fusion protein sequestering HAT activity and diminishing H3K18, H4, and H4K8 acetylation, histone marks associated with active gene expression, all of which can be reversed by HDACi treatment [285]. HDACi treatment restores normal levels of histone hyperacetylation and allows the resumption of squamous differentiation programs [284, 285]. Finally, HDACi treatment has also been demonstrated to induce differentiation of colorectal cancer cells assessed by the formation of dome like structures, which is a characteristic of increased water absorption [286] and improved tight junction function [287]. In addition to inducing these morphological changes, HDACi treatment also induces expression of a number of differentiation markers including intestinal alkaline phosphatase [269, 288, 289], E-cadherin [290-292], villin [10, 293] and gelsolin [294-296].

1.9 Mechanisms by which HDACi regulate gene expression

HDACs regulate gene expression by deacetylating DNA-bound histone proteins as well as transcription factors (Figure 1.4). Conversely, by inhibiting the activity of HDACs in the cell, HDAC-inhibitors alter the balance between the activity of HATs and HDACs in favour of HATs which can alter transcription in several ways [10, 11]. First, altering the balance in favour of HATs leads to histone hyper-acetylation and a more open chromatin conformation (Figure 1.5). This facilitates transcription by increasing the accessibility of the core RNA polymerase complex to the DNA template,

resulting in transcriptional activation [11, 198]. Altering the balance in favour of HATs can also induce hyper-acetylation of transcription factors. This can modulate their DNA binding capacity [29], their recognition by BRD-containing epigenetic readers [297], their ability to homo [298] and hetero-dimerise [299], and their subcellular localisation [300]. Finally, there are also studies which demonstrate that acetylation of a specific lysine residue influences its ability to undergo other post-transcriptional modifications. For example, Vervoots *et al* demonstrated that cAMP-response-element-binding protein (CBP) acetylates c-MYC which decreases its ability to be ubiquitinated and degraded ultimately resulting in increased c-MYC levels [301, 302].

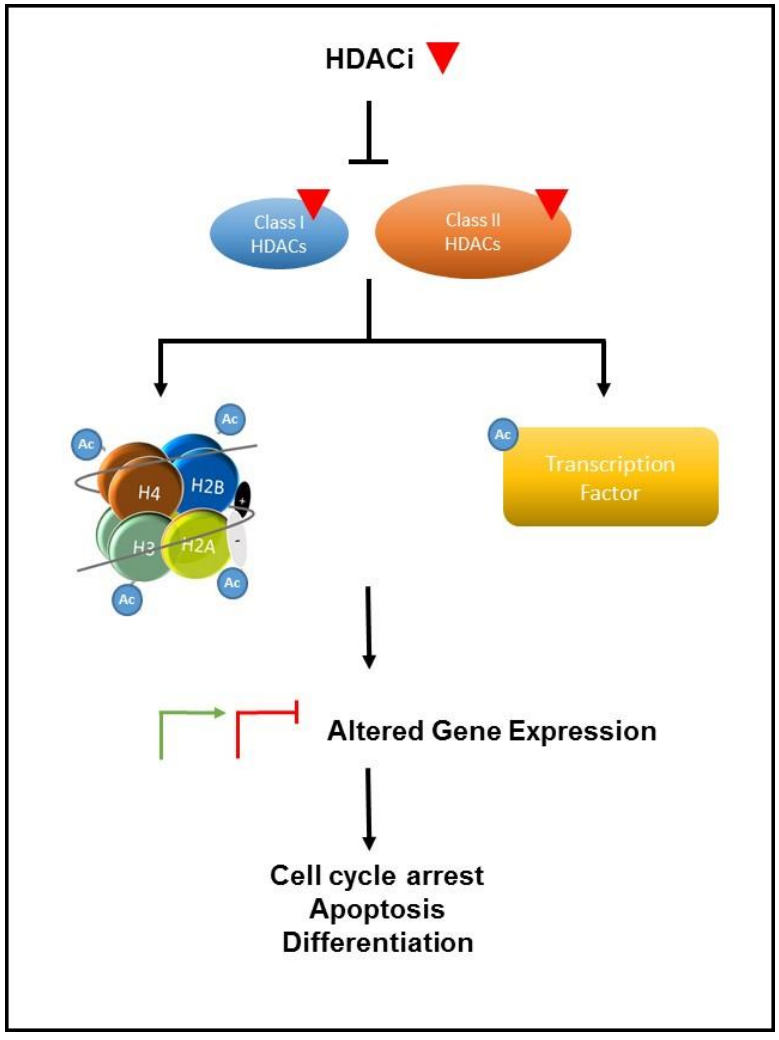

Figure 1.4: HDAC inhibitors modify gene expression through induction of histone and transcription factor hyper-acetylation.

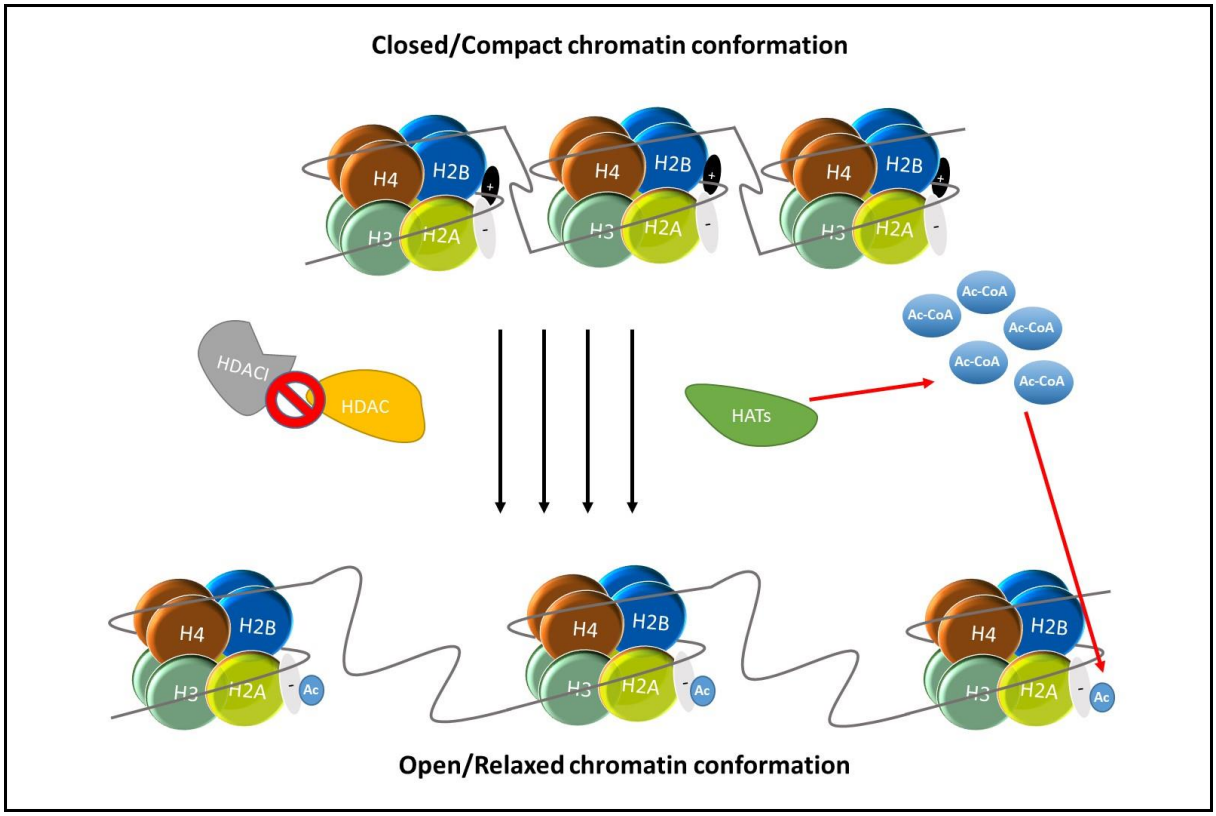

Figure 1.5: HDACi induce histone hyper-acetylation by inhibiting HDACs and allowing HATs to transfer an acetyl group from acetyl onto histones and inducing a more open chromatin conformation.

1.9.1 Review of gene expression changes induced by HDACi

As a result of both histone and transcription factor acetylation, HDACi treatment induces extensive transcriptional changes in tumour cells. Several studies have investigated the similarities and differences in these transcriptional changes both within and between different tumour types.

A study by LaBonte *et al* [303], compared the transcriptional changes induced by HDACi in the colorectal cancer cell lines, HT29 and HCT116, and observed a 35% overlap in the percentage of genes altered in expression. A similar study conducted by our own laboratory in SW403 and HCT116 colorectal cancer cells identified a 64% overlap (Figure 1.6). A similar study performed in hepatoma cell lines, compared HDACi-induced transcriptional changes between 6 lines. Although they did not publish the percentage of overlap between the lines, they did find 57 genes which were consistently induced and 119 which were consistently repressed [304]. Collectively,

these studies suggest that HDACi control the transcriptional regulation of a common set of genes within the same tumour type.

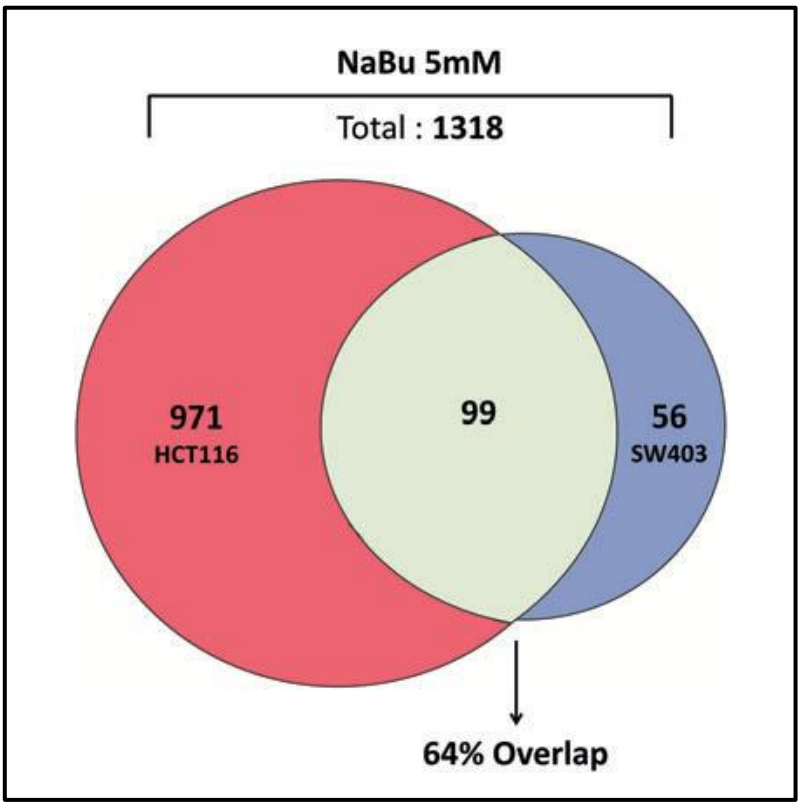

Figure 1.6: Overlap of gene expression changes following HDACi treatment of two colorectal cancer cell lines. Genes induced or repressed by two-fold or more were included in the analysis [11].

To investigate the extent of overlap in HDACi regulated genes between tumour types, Monks *et al* compared the HDACi-induced gene expression changes in colorectal cancer cell lines with the changes induced in seven cell lines derived from other tumour types [305]. A total of 25 genes were found to be similarly regulated by HDACi treatment across the 8 cell lines, including several key cell cycle regulators (CCNA2, CCNB1, and TYMS) which were consistently repressed upon HDACi (Belinostat) treatment [305]. Similarly, Glaser *et al* identified 13 genes which were commonly regulated upon HDACi treatment in breast and bladder cancer cell lines [160]. Once again, several of these genes were found to encode cell cycle regulators (TYMS and CTP synthase). However, the proportion of genes which are commonly regulated by HDACi across different tumour types only represent 1-2% of the total genes modified by HDACi in these cell lines. This indicates that most HDACi-induced transcriptional

changes are tumour type specific, but there still remains a core sub-set of genes which are consistently altered by HDACi regardless of tumour type. A list of genes consistently altered in expression by HDACi in at least two tumour types is listed in (Table 3) [11]. As indicated in Table 3, one gene that is consistently induced by HDACi in the majority of cell lines investigated to-date is p21 (CDKN1A) [306-308].

Table 3. Genes regulated by HDACi in at least two tumour types.

Genes Induced	Genes Repressed
P21	TYMS
CTGF	CTPS
H1F0	MCM3
IGFBP3	MCM7
GADD45	Cyclin D1
BMF	CCNA2
Gelsolin	Myc
BAK	Survivin
APAF1	FLIP
ATF3	TRX

1.10 Transcriptional response associated with apoptotic sensitivity to HDACi

While HDACi-induced apoptosis has been associated with altering the balance between pro- and anti-apoptotic genes in favour of pro-apoptotic genes, the changes induced vary significantly both within and between tumour cell types. Furthermore, the specific transcriptional mechanisms by which these genes are induced have not been clearly elucidated.

To address this, Wilson *et al* analysed the HDACi-induced transcriptional changes associated with apoptotic sensitivity to these agents [309]. The study utilised microarray profiling to compare the gene expression changes induced by HDACi in five sensitive versus five resistant cell lines and identified 48 genes which were preferentially induced and 44 genes which were preferentially repressed in the sensitive lines [309]. Notably, they found that the genes preferentially induced by HDACi in sensitive lines were enriched for immediate-early genes including several members of the AP-1 transcription factor complex (*FOS, JUN and ATF3*) and stress-response genes (*Gadd45β, Ndrg4, Mt1B, Mt1E, Mt1F, Mt1H,* and *MtIX*). As immediate-early and stress-response genes have been previously shown to be co-

ordinately regulated [310], they investigated the mechanism of induction of these genes by HDACi identifying a key role for the Sp1 and Sp3 transcription factors. This was established by knockdown experiments, whereby simultaneous knockdown of Sp1 and Sp3 attenuated HDACi induction of the immediate-early and stress response genes as well as HDACi-induced apoptosis [309].

1.10.1 HDACi-induce immediate-early genes across multiple tumour types.

To determine whether the induction of immediate-early and stress response genes are associated with HDACi-induced apoptosis in other tumour types, a screen was first undertaken in my laboratory to identify cancer cell lines which were sensitive and resistant to HDACi [311]. Fifty human cancer cell lines containing representative lines from common solid tumours, as well as CTCL and multiple myeloma cell lines where HDACi are currently clinically utilised, were screened for sensitivity to Vorinostat-induced apoptosis. This screen identified multiple sensitive (A549-lung carcinoma, HCT116 and SW948-colorectal cancer, AGS-gastric cancer, SK-MEL28-melanoma and MCF-7-breast cancer) and resistant (PC3-prostate cancer, U87-glioblastoma and PANC1-pancreatic cancer) cell lines from a range of tumour types. Notably, the screen also revealed that grouping the cell lines into haematological vs solid tumours re-capitulated what is clinically observed, in that haematological cell lines were significantly more sensitive to HDACi compared cell lines derived from solid tumours.

Having identified HDACi sensitive cancer cell lines from a range of tumour types, the laboratory next sought to determine whether the HDACi-induced transcriptional response identified in colorectal cancer cell lines was also induced in cell lines derived from other tumour types. As observed in colorectal cancer cells, Vorinostat induced expression of the IE genes c-FOS, c-JUN, ATF3, EGR1 and EGR3, and the stress response gene GADD45β in 8 sensitive cell lines derived from a range of tumour types, including melanoma (SK-MEL-28), breast (MCF7), lung adenocarcinoma (A549), gastric (AGS), CTCL (HH) and multiple myeloma (KG1 and RPMI-8226).

1.10.2 HDACi-induced apoptosis correlates with the magnitude of induction of c-FOS, c-JUN and ATF3

Finally, to determine if induction of these genes correlated with HDACi-induced apoptosis across tumour types, the magnitude of induction of *c-FOS, c-JUN, ATF3, EGR1, EGR3* and *GADD45B* in response to 24h Vorinostat treatment was determined in each of the 50 cell lines, and correlated with the corresponding magnitude of apoptosis induction. Of the 6 genes analysed, the magnitude of induction of c-*FOS*, c-*JUN* and *ATF3* correlated significantly with the magnitude of induction of apoptosis across the 50 cell lines.

1.11 AP-1 transcription factor complex

The three genes identified which positively correlate with HDACi-induced apoptosis encode for proteins which are members of the AP-1 transcription factor complex [312]. AP-1 belongs to the dimeric basic region-leucine zipper (bZIP) group of transcription factors and is composed by hetero- or homo-dimerisations of members of the FOS (c-FOS, FosB, Fra-1, and Fra2), JUN (c-JUN, JunB, and JunD), ATF (ATF2, ATF3, B-ATF3, JDP1, and JDP2) and MAF (c-Maf, MafB, MafA, MafG/F/K, and Nrl) family of proteins.

The AP-1 transcriptional complex is induced in response to a wide range of stimuli including growth factors, cytokines, and physical and chemical stresses [313]. These stimuli typically activate the mitogen activated protein kinase (MAPK) signalling pathways (ERK, JNK and p38) which in turn induce AP-1 transcriptional activity in several ways [314]. For example, growth factor-mediated activation of the ERK pathway results in the activation of ternary complex factors (TCFs) such as ELK-1, SAP-1 or NET, that bind to the promoter of *Fos* to further promote transcription [315, 316]. Stimulation of the JNK and p38 pathways can also induce AP-1 activation [314]. Specifically, activation of JNK induces phosphorylates c-JUN, which enhances its transcriptional activity [317]. In addition, JNK can also phosphorylate and activate ATF2, which can dimerise with c-JUN and bind to AP-1 sites in the c-JUN promoter to also promote c-JUN transcription [318]. Finally, the p38 MAPK pathway can also activate the AP-1 transcription factor complex via similar mechanisms. Specifically,

activation of p38 phosphorylates ATF2, MEF2C and TCFs which in turn can drive transcription of c-FOS and c-JUN [319].

A class of anti-cancer therapeutics which induces AP-1 activity via activation of the JNK and p38 MAPK pathways are proteasome inhibitors [320-322]. Proteasome inhibitors are approved for the treatment of multiple myeloma, but were initially used as a tool by cell biologists to identify proteins which were targeted for degradation by the 26S proteasome [323]. Inhibition of the proteasome results in the abnormal accumulation of proteins targeted for destruction, which induces cellular stress and activation of the JNK and p38 stress response pathways [324, 325]. Consistent with induction of these pathways, several studies have shown that proteasome inhibitor treatment induces expression of c-FOS, c-JUN and ATF3 in various cancer cells [326-328].

Given the large number of AP-1 homo and hetero-dimers which can be formed, it is not surprising that AP-1 transcription factor complex regulates a wide range of cellular processes including proliferation, apoptosis, survival and differentiation [329, 330]. As HDACi induced apoptosis positively correlates with induction of three members, c-FOS, c-JUN and ATF3, their function is discussed is more detail below.

1.11.1 FOS (c-FOS)

The FOS family consists of 5 members, c-FOS, FosB, FosB2, and the Fos-related proteins Fra-1 and Fra-2. All Fos proteins contain a hydrophobic bZIP motifs, which comprises a leucine zipper region that mediates heterodimerization with Jun, and a basic region that mediates DNA binding [331]. While all family members contain a bZIP domain, c-FOS and FosB also contain transactivation motifs. These transactivation motifs play a role in transcriptional activation, acting to stabilise the assembly of the pre-initiation complex [332]. The *c-fos* gene comprises of four exons, which encode a 381 amino acid, 55kDa protein [333, 334]. Fos proteins cannot form homodimers, but can heterodimerize with Jun family proteins and can also interact with other transcriptional co-activators such as creb-binding protein (CBP) and the GATA-4 transcription factor [335-337]. While the level of expression of c-FOS is low

or undetectable in most normal cells its induction is associated with a range of cellular processes including cell cycle progression and cellular differentiation [313].

In the context of its role in cancer development and progression, evidence exists for c-FOS acting as either an oncogenic driver or a tumour suppressor, depending on the cell type. The oncogenic properties of c-FOS were initially reported by Grigoriadis *et al*, where the overexpression of c-FOS induced osteosarcomas by transforming chondroblasts and osteoclasts in transgenic mice [338]. Furthermore, knockout of c-FOS reduces proliferation and invasion of squamous carcinoma cells [339] and supresses the growth of human colorectal carcinomas grown as xenografts in immune-compromised mice [340]. Conversely, other studies have reported that c-FOS can also act as a tumour suppressor with the overexpression of c-FOS inducing apoptosis in $p53^{+/+}$ colorectal cancer cells and also in CD43+ pro-B cells [341, 342]. Correlative studies also support a tumour suppressor function with low levels of c-FOS expression associated with worse outcome in ovarian [343, 344] and in gastric cancers [345].

1.11.2 JUN (c-Jun)

The human *c-jun* protein is composed of 334 amino acids and comprises three highly conserved domains: a bZIP domain, a basic region and a transactivation domain. The bZIP domain contains two parallel α-helices that form a coiled coil in the C-terminus and is responsible for dimerization of with other AP-1 proteins [346]. In normal cells, c-JUN is required for progression through the G1 phase of the cell cycle with cells lacking c-JUN undergoing G1 arrest due to increased expression of p53 and p21 [347-349]. c-JUN also plays an important role in apoptosis, although both pro and anti-apoptotic functions have been demonstrated. For example, serine 63/73 phosphorylated c-JUN co-operates with NF-κB to prevent TNFα induced apoptosis [348]. Conversely, several studies have demonstrated that c-Jun overexpression leads to increased apoptosis in a variety cell types [350-352].

In the context of cancer, c-JUN has been described to be an important factor in liver tumour development. In a model of carcinogen-induced liver cancer, mice with c-JUN

knocked out in the liver showed decreased tumour formation and increased survival compared to wild-type mice [353]. Conversely, the overexpression of c-JUN in breast cancer xenografts induces increased invasiveness, tumour formation and metastasis in nude mice [354, 355]. In regards to c-JUN expression in tumour samples, Szabo et al found that c-JUN was expressed in 31% of primary and metastatic lung tumours compared to normal airway and alveolar epithelial cells, although expression was not associated with outcome [356]. Comparatively, in invasive breast cancers, phosphorylated c-JUN was predominantly expressed in cells at the invasive front in 38% cases. However, as for lung cancers, expression of c-JUN was not correlated with outcome [357].

One of the few studies looking into c-JUN regulation by HDACi in cancers, demonstrated that HDACi treatment of K-562 bone marrow chronic myelogenous leukemia cells induced c-JUN. However, HDACi treatment of c-JUN overexpressing K-562 cells did not potentiate HDACi-induced growth inhibition or apoptosis [358]. Conversely, HDACi treatment of neuroblastoma cells demonstrated a repression in c-JUN and Fra-1 levels. In the study by He *et al*, they demonstrated that co-overexpression of c-JUN and Fra-1 promoted proliferation and antagonised HDACi-mediated anti-proliferative effects [359].

1.11.3 ATF (ATF3)

Finally, the preliminary findings in my laboratory also demonstrated that expression of activating transcription factor 3 (ATF3) is induced by HDACi, and that the magnitude of its induction correlates significantly with induction of apoptosis across multiple cell lines. ATF3 is a member of the activating transcription/cyclic AMP responsive element-binding (CREB) family of transcription factors that share the leucine zipper DNA-binding motif and bind to the CREB/ATF sequence *TGACGTCA*.

ATF3 levels are mostly un-detectable in cells under normal conditions but is induced by a variety of stress-inducing stimuli including DNA damage inducing agents and hypoxia [360{Hai, 2001 #1672]}. Similar to the other AP-1 complex members, ATF3 is also responsible for the regulation of numerous cellular processes including proliferation, invasion, and in some cases apoptosis, depending on cell type [361,

362]. Numerous xenograft studies have also demonstrated that depending on the cancer cell type, the expression of ATF3 can act as either a tumour suppressor or an oncogene [361, 363-365]. For example, the dual role of ATF3 in cancer was demonstrated by Yin *et al* where overexpression of ATF3 in aggressive MCF101a breast cancer cells protected the cells from apoptosis and promoted their metastatic potential, while in un-transformed MCF10a cells the gain of ATF3 induced apoptosis [366].

In regards to the oncogenic role of ATF3, transgenic mice overexpressing ATF3 in their mammary glands develop mammary carcinomas [367]. Similarly, transgenic mice overexpressing ATF3 in basal epithelial cells develop epidermal hyperplasia, hair follicle anomalies and neoplastic lesions of the oral mucosa including squamous cell carcinoma [368]. The oncogenic properties of ATF3 have also been demonstrated in prostate cancer cells, where its overexpression increased proliferation and metastasis [369]. This oncogenic role of ATF3 was further demonstrated in colorectal cancer [363] and Hodgkin lymphoma [370] cells where attenuation of ATF3 decreased cell proliferation and viability. The expression pattern of ATF3 in tumour specimens also supports an oncogenic role, with elevated expression of ATF3 observed in breast [366], prostate [371], and Hodgkin's lymphoma [370].

Converse to the oncogenic properties of ATF3 discussed above, there are also several studies which demonstrate the tumour suppressor role of ATF3. For example *Atf3*$^{-/-}$ MEFs, upon Ras transformation demonstrated increased growth rates, increased colony formation, and formed larger tumours when injected into mice when compared to the WT MEFs [364]. Similarly, the overexpression of ATF3 in colorectal [372], prostate [373], oesophageal squamous cell carcinoma [374] and ovarian [375] cancer cells resulted in decreased proliferation and invasion properties, and in the case of ovarian and prostate even increased apoptosis. This tumour suppressor role was further demonstrated in colorectal cancer by Bottone *et al* where ATF3 overexpression reduced the size of HCT116 colorectal cancer xenografts [361]. In line with these results, several of the studies mentioned above also performed ATF3 knockdown studies which resulted in increased proliferation and migration, and tumour growth in mice xenograft models. Furthermore, the anti-tumour effect of ATF3 is supported by

the observation that a wide range of anti-tumour agents including curcumin [376], non-steroidal anti-inflammatory drugs [361] and HDACi [377, 378] induce ATF3.

Finally, ATF3 has been reported to act as both a transcriptional activator as well as a transcriptional repressor [379-382]. In terms of its role as a transcriptional activator, ATF3 has been shown to induce expression of *MAL2, RGS2* and *GPR110* [383]. Conversely, its role as a transcriptional repressor is demonstrated by its ability to transcriptionally repress targets such as p53 [382] and to repress activity of a reporter construct containing 3 contiguous ATF3 binding sites [381].

1.12 Hypothesis

HDACi are an effective treatment option for specific haematological malignancies, however their activity in solid tumours is more limited. A detailed understanding of the mechanisms which drive HDACi-induced apoptosis could provide important biological insight into the molecular basis for the differential cellular responses to these agents, which in turn could help identify rational drug combinations which overcome inherent resistance to these agents.

Prior work undertaken in my laboratory has demonstrated that HDACi-induced apoptosis across a range of tumour types is significantly correlated with induction of the immediate-early genes c-FOS, c-JUN and ATF3. These genes encode transcription factors which form the AP-1 transcription factor complex.

Based on these findings, we hypothesise that the induction of AP-1 transcription factor components c-FOS, c-JUN or ATF3 are critical determinants of HDACi-induced apoptosis. In turn, this thesis will investigate if induction of the AP-1 components alters the expression of specific pro-and or apoptotic genes to drive HDACi-induced apoptosis, and if this information can be utilized to develop rational drug combinations to overcome inherent resistance to HDACi.

1.12.1 Aims

1. To validate the finding that induction of c-FOS, c-JUN and ATF3 is a consistent feature of HDACi-induced apoptosis, independent of tumour type, and to determine whether the induction of these genes is required for HDACi induced apoptosis.
2. To determine if HDACi-induction of c-FOS, c-JUN and or ATF3 alters the expression of pro and or anti-apoptotic genes.
3. Based on the findings above, to identify rational drug combinations which can overcome inherent resistance to HDACi.

Chapter 2: Methods

2.1 Cell Culture

2.1.1 Cell Lines

All cancer cell lines were obtained from the American Type Culture collection (ATCC, USA), or from other sources as cited in Table 4. Cells were cultured in Dulbecco's Modified Eagles Medium (DMEM)-F12 (GIBCO BRL, Australia), supplemented with 10% Fetal Calf Serum (FCS) (PAA Laboratories, Australia), 1% GlutaMAX™ Supplement - 200 mM L-alanyl-L-glutamine dipeptide in 0.85% NaCl (GIBCO BRL, Australia), 1% Penicillin Streptomycin (PenStrep) - 10,000U/ml (GIBCO BRL, Australia) and 1% 1M HEPES (N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid) (GIBCO BRL, Australia), unless otherwise stated (Table 4) and maintained in a 37°C incubator with 5% CO_2. Cells were passaged when approximately 80% confluent and seeded 12-24 hours prior to drug treatment. All cells were in their exponential growth phase when treated with drug. Adherent cells were passaged by removing the old medium and washing with Dulbecco's phosphate-buffered saline (DPBS, no calcium and no magnesium [KCl 200mg/L, KH_2PO_4 200mg/L, NaCl 8000mg/L, Na_2HPO_4-$7H_2O$ 2160mg/L]) (GIBCO BRL, Australia). TrypLE™ Express Enzyme (Life technologies™, Australia) was then added to the flasks (3ml for a T25 and T75 flask and 5mL for a T175 flask) to induce cell detachment. Suspension lines were passaged by taking a 3mL aliquot of the old cell suspension and adding 7mL of fresh medium.

Table 4. Cancer cell lines used and their growth medium.

Cell line	Description	Media
SK-MEL-28	Adherent human malignant melanoma cell line, with an epithelial-like morphology.	DMEM-F12 +Supplements
SK-MEL-3	Adherent human malignant melanoma cell line, with an fibroblast-like morphology.	DMEM-F12 +Supplements
MCF7	Adherent human breast cancer cell line, with an epithelial morphology.	RF-10 +Supplements
PANC-1	Adherent human pancreatic cancer cell line, with an epithelial morphology.	DMEM-F12 +Supplements
PC3	Adherent human prostate cancer cell line; derived from metastatic bone site, with an epithelial morphology.	DMEM-F12 +Supplements
U-87 MG	Adherent human glioblastoma cell line, with an epithelial-like morphology.	DMEM-F12 +Supplements
SW948	Adherent human colorectal cancer cell line, with an epithelial morphology.	DMEM-F12 +Supplements
HCT116	Adherent human colorectal cancer cell line, with an epithelial morphology.	DMEM-F12 +Supplements
A549	Adherent human lung cancer cell line, with an epithelial morphology.	DMEM-F12 +Supplements
AGS	Adherent human gastric cancer cell line, with an epithelial morphology.	DMEM-F12 +Supplements
HH	Suspension human CTCL cancer cell line, with a lymphoblast morphology.	RF-10 +Supplements
U266	Suspension human multiple myeloma cell line, with a lymphoblast morphology.	RF-10 +Supplements
OPM2	Suspension human multiple myeloma cell line, with a lymphoblast morphology.	RF-10 +Supplements

MEF WT	Wild type mouse embryonic fibroblasts cells	MEM +Supplements
MEF ATF3$^{-/-}$	ATF3 knockout mouse embryonic fibroblasts cells	MEM +Supplements
MEF WT	Wild type mouse embryonic fibroblasts cells	DMEM +Supplements +250 µM L-asparagine +50 µM 2-mercaptoethanol
MEF FLAG-tagged hBCL-XL	Flag-tagged human BCL-X_L overexpressing mouse embryonic fibroblasts cells	DMEM +Supplements +250 µM L-asparagine +50 µM 2-mercaptoethanol

2.1.2 Cell counting & seeding

Cells were trypsinised and re-suspended in 5-10mL of media for cell counting. Trypan blue (Bio-Rad, USA) exclusion was used to ensure only live cells were counted. 10 µL of the cell suspension with an equal volume of Trypan blue was loaded onto a Bio-Rad Cell counting slide (Bio-Rad, USA) and cells counted using a Bio-Rad TC10 Automated cell counter (Bio-Rad, USA). The seeding density for each cell line was optimized to ensure that cells reached approximately 80% confluence at the time of completion of the experiment. Seeding densities used for each cell line are listed Table 5.

Table 5. Seeding densities of all cancer cell lines used.

Cell line	Plate size	Seeding density
SK-MEL-28	6-well plate	$3x10^5$
	12-well plate	$1x10^5$
	24-well plate	$4x10^4$
SK-MEL-3	6-well plate	$3x10^5$
	12-well plate	$1x10^5$
	24-well plate	$4x10^4$
MCF7	6-well plate	$3x10^5$
	12-well plate	$1x10^5$
	24-well plate	$4x10^4$
PANC-1	6-well plate	$3x10^5$
	12-well plate	$1x10^5$
	24-well plate	$4x10^4$
PC3	6-well plate	$3x10^5$
	12-well plate	$1x10^5$
	24-well plate	$4x10^4$
U-87 MG	6-well plate	$3x10^5$
	12-well plate	$1x10^5$
	24-well plate	$4x10^4$
SW948	6-well plate	$4x10^5$
	12-well plate	$2x10^5$
	24-well plate	$5x10^4$
HCT116	6-well plate	$3x10^5$
	12-well plate	$1x10^5$
	24-well plate	$4x10^4$
A549	6-well plate	$1.5x10^5$
	12-well plate	$6x10^4$
	24-well plate	$2.5x10^4$
AGS	6-well plate	$3x10^5$
	12-well plate	$1x10^5$
	24-well plate	$4x10^4$
HH	6-well plate	$1x10^6$

	12-well plate	$5x10^5$
U266	6-well plate	$1x10^6$
	12-well plate	$5x10^5$
OPM2	6-well plate	$1x10^6$
	12-well plate	$5x10^5$
MEF WT	6-well plate	$3x10^5$
	12-well plate	$1x10^5$
MEF ATF3$^{-/-}$	6-well plate	$3x10^5$
	12-well plate	$1x10^5$
MEF WT	6-well plate	$3x10^5$
	12-well plate	$1x10^5$
MEF FLAG-tagged hBCL-X_L	6-well plate	$3x10^5$
	12-well plate	$1x10^5$

2.2 Drug Treatments

After seeding, cells were incubated overnight at 37^{o}C in 5% CO_2 and subjected to drug treatment the following day. All drugs used in this thesis, their function, diluent and concentration ranges are listed in Table 6.

Table 6. Function, source, diluent and concentration ranges of drugs used in this thesis.

Compound Name	Function/Target	Supplier	Diluent & Concentration
SAHA/Vorinostat	Pan-HDACi	Selleckchem, USA	DMSO 2.5-5 µM
Panobinostat/LBH-589	Pan-HDACi	Selleckchem, USA	DMSO 10-50 nM
Depsipeptide	Class I&II HDACi	Selleckchem, USA	Water 10-50 nM
MS-275/Entinostat	HDAC 1,2,3&8 inhibitor	Selleckchem, USA	DMSO 1-5 µM
Belinostat/PXD101	Pan-HDACi	Selleckchem, USA	DMSO 1–2.5 µM
Valproic Acid (VPA)	Class I&IIa HDACi	Selleckchem, USA	DMSO 5-10 mM
Sodium Butyrate	Class I&IIa HDACi	Selleckchem, USA	Water 1–5 mM
Bortezomib	Proteasome inhibitor	Selleckchem, USA	DMSO 1–10 nM
Mithramycin	Sp1&Sp3 inhibitor	Selleckchem, USA	DMSO 1 µM
PMA	Protein Kinase C agonist	SIGMA ALDRICH, USA	DMSO 100 nM
SB203580	p38 inhibitor	Selleckchem, USA	DMSO 100 nM
ABT-737	BH3 mimetic inhibitor	Selleckchem, USA	DMSO 1-10 µM

ABT-199	Bcl-2 inhibitor	Selleckchem, USA	DMSO 1-10 µM
A-1331852	Bcl-xL inhibitor	WEHI	DMSO 1-10 µM
ABT-263	Bcl-xL inhibitor	Selleckchem, USA	DMSO 0.1-10 µM

2.3 RNA extraction, cDNA synthesis and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

2.3.1 Extraction of RNA

Following drug treatment, cells were harvested by scraping and pelleted by centrifugation at 300 x *g*. A minimum of $1.5x10^6$ cells per sample were collected for RNA extraction using the High Pure RNA Isolation Kit (ROCHE, Germany). Cell pellets were re-suspended in 200 µl PBS and then lysed with 400 µl of Lysis Buffer by vortexing for 15 seconds. The lysed cell suspension was then transferred to a High Pure Filter Tube and then inserted into a collection tube. The entire tube was then centrifuged in a table-top centrifuge for 15 seconds at 8000 x g. After centrifugation, the fluid collected in the collection tube was discarded and re-combined with the High Pure Filter Tube. For each sample, 90µl of DNase I Incubation Buffer and 10 µl DNase I was added to the top part of the High Pure Filter Tube, ensuring coverage of the fleece part of the tube. Each sample was incubated for 15 min at +15 to +25°C. 500 µl of Wash Buffer I was then added to the High Pure Filter Tube and centrifuged for 15 s at 8,000 x g, with the flowthrough in the collection tube discarded. 500 µl of Wash Buffer II was then added to the High Pure Filter Tube and centrifuged for 15 s at 8,000g. The flowthrough was once again discarded and 200 µl of Wash Buffer II was added to the High Pure Filter Tube, and centrifuged for 2 minutes at maximum speed (approx. 13,000 x g) to remove any residual Wash Buffer. 30-50 µl of Elution Buffer was then added to the High Pure Filter Tube/1.5mL eppendorf tube and centrifuged for 1 minute at 8000 x g to elute the final RNA product which was stored at -80°C for further cDNA synthesis.

2.3.2 Assessment of RNA concentration and quality

To determine the concentration and purity of the extracted RNA, spectrophotometric analyses were performed using the NanoDrop ND-2000

instrument (NanoDrop Technologies, United States). Purity of the extracted RNA was assessed by computing the 260 nm/280 nm absorbance ratio, and samples with ratio> 1.8 were considered to be of high purity and suitable for cDNA synthesis.

2.3.3 cDNA synthesis

cDNA synthesis was performed using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostic, Germany), by combining 1µg of total RNA with 0.5µl of Random Hexamer (600 pmol/µl) and 5.5µl of High grade PCR H_20, on ice, in 0.2mL Eppendorf PCR Tubes (Eppendorf, USA). The mixture was incubated at 65^{o}C for 10 minutes in a MasterCycler® (Eppendorf, USA) thermal cycler, then held at 4^{o}C. A reverse transcriptase master mix containing 2µl of 5x Transcriptor High Fidelity Reaction Buffer, 1µl of 10mM PCR grade dNTP mix, 0.5 µl of 100mM DTT, 0.25 µl of 40U/µl Protector RNase Inhibitor and 0.55 µl of 10U Transcriptor High Fidelity Reverse Transcriptase (blend of a recombinant reverse transcriptase and a proofreading mediating enzyme), was subsequently added to the 5.7µl RNA mixture. The 10µl reaction was then incubated at 48^{o}C for 60 minutes in the MasterCycler® (Eppendorf, USA) thermal cycler, followed by incubation at 85^{o}C for 5 minutes to inactivate the enzyme. Samples were cooled to 4^{o}C, and diluted 1:10 with high grade PCR H_20 and stored at 80^{o}C for use in subsequent real-time PCR experiments.

2.3.4 Quantitative Real-Time Polymerase Chain Reaction (Q-RT-PCR)

Quantitative real-time PCR analysis was performed using either the 7500 Fast Real Time PCR System or the ViiA™ 7 384 well block Real-Time PCR System and data analysed using the respective software of each system (Applied Biosystems, USA). The oligonucleotides/primers used to detect expression of specific genes, are listed in Table 3. In all cases, expression levels of genes of interest were normalized against the expression of beta actin (ACTB) housekeeping gene using the delta C_T method (described below).

7500 Fast Real Time PCR System

For qRT-PCR analyses using the 7500 Fast Real Time PCR System, 10µl reactions were prepared consisting of: 1.5 µL of forward primer (50 nM final concentration), 1.5

µL of reverse primer (50 nM final concentration), 5 µL of 2 x SBYR Green PCR Master Mix (Roche Applied Sciences) and 2 µL of template cDNA. Samples were loaded in triplicate onto a MicroAmp® Optical 96-Well Reaction Plate (Applied Biosystems, USA) and the plate centrifuged to ensure components of the reaction were concentrated at the base of the wells. Plates were then run on the 7500 Fast Real Time PCR System using the following running conditions:

1. An initial incubation at 95^{o}C for 10 minutes to denature the sample.
2. Forty-five amplification cycles using a 2-step cycling protocol consisting of a 15 second 95^{o}C denaturing step and a 1 minute 60^{o}C annealing and extension step.

ViiA™ 7 Real-Time PCR System – 384 well block system

For qRT-PCR analyses using the ViiA™ 7 384 well block Real-Time PCR System, 5µl reactions were prepared consisting of: 0.75 µL of forward primer (50 nM final concentration), 0.75 µL of reverse primer (50 nM final concentration), 2.5 µL of 2 x SBYR Green PCR Master Mix (Roche Applied Sciences) and 1 µL of template cDNA. Samples were prepared in a MicroAmp® Optical 384-Well Reaction Plate with Barcode (Applied Biosystems, USA). The plate was centrifuged and run on the ViiA™ 7 Real-Time PCR System under the following conditions:

1. A denaturing step was initially performed (95^{o}C for 10 minutes).
2. 50 amplification cycles, using a 2-step cycling protocol which consists of a 15 second 95^{o}C denaturing step and a 1 minute 60^{o}C annealing and extension step.

2.3.5 Primers

All primers were obtained from Sigma-Aldrich, Australia. Primers were diluted to 500 nM in nuclease-free H_2O and stored at –20°C.

Table 7. Primer sequences used in all qRT-PCR analysis.

Primer Name	Sequence
c-FOS Forward	ATCAAGGGAAGCCACAGACA
c-FOS Reverse	ATCAAGGGAAGCCACAGACA
c-JUN Forward	TGACTGCAAAGATGGAAACG
c-JUN Reverse	TGAGGAGGTCCGAGTTCTTG
ATF3 Forward	GTGCCGAAACAAGAAGAAGG
ATF3 Reverse	GTGCCGAAACAAGAAGAAGG
EGR1 Forward	ATCCCCGACTACCTGTTTCC
EGR1 Reverse	GTTTGATGAGCTGGGACTGG
EGR3 Forward	ATCTGTGGGGAGAAAGAGCA
EGR3 Reverse	ATGATGGTGGGAAGGAGAAA
GADD45β Forward	ACCTGCATTGTCTCCTGGTC
GADD45β Reverse	TGGCAGCAACTCAACAGATT
Bcl-xL Forward	CTGCTGCATTGTTCCCATAG
Bcl-xL Reverse	TTCAGTGACCTGACATCCCA
ACTB Forward	CACCTTCACCGTTCCAGTTT
ACTB Reverse	GATGAGATTGGCATGGCTTT

2.4 Western Blot analysis

2.4.1 Cell lysate preparation

Following drug treatment, the medium was removed, cells were washed in cold DPBS and harvested by scraping on ice in cold DPBS. Cells were pelleted by centrifugation at 300 x *g* for 5 minutes at 4^{o}C in a micro-centrifuge (Thermoscientific, USA). Proteins were extracted following the lysis of cells in 250µL of NP-40 lysis buffer [1% NP-40, 50mM Tris-HCL pH 7.5, 150mM NaCl, 0.5% sodium deoxycholate, 1mM EDTA, Protease Inhibitor Cocktail, 1 tablet/10mL, Phosphatase Inhibitor cocktail, 1 tablet/10mL, 3M Urea] (all products from SIGMA ALDRICH, USA), by incubation at 4^{o}C, with gentle agitation. Samples were subsequently sonicated at 15 pulses/second for 10 minutes and the insoluble contents removed by centrifugation at 17,000 x *g* for 15 minutes at 4^{0}C. The supernatant containing the extracted proteins were then transferred to fresh tubes and stored at -80^{0}C.

2.4.2 Extraction of Histone proteins

Total histones were extracted from cell lines treated with or without the HDAC inhibitor Vorinostat for 24 hours. For histone extraction, approximately 4x10^{6} cells were harvested as described above. Larger number of cells were required for this procedure to ensure sufficient amounts of histone proteins were collected. Pelleted cells were re-suspended in 100 µL of cold histone lysis buffer [10mM HEPES pH 7.9; 1.5mM $MgCl_2$; 10mM KCl, 0.5mM DTT and protease inhibitor cocktail tablets (1tablet/10mL)] (Roche, Germany). 4 µL of 5mM sulphuric acid (H_2SO_4) (SIGMA ALDRICH, USA) was then added to each sample and vortexed immediately. Samples were incubated on ice for 1 hour with intermittent vortexing, and centrifuged at 17,000 x *g* for 10minutes at 4^{o}C. The supernatant was harvested and diluted with acetone at a ratio of 9:1, and incubated overnight at -20^{o}C. Samples were then centrifuged at 17,000 x *g* for 10 minutes at 4^{o}C, and the supernatant removed. The insoluble pellet containing histone proteins was washed with 70% ethanol and air dried. Histone protein pellets were re-suspended in 100 µL of Milli-Q water and sonicated at 15 pulses/second for 10 minutes on ice. Remaining un-dissolved particles were removed by further centrifugation at 17,000 x g for 5 minutes.

2.4.3 Determination of protein concentration

The Bradford assay was used to determine the concentration of extracted proteins. Protein lysates (1µL) was diluted with 9 µL of MilliQ water in a 90-well flat bottom plate (BD Falcon, Australia). 190µL of Bradford Reagent (Bio-Rad Laboratories, USA) was added to the sample and incubated at room temperature for 5 minutes. Absorbance was measured at 595 nm using a Molecular Devices $VERSA_{max}$ micro-plate reader (Sunnyvale, United States). Protein concentrations were determined against a standard curve generated from known concentrations of bovine serum albumin (Sigma Aldrich, USA), incubated in parallel with Bradford Reagent.

2.4.4 Resolution of proteins on Bis-Tris gels

30 µg of protein in lysis buffer was combined with a corresponding volume of 4X sample buffer (SB) (Novex, USA) and 10X sample reducing agent (Novex, USA). 20 µg of protein was then resolved using NuPAGE® Novex® 10% Bis-Tris precast 10 or 15 well gels (Invitrogen, USA). Gels were run in a XCell SureLock™ Mini-Cell tank (Novex, USA), using 1 X MES SDS Running Buffer (Invitrogen, USA), with the addition of 500 µL of NuPAGE® Antioxidant to the inner chamber. Novex® Sharp Pre-Stained Protein Standards (Invitrogen, USA) were used as protein weight markers for all western blots performed. Gels were initially run at 100 Volts to stack the proteins and subsequently increased to 120 Volts for the remaining run. Proteins were then transferred to PVDF membranes (iBlot® PVDF Transfer Stacks) (Novex, USA) using the iBlot® Dry Blotting System (Invitrogen, USA) at 20 Volts for 8 minutes.

PVDF membranes were blocked with Odyssey PBS Blocking Buffer [PBS based, highly purified non-mammalian proteins] (LICOR, USA), a for 30-60 minutes at room temperature. Blots were then incubated with primary antibodies diluted in a 50% blocking buffer and 50% PBS/T (PBS plus 0.1% Tween 20) solution, overnight at 4^0C. Blots were then washed 3 times for 10 minutes each with PBS/T, and incubated with a species-appropriate fluorescent IRDye-conjugated secondary antibody (LICOR, USA) diluted in the 50% blocking buffer and 50% PBS/T solution, for 1 hour at room temperature in the dark. Blots were once again washed 3 times for 10 minutes each with PBS/T and dried in the dark. Dried blots were then scanned and analysed using

the Odyssey Classic Infrared Imaging system and visualized using the Odyssey software (LICOR, USA). Primary and secondary antibodies used, their dilutions, incubation conditions and suppliers are listed in Table 8 and Table 9, respectively.

2.4.5 Primary and secondary antibodies used in Western blotting

Table 8. Primary antibodies used. O/N denotes overnight.

Primary Antibodies	Supplier	Condition		
Anti-c-FOS #4384 (Rabbit)	Cell Signalling, USA	1:5000	4^0C	O/N
Anti-c-JUN #9165 (Rabbit)	Cell Signalling, USA	1:5000	4^0C	O/N
Anti-ATF3 C19 #SC-188 (Rabbit)	Santa Cruz, USA	1:2500	4^0C	O/N
Anti-Sp1 #07-645(Rabbit)	Millipore, USA	1:10000	4^0C	O/N
Anti-Sp3 #07-107(Rabbit)	Millipore, USA	1:10000	4^0C	O/N
Anti-p-p38 #9211 (Rabbit)	Cell Signalling, USA	1:2500	4^0C	O/N
Anti-Cleaved Caspase 3 ASP175 #9661 (Rabbit)	Cell Signalling, USA	1:5000	4^0C	O/N
Anti-Ac-Histone H3 #382158 (Rabbit)	Millipore, USA	1:20000	4^0C	O/N
Anti-H3 C-16 #SC-8654 (Goat)	Santa Cruz, USA	1:5000	4^0C	O/N
Anti-β-Tubulin #Ab6046 (Rabbit)	Abcam, USA	1:15000	4^0C	O/N

Table 9. Secondary antibodies used. R/T denotes room temperature.

Secondary Antibodies	Supplier	Condition		
IRDye Mouse 680RD	LICOR, USA	1:20000	RT	1 Hour
IRDye Rabbit 680RD	LICOR, USA	1:20000	RT	1 Hour
IRDye Mouse 800RD	LICOR, USA	1:20000	RT	1 Hour
IRDye Rabbit 800RD	LICOR, USA	1:20000	RT	1 Hour
IRDye Goat 6800RD	LICOR, USA	1:20000	RT	1 Hour

2.5 Fluorescence Activated Cell Sorting (FACS)

Cell cycle changes and the percentage of apoptotic cells induced following drug treatments were determined by staining cells overnight with propidium iodide (PI)

[50µg/ml PI diluted in citrate/Triton buffer (0.1% Sodium citrate, 0.1% Triton X-100) (Sigma-Aldrich Corporation, USA)], followed by fluorescence activated cell sorting (FACS). At the completion of the treatment period, cells were scraped in their individual wells and 200 µL of the cell-media suspension (which included adherent and floating cells) centrifuged in a 96-well plate at 300 x *g* for 5 minutes. The medium was discarded, cell pellets re-suspended in 200 µL of PI buffer and incubated in the dark, overnight at 4ºC. The following day PI-stained cells were analysed using a FACS Canto II flow-cytometer (BD Biosciences, USA) equipped with a high throughput sampler (HTS). A total of 10,000 events (cells) were recorded for each sample. Single cells were initially gated from debris and clumped cells using the forward (FSC-A) and side scatter (SSC-A) parameters. This population was then further gated to exclude debris and clumped cells based on the area and width of the PE-Cy5 signal (PE-Cy5-W vs Pe-Cy5-A). The previous gate is then displayed in a count vs PE-Cy5-A plot summarizing the distribution of cells in the gated population with different DNA contents. The FlowJo software (FlowJo-LLC, USA) was used to assess any cell cycle changes, particularly the changes in the percentage of sub-diploid DNA content which were considered to be apoptotic cells.

2.6 Transient transfections and luciferase reporter assays

BCL-X_L luciferase promoter reporter activity following ATF3 overexpression and Vorinostat treatment was measured using the Dual-Luciferase Reporter Assay System (Promega, USA), which measures Firefly and Renilla luciferase activities in a sample.

2.6.1 DNA transfections

All transfections were performed in cells seeded in 24-well plates in pen-strep free DMEM medium supplemented with 10% FCS and 1% Glutamax. Prior to transfection the medium was replaced with 400 µL/well of fresh pen-strep free DMEM. Source of the BCL-X_L reporter and ATF3 overexpression constructs used are listed in Table 10. Transfection mixtures were prepared as follows.

Reporter Construct Transfection Mixture

Mixture A

- Reporter construct 0.5µg
- Renilla 0.1µg
- Volume adjusted to 50µL with OPTI-MEM
- Incubated at room temperature for 5 minutes

Mixture B

- 2µL of Lipofectamine 2000
- 48µL of OPTI-MEM
- Incubated at room temperature for 5 minutes

Mixtures A & B were then combined, incubated at room temperature for 20 minutes and added to cells growing in a 24-well plate in 400µL of medium (pen-strep free). Cells were incubated with the transfection mixture for 24-hours following which in the appropriate experiments, drug treatment was commenced. Following 24-hours of drug treatment cells were collected and prepped for luciferase assay.

2.6.2 Reporter constructs

Table 10. List of reporter constructs used and their respective sources.

Reporter Constructs	Supplier
pGL3	Dr Ni Chen, Sichuan University
pGL3-Bcl-xL-1281	Dr Ni Chen, Sichuan University
pCG	Professor Tsonwin Hai, Ohio State University
pCG ATF3	Professor Tsonwin Hai, Ohio State University

2.6.3 Dual luciferase reporter assays

At the completion of the drug treatment period, cells were lysed with 100µL of 1X passive lysis buffer provided in the Dual-Luciferase Reporter Assay kit (Promega, USA) by incubation at room temperature for 20 minutes with regular agitation. 20 µL of cell lysate was transferred into 96-well Solid White Polystyrene plates (CORNING, USA) and luciferase assays were performed using a SpectraMax Luminescence spectrometer using the in-built Luminescence Assay_Flash > Dual-Luciferase Spectra

MaxL protocol. This protocol dispenses 25µL of Luciferase Assay Reagent II (PROMEGA, USA) which is the substrate for Firefly luciferase, and reads the luminescence signal generated. 25 µL of Stop&Glo reagent (PROMEGA, USA) is then dispensed into the same well to quench the firefly luciferase reaction and concomitantly activate *Renilla* luciferase activity, followed by reading of the luminescence signal. *Renilla* values were not used to control for transfection efficiency in experiments involving HDACi treatment due these drugs strongly inducing *Renilla* reporter activity. To account for this, untreated and HDACi-treated cells were always transfected with the same transfection master mix, and samples were corrected for total cellular protein instead to account for the reduction in cell number induced by HDACi treatment.

2.7 Small interfering RNA (siRNA)-mediated-knock-down of gene expression

2.7.1 siRNA Transfection protocol

Expression of target genes of interest were downregulated by transient transfection of various siRNAs listed in Table 11. A reverse transfection protocol was used for all siRNA transfections, which were performed in cells seeded 12-well plates according to the seeding densities listed in Table 5. All transfections were performed such that the final concentration of siRNA per well was 25 nM. ON-TARGET Plus siRNA (GE Dharmacon, USA) was used as a control for all siRNA experiments. The transfection mixtures were prepared for each well as described below.

siRNA Transfection Mixture

Mixture A

- 1.25µL of 20µM siRNA stock
- 98.75µL of OPTI-MEM
- Incubate at room temperature for 5 minutes

Mixture B

- 2µL of RNAi Max
- 98µL of OPTI-MEM

- Incubated at room temperature for 5 minutes

Mixture A & B were combined and incubated at room temperature for 20 minutes, then added to cells cultured in 800µL medium for 48 hours. Fresh medium was then added and cells subjected to drug treatments.

Table 11. Sequences of siRNAs used. ORF-Open reading frame, UTR-Untranslated region.

siRNA Target	Targeted Reg.	Sequence	Catalogue No.
c-FOS ON-TARGET PLUS	Pooled	GGGAUAGCCUCUCUUACUA ACAGUUAUCUCCAGAAGAA GAACCUGUCAAGAGCAUCA GCAAUGAGCCUUCCUCUGA	L-003265-00-0005
c-JUN ON-TARGET PLUS	Pooled	GAGCGGACCUUAUGGCUAC GAACAGGUGGCACAGCUUA GAAACGACCUUCUAUGACG UGAAAGCUCAGAACUCGGA	L-003268-00-0005
ATF3 ON-TARGET PLUS	Pooled	GAGCUAAGCAGUCGUGGUA GCAAAGUGCCGAAACAAGA AGAAGCAGCAUUUGAUAUA CGAGAAAGAAAUAAGAUUG	L-008663-00-0005
ATF3#1 ON-TARGET PLUS	3'UTR, ORF	GAGCUAAGCAGUCGUGGUA	J-008663-05-0005
ATF3#2 ON-TARGET PLUS	ORF	GCAAAGUGCCGAAACAAGA	J-008663-05-0005
NON-TARGETING ON-TARGET PLUS	Pooled	UGGUUUACAUGUCGACUAA GGUUUACAUGUUUUCUGA UGGUUUACAUGUUUUCCUA UGGUUUACAUGUUGUGUGA	D-001810-10-50

2.8 Plasmid preparation

2.8.1 Bacterial transformation

Bacterial transformation was performed using One Shot® TOP10 Competent cells (Thermofisher, USA). 5 µL of the DNA was added to 100 µL of competent cells and incubated on ice for 30 minutes. Cells were then heat shocked by incubation at 42ºC for 30 seconds and placed on ice. 250 µL of pre-warmed S.O.C medium was then added to each vial and cells incubated at 37ºC shaker 1 hour. 100 µL of the bacterial culture was then spread onto LB agar plates containing 0.1mg/mL ampicillin, the plates inverted and incubated overnight at 37ºC.

2.8.2 Minipreps

Individual ampicillin resistant colonies were picked and cultured overnight in 5mL of LB broth containing 0.1mg/mL ampicillin. The following day, 1mL of the bacterial culture was frozen at -80ºC in lysogeny broth (LB) containing 30% glycerol. The remaining bacterial stock was subjected to plasmid DNA extraction using the PureLink® HiPure Plasmid Miniprep Kit (Thermofisher, USA) as per manufacturer's protocol. Purified plasmid DNA were eluted in 50µl of nuclease-free H_2O and stored at -20°C. The presence of DNA and identity of the plasmid was determined by restriction enzyme digestion and resolution and visualization of the digests on agarose gels.

2.8.3 Maxipreps

Once the proper identity of the plasmids were confirmed, large scale plasmid preparation was performed using the PureLink® HiPure Plasmid Maxiprep Kit. Two-hundred µl from the frozen miniprep culture was added to 250mL of LB broth containing 0.1mg/mL of ampicillin (Millipore, USA) and incubated at 37°C with continuous shaking for approximately 18 hours. Bacterial cells were then harvested by centrifugation at 6000 rpm for 15 minutes at 4°C in an Allegra® X-12R centrifuge. Plasmid DNA was extracted as per the manufacturer's instructions and re-suspended in 200 µl of nuclease-free H_2O and stored at -20°C. The concentration of plasmid DNA

was measured using a Nanodrop® Spectrophotometer (ND-2000), and additional restriction enzyme digests performed to further confirm the proper identity of the plasmid.

2.9 Mouse Xenograft experiments

2.9.1 Mice

BALB/c nu/nu immune-deficient mice (developed through crosses and back-crosses between BALB/cABom-nu and BALB/cAnNCrj-nu at Charles River Laboratories, Japan) were purchased from the Animal Resource Centre (Perth, Western Australia) and maintained at the Bio-Resources Facility (BRF) at Austin Health. Mice were housed in micro-isolators and used between 6-12 weeks of age. All animal experiments were approved by the Austin Health Animal Ethics Committee and carried out in accordance with the guidelines of University of Melbourne and conformed to the National Health and Medical Research Council's code of practice for the care and use of animals for scientific purposes. Euthanasia was performed by overdose with Isoflurane and subsequent cervical dislocation.

2.9.2 Tumour Xenograft injections

The glioblastoma line, U87 was used in the mouse xenograft study. Cells were trypsinised and cell counts performed 45 minutes prior to injection into mice to ensure maximal cell viability. A total of $3x10^6$ cells were pelleted at 300 x *g* and re-suspended in 75uL of medium and combined with an equal volume of ice-cold Matrigel (BD Biosciences, Australia). This suspension was kept on ice during transport to the animal facility. Using ice cold 27-gauge insulin needles (TERUMO, Japan), the cell suspension was injected subcutaneously into the left and right flanks of the BALB/c nu/nu mice. Tumours were allowed to establish for 4 days before commencing drug treatments.

2.9.3 Drug treatment of mice

All drugs were prepared in the laboratory prior to transport to the animal facility. A full description of the drugs used, their concentrations, vehicles and methods of delivery are listed in the Table 7. Mice were randomized into 4 groups to receive Vehicle only (DMSO Intra-peritoneal injection and Phosal50 mixture gavage), ABT-263, Vorinostat or the combination of ABT-263 plus Vorinostat. Mice injected every day for 5 days with a 2 day break in between.

2.9.4 Monitoring of mice and tumour measurements

Mice were weighed daily and monitored every day for changes in physical appearance or activity. Tumours were measured by a single investigator (Mr. Ian Luk) blinded to the identity of the mouse throughout the duration of the study. At the end of the study mice were culled by Isolfurane overdose (ABBOTT, AUSTRALIA) followed by cervical dislocation. Tumours were excised, photographed, measured and weighed, and subsequently paraffin-embedded for future IHC analysis and parts were snap frozen for further analysis.

Chapter 3: Determination of the role of c-FOS, c-JUN and ATF3 in HDACi-induced apoptosis

3.1 Introduction

Our laboratory previously identified a 48 gene signature which was preferentially induced by HDACi treatment in colorectal cancer cell lines that were sensitive to these agents [311]. This gene signature was enriched for immediate-early genes, including several members of the AP-1 transcription factor complex (*c-FOS, c-JUN* and *ATF3*) and stress-response genes (*Gadd45B, Ndrg4, Mt1B, Mt1E, Mt1F, Mt1H,* and *MtIX*).

To determine if the induction of these genes was linked to HDACi-induced apoptosis independent of tumour type, our laboratory performed a screen of 50 cancer cell lines derived from a range of haematological and solid tumours, for their apoptotic responses to Vorinostat. This identified multiple HDACi-sensitive and resistant cell lines from various tumour types. As observed in colorectal cancer cells, Vorinostat induced mRNA expression of the immediate early genes c-FOS, c-JUN, ATF3, EGR1 and EGR3, and the stress-response gene GADD45B in multiple HDACi-sensitive cell lines derived from a range of tumour types, including melanoma, breast, lung adenocarcinoma, gastric, CTCL and multiple myeloma. Furthermore, the magnitude of induction of c-FOS, c-JUN and ATF3, but not EGR1, EGR3 or GADD45B, correlated significantly with the magnitude of apoptosis induction across the 50 cell lines.

Having demonstrated that HDACi preferentially induce mRNA expression of c-FOS, c-JUN and ATF3 in HDACi sensitive cell lines [309], we first sought to confirm these findings and determine if their induction was also evident at the protein level. Secondly, we sought to determine whether induction of these genes was observed in response to treatment with HDACi from multiple different chemical classes. Thirdly, we sought to determine the mechanism by which HDACi induce expression of these genes in diverse tumour types. Finally, and most importantly, the aim was to determine whether HDACi-induction of c-FOS, c-JUN and ATF3 was a direct requirement for HDACi-induced apoptosis and to investigate this question in cell lines derived from multiple tumour types.

3.2 Results

3.2.1 Confirmation of HDACi sensitive and resistant cancer cell lines

A prior screen undertaken in my laboratory assessed sensitivity to HDACi-induced apoptosis in 50 cancer cell lines derived from various tumour types, and identified multiple HDACi sensitive and resistant cell lines. To confirm those findings, 3 representative sensitive and resistant cell lines were selected from this panel and their differential sensitivity to Vorinostat were determined (Figure 3.1). The HDACi sensitive cell lines selected were a lung cancer line (A549), a gastric cancer line (AGS) and a colorectal cancer line (HCT116), while the HDACi resistant cohort consisted of a pancreatic cancer line (PANC1), a prostate cancer line (PC3) and a glioblastoma line (U87). The six cancer cell lines were selected to cover a broad spectrum of tumour types.

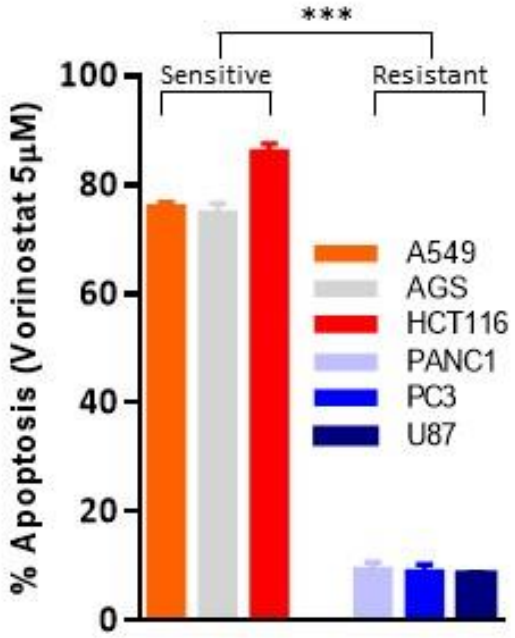

Figure 3.1: Apoptotic sensitivity of 3 representative HDACi sensitive (A549, AGS, HCT116) and resistant (PANC1, PC3, U87) cancer cell lines to 72 hours Vorinostat (5□M) treatment. Apoptosis was determined by propidium-iodide (PI) staining and FACS analysis. Values shown are mean ± SEM from 2 independent experiments, each performed in triplicate. (***P<0.0001)

3.2.2 HDACi induce c-FOS, c-JUN and ATF3 protein expression in multiple tumour types

Studies from my laboratory also established a statistically significant correlation between the magnitude of HDACi-induced apoptosis and the magnitude of HDACi-induction of c-FOS, c-JUN and ATF3 mRNA expression across the cell line panel. To confirm this finding at the protein level, the effect of HDACi treatment on c-FOS, c-JUN and ATF3 protein expression was examined in the three representative HDACi sensitive cell lines. A549, AGS and HCT116 were treated with Vorinostat for 8 to 48 hours and protein expression determined by Western blot. Consistent with the previously observed induction of mRNA expression of these genes, Vorinostat induced a sustained induction of c-FOS, c-JUN and ATF3 protein in all 3 cell lines (Figure 3.2).

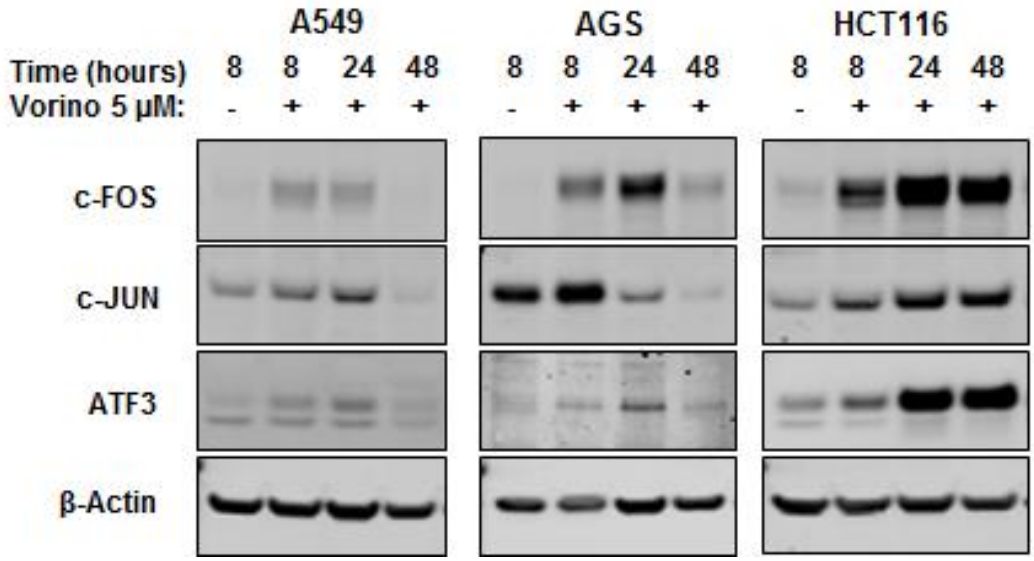

Figure 3.2: Induction of c-FOS, c-JUN and ATF3 protein in response to Vorinostat (Vorino) treatment in 3 HDACi sensitive cell lines derived from different tumour types.

Next, to determine whether induction of these proteins occurred preferentially in cell lines sensitive to HDACi, we selected 2 representative HDACi sensitive (A549 and HCT116) and 2 representative HDACi resistant (PANC1 and PC3) cancer cell lines to analyse HDACi-induced c-FOS, c-JUN and ATF3 protein induction. All 4 cell lines were treated with Vorinostat for 24 hours and the induction of the three targets were examined by western blot. The induction of c-FOS, c-JUN and ATF3 protein upon Vorinostat treatment was observed overall in the 2 HDACi sensitive lines, with

induction of only one of the three genes (c-JUN) observed in one of the resistant cell lines (PANC1) (Figure 3.3).

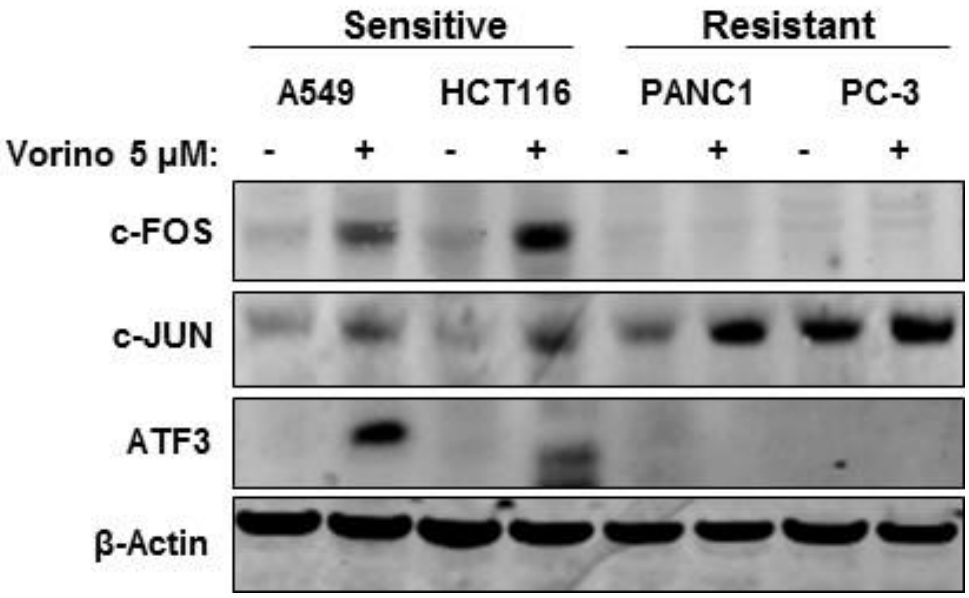

Figure 3.3: Induction of c-FOS, c-JUN and ATF3 protein following 24 hour Vorinostat (Vorino) treatment in representative HDACi sensitive (A549 and HCT116) and resistant (PANC-1 and PC-3) cancer cell lines.

3.2.3 Effect of other HDACi on c-FOS, c-JUN and ATF3 mRNA induction

Next, we also wanted to determine if the induction of c-FOS, c-JUN and ATF3 mRNA expression in response to HDACi treatment occurred in response to treatment with different structurally distinct classes of HDACi. To address this, the HH CTCL cell line, for which these drugs are approved for, was treated with HDACi belonging to each major chemical class across a range of concentrations and induction of c-FOS, c-JUN and ATF3 mRNA determined by qRT-PCR. c-FOS mRNA levels were induced 8-2000 fold, c-JUN mRNA levels were induced from 2-128 fold and ATF3 mRNA levels were induced from 2-16 fold, following treatment with the highest doses of the hydroxamic acids Panobinostat, Belinostat and TSA, the cyclic tetrapeptide Depsipeptide, the benzamide MS275 and the short-chain fatty acid VPA. In each case, gene expression occurred in a concentration-dependent manner (Figure 3.4).

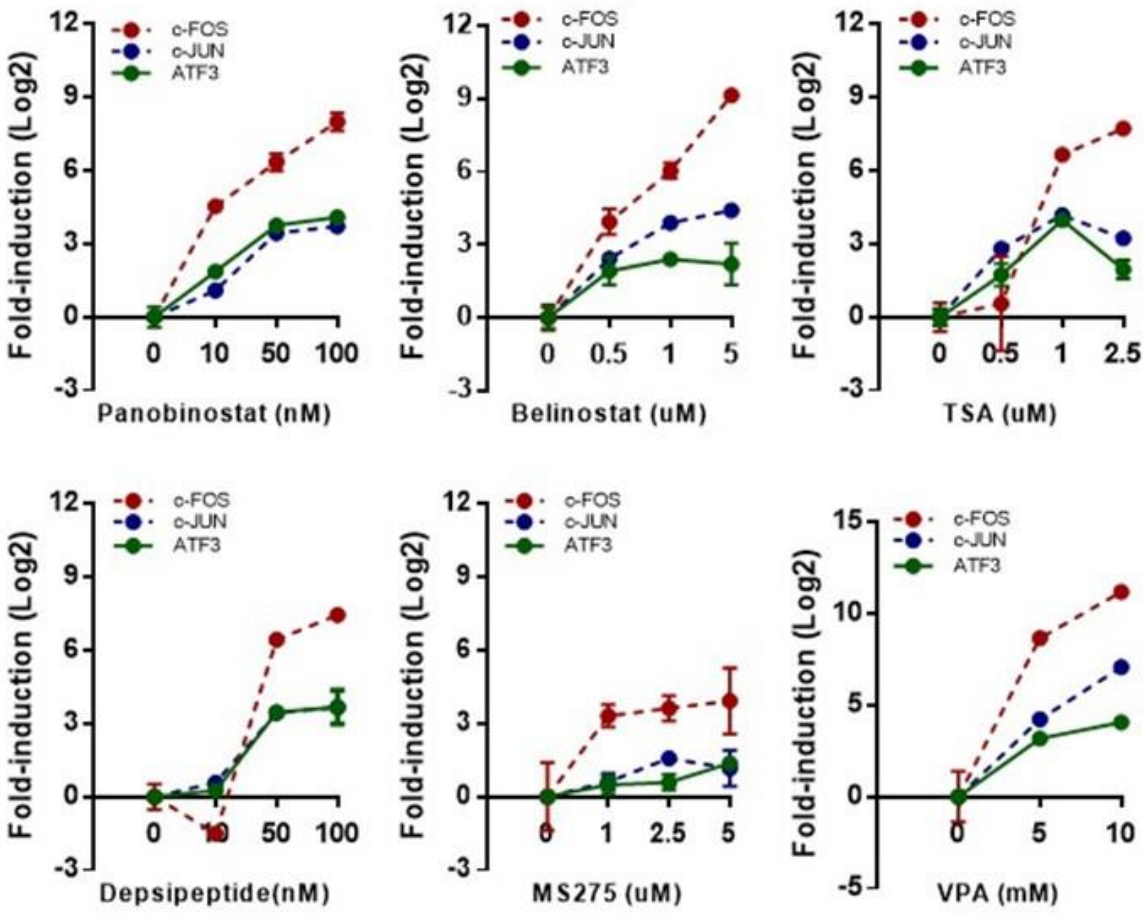

Figure 3.4: Effect of the HDACi Panobinostat, Belinostat, trichostatin A (TSA), Depsipeptide, MS-275 and valproic acid (VPA on c-FOS, c-JUN and ATF3 mRNA induction in the CTCL cell line, HH. Cells were treated with each HDACi for 24 hours and gene expression determined by q-RT-PCR. Values shown are mean ± SEM from 3 independent experiments, each performed in triplicate.

3.2.4 The differential induction of c-FOS, c-JUN and ATF3 between sensitive and resistant cell lines is not due to differences in HDAC inhibition

We next determined whether the mechanistic basis for the differential induction of c-FOS, c-JUN and ATF3 between sensitive and resistant cell lines was a consequence of differences in the extent of HDAC inhibition. To address this, the 3 sensitive and resistant cell lines investigated above were treated with Vorinostat for 24 hours and the extent of histone acetylation measured by western blot using a pan-acetyl Histone H3 antibody (Figure 3.5A). Histone H3 acetylation was induced to a similar extent following Vorinostat treatment in both sensitive and resistant lines (p-value=0.932) (Figure 3.5B). These findings demonstrate that the differential induction

of c-FOS, c-JUN and ATF3 between sensitive and resistant cell lines is not due to differential inhibition of HDAC activity.

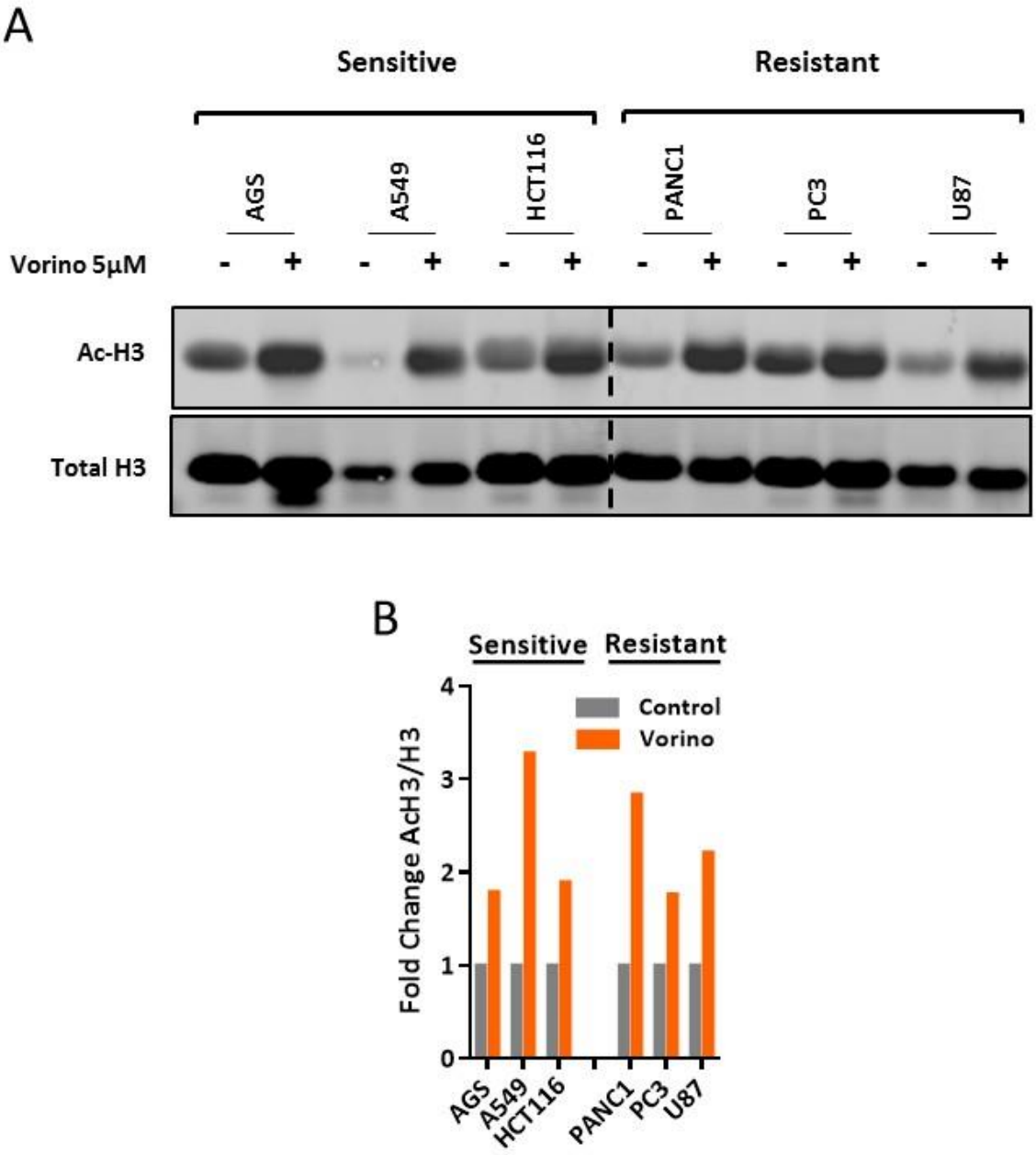

Figure 3.5: (A) Induction of histone acetylation following 24 hours Vorinostat treatment in 4 representative sensitive and resistant cell lines. Histone acetylation was assessed by extraction of total histones and assessment of pan-acetylated histone H3 (Ac. H3) by Western blot. Separate blots were probed for total histone H3 to control for loading. (B) Densitometric analysis of Ac-H3/H3 ratio.

3.2.5 Induction of ATF3 but not c-FOS or c-JUN is required for HDACi-induced apoptosis

Our studies to-date have established a correlation between the magnitude of induction of c-FOS, c-JUN and ATF3 and HDACi-induced apoptosis, which transcends tumour type. However, whether induction of these genes is directly required for HDACi-induced apoptosis has not been previously investigated. Therefore, to determine if induction of c-FOS, c-JUN or ATF3, are direct drivers of HDACi-induced apoptosis, the expression of each of these genes were knocked-down using pooled siRNAs (containing a pool of 4 individual siRNAs) in the 3 representative HDACi sensitive cell lines, prior to Vorinostat treatment. Western blot analysis demonstrated that transfection with c-FOS, c-JUN and ATF3-targeting siRNAs significantly attenuated Vorinostat-induction of their respective targets in all 3 cell lines (Figure 3.6).

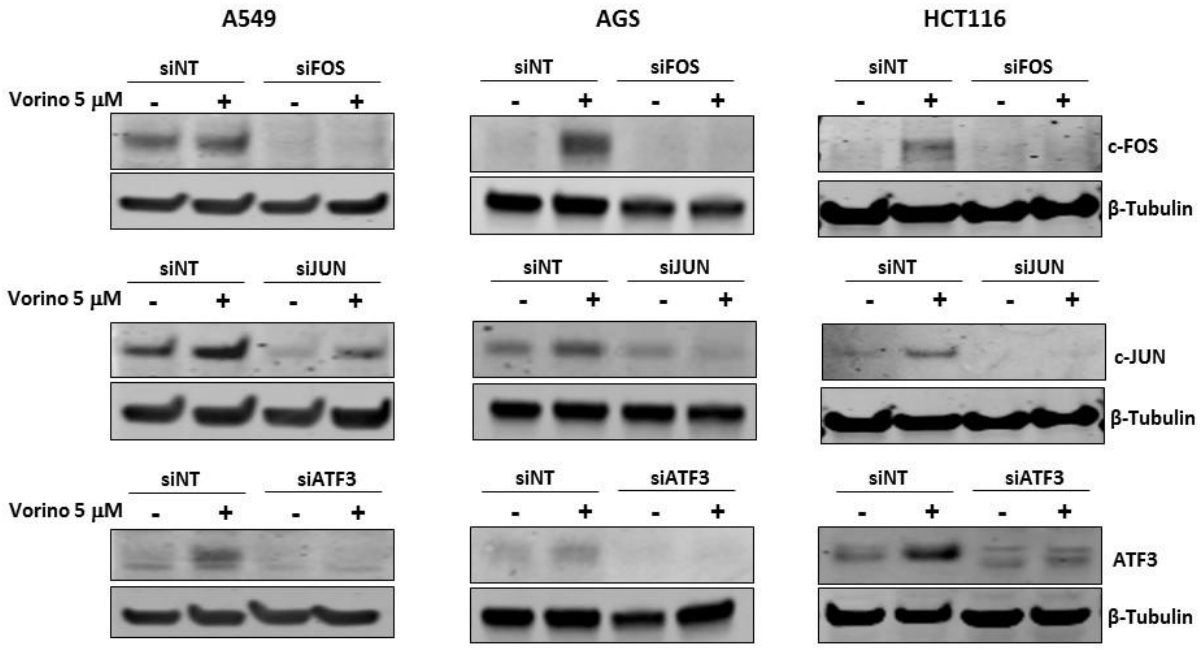

Figure 3.6: Knockdown efficiency of c-FOS, c-JUN and ATF3 in the HDACi sensitive cell lines A549, AGS and HCT116. Cell lines were transiently transfected with a non-targeting siRNA or c-FOS, c-JUN or ATF3-targeting siRNAs for 48 hours, following which they were treated with Vorinostat for 24 hours. Knockdown efficiency of c-FOS, c-JUN and ATF3 protein was determined by Western blot.

The effect of c-FOS, c-JUN and ATF3 knockdown on Vorinostat-induced apoptosis was next investigated in the 3 cell lines. Knockdown of c-FOS failed to inhibit HDACi-induced apoptosis in any of the cell lines. Notably, an enhancement of HDACi-induced apoptosis from 6.93±0.29-fold to 14.40±0.72-fold was observed in the AGS gastric cancer cell line; however, this effect was not observed in the A549 lung cancer or HCT116 colon cancer cell lines (Figure 3.7). Likewise, knockdown of c-JUN had minimal effect on HDACi-induced apoptosis in all 3 cell lines. Conversely, knockdown of ATF3 significantly attenuated HDACi-induced apoptosis in all 3 cell lines (Figure 3.7). In A549 cells HDACi-induced apoptosis was reduced from 15.12±0.24-fold in cells transfected with a non-targeting siRNA to 9.11±1.10-fold following ATF3 knockdown. Similarly, HDACi-induced apoptosis was reduced from 6.93±0.29-fold to 4.83±0.22-fold in AGS cells and from 9.85±1.95-fold to 4.63±0.47-fold in HCT116 cells (Figure 3.7).

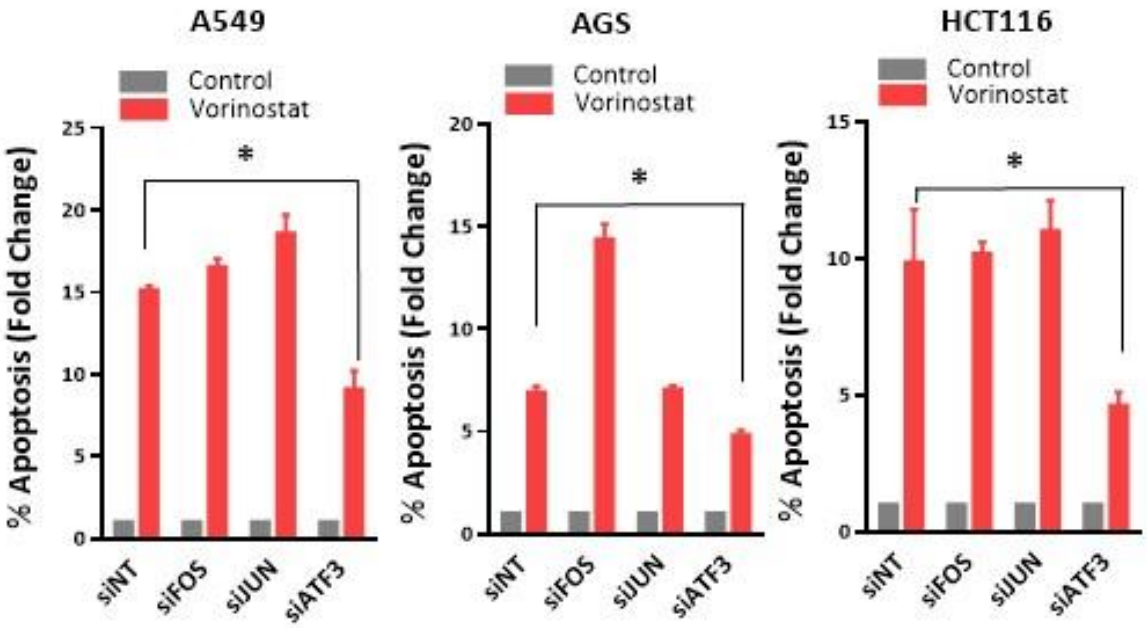

Figure 3.7: Effect of c-*FOS, c-JUN* and *ATF3* knockdown on HDACi-induced apoptosis. The HDACi sensitive cell lines A549, AGS and HCT116 were transiently transfected with a non-targeting siRNA or c-FOS, c-JUN or ATF3-targeting siRNAs for 48 hours, following which cells were treated with 5 µM Vorinostat for 24 hours. Apoptosis was determined by FACS analysis of cells stained with PI. Values shown are mean ± SEM from a representative experiment performed in triplicate. *P<0.05, Student's t-test.

To validate this finding with independent ATF3-targeting siRNAs, we repeated the experiments using the pooled *ATF3* siRNA as well as with 2 individual *ATF3*-targeting siRNAs that were part of the pool. The pool, as well as the 2 individual siRNAs effectively attenuated HDACi-induction of ATF3 in all 3 cell lines (Figure 3.8A). As observed with the pool of ATF3-targeting siRNAs, knockdown of ATF3 using the 2 individual ATF3-targeting siRNAs also significantly attenuated HDACi-induced apoptosis in all 3 cell lines. In A549 cells, the magnitude of HDACi-induced apoptosis was reduced from 23.60±0.70-fold in cells transfected with a non-targeting siRNA to 19.07±0.98-fold, 12.63±1.27-fold, and 9.70±0.50-fold following transfection with the pool, ATF3 #1 and siATF3 #2, respectively. Similarly, in AGS cells, apoptosis was attenuated from 21.32±0.32-fold in cells transfected with a non-targeting siRNA to 9.30±2.15-fold, 7.26±0.31-fold and 10.00±0.46-fold following transfection with the pool, ATF3 #1 and ATF3 #2 respectively, while finally in HCT116 cells apoptosis was attenuated from 6.93±0.28-fold to 4.83±0.22-fold, 3.87±0.34-fold, and 4.20±0.50-fold respectively (Figure 3.8B).

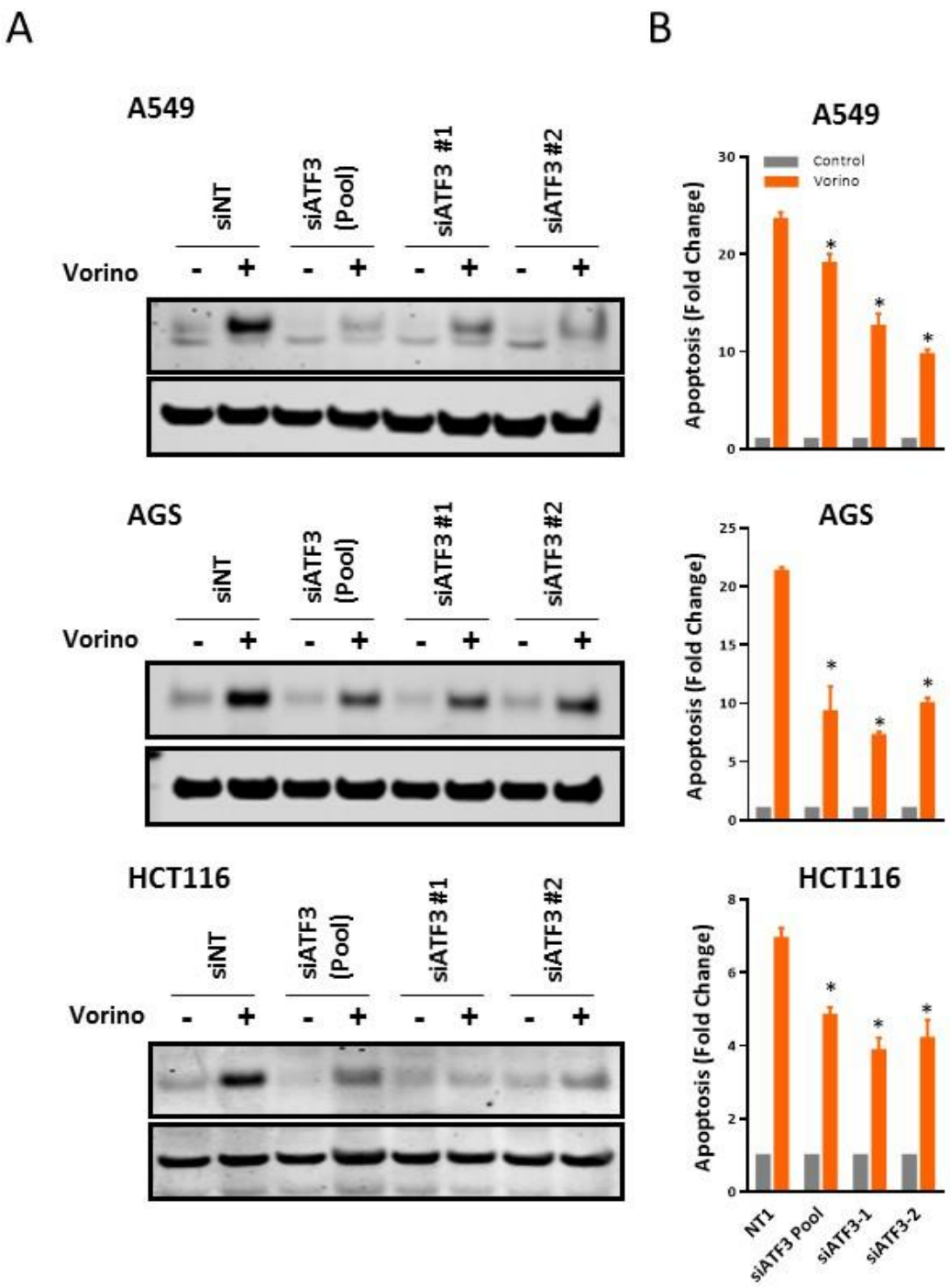

Figure 3.8: Effect of ATF3 knockdown with a pool of 4 ATF3-targeting siRNAs or 2 individual ATF3-targeting siRNAs, on HDACi-induced apoptosis. The HDACi-sensitive cell lines A549, AGS and HCT116 were transiently transfected with a non-targeting siRNA, a pool of 4 ATF3-targeting siRNAs (siATF3-pool) or 2 individual ATF3 targeting siRNAs, siATF3 #1 and siATF3 #2 for 48 hours followed b treatment with Vorinostat 5 µM for 24 hours. (A) Assessment of knockdown efficiency of ATF3 protein expression by Western blot. (B) Assessment of apoptotic responses by FACS analysis of PI stained cells. Values shown are represented mean ± SEM from 3 independent experiments, each performed in triplicate. *P<0.05, Student's t-test.

3.2.6 ATF3 is required for HDACi-induced apoptosis of mouse embryonic fibroblasts

To confirm the role of ATF3 in HDACi-induced apoptosis using an independent model system, we used mouse embryonic fibroblasts (MEFs) derived from wild-type (WT) and ATF3 knockout (ATF3$^{-/-}$) mice. We first treated WT and ATF3$^{-/-}$ cells with Vorinostat and measured the induction of mouse *ATF3* mRNA expression by qRT-PCR. Vorinostat induced ATF3 mRNA expression 2.3-fold in WT MEFs but as expected, failed to induce ATF3 mRNA expression in ATF3$^{-/-}$ cells (Figure 3.9A). We next examined the effect of HDACi treatment on apoptosis induction in the WT and ATF3$^{-/-}$ lines. Cells lacking ATF3 underwent significantly reduced apoptosis following treatment with Vorinostat or the short-chain fatty acid-based HDACi, sodium butyrate, compared to WT cells. The attenuated apoptotic response was observed across a range of concentrations of Vorinostat (2.5-20 µM) (Figure 3.9B) and sodium-butyrate (2.5-20mM) (Figure 3.9C).

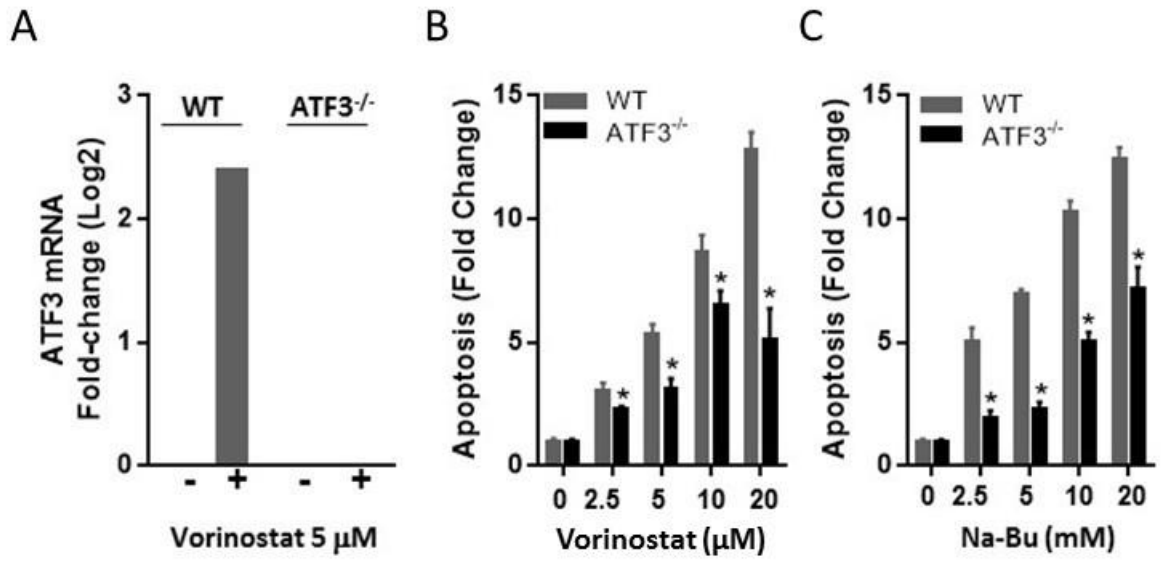

Figure 3.9: Effect of HDACi-induced apoptosis in ATF3$^{-/-}$ MEFs. (A) Induction of ATF3 mRNA following Vorinostat treatment of WT and ATF3$^{-/-}$ MEFs. Cells were treated with Vorinostat for 24 hours and ATF3 mRNA expression assessed by q-RT-PCR. (B-C) Apoptotic response to increasing doses of (B) Vorinostat or (C) sodium butyrate (NaBu). Cells were treated with Vorinostat or NaBu for 72 hours and apoptosis determined by PI staining and FACS analysis. Values shown are mean ± SEM from 3 independent experiments, each performed in triplicate. *P<0.05, Student's t-test.

3.3 Discussion

HDACi induce a range of anti-tumour effects on cancer cell lines including growth arrest, autophagy, differentiation and apoptosis in multiple cancer types [10, 44, 116], and are an established treatment option for CTCL and more recently for multiple myeloma [117-121]. The clinical activity of these agents also continues to be researched and tested in trials, mostly in combination, in a range of tumour types, including solid tumours where their single agent activity is limited.

We undertook this study to investigate the mechanisms of action of HDACi in order to subsequently facilitate the identification of predictive biomarkers of HDACi response, which is required to enhance the clinical use of these agents. We previously demonstrated that HDACi-induced apoptosis in HDACi-sensitive cells is associated with a specific transcriptional response involving the upregulation of multiple members of the AP-1 complex, specifically c-FOS, c-JUN and ATF3. We are now able to demonstrate that HDACi induction of this transcriptional response robustly occurs in multiple tumour types, significantly correlates with apoptotic sensitivity, and occurs independent of tumour type.

Of these three AP-1 genes we also now establish a direct role for the basic-region leucine zipper (bZIP) transcription factor ATF3 in mediating HDACi-induced apoptosis in multiple tumour cell types. Our finding that ATF3 plays a pro-apoptotic role in HDACi-induced apoptosis is consistent with a number of previous observations. First, ATF3 overexpression alone has been shown to induce apoptosis in prostate [384] and ovarian cancer cells [385], while fibroblasts derived from ATF3 knockout mice are refractory to UV-induced apoptosis [386]. ATF3 induction is also required for apoptosis triggered by stimuli including ER stress [387], anoxia [388], and the cytotoxic agents 5FU [389], Etoposide, Camptothecin [390] and Cisplatin [391]. Our finding that HDACi induced ATF3 expression is also consistent with a report by Liu *et al* [392]. Lui *et al* went on to further demonstrate that the induction of ATF3 by HDACi drove subsequent expression of death receptor 5 (DR5), and sensitised cells to an agonistic anti-DR5 antibody. Furthermore, this sensitisation was attenuated by knockdown of ATF3 [392]. Consistent with the current findings, ATF3 induction by HDACi has also been observed in breast, prostate, ovarian and lung cancer cell lines [391, 393], and HDACi have

been shown to further enhance Cisplatin-induced ATF3 expression [391]. Notably, the enhanced apoptotic effect induced by combination treatment of A549 lung cancer cells with cisplatin and the HDACi M344 was also partially attenuated by ATF3 knockdown [391]. Finally, during the course of these studies, Sooraj *et al* demonstrated that the HDACi Pracinostat induces ATF3 expression in bladder cancer cell lines, and that ATF3 knockdown attenuated HDACi-induced apoptosis [393]. However, whether the magnitude of ATF3 induction correlated with the extent of apoptosis was not investigated. Furthermore, the downstream mechanisms by which ATF3 induction drives apoptosis was not investigated, a key question that we address in the following chapter.

Therefore, in the current study we build on a number of prior findings by demonstrating a statistically significant correlation between the magnitude of ATF3 induction and HDACi-induced apoptosis across multiple cell lines derived from a wide range of tumour types. Furthermore, direct evidence that ATF3 induction is required for HDACi-induced apoptosis is demonstrated through the resistance of ATF3-deficient MEFs to HDACi-induced apoptosis and the attenuation of HDACi-induced apoptosis following ATF3 knockdown in multiple cell lines. The underlying reason to why there is a difference in HDACi-induction of c-FOS, c-JUN and ATF3 between HDACi sensitive and resistant lines is yet to be defined. To investigate this, our lab also previously demonstrated that the promoters of c-FOS, c-JUN and ATF3 are GC rich and harbor multiple binding sites for the Sp1/Sp3 family of transcription factors [394]. It was also established that Sp1 and Sp3 physically localised to these promoters in colorectal cancer cells, and that HDACi induction of these genes could be inhibited using the Sp1 and Sp3 inhibitor Mithramycin, or by siRNA-mediated knockdown of Sp1 and Sp3 [394].

While these findings demonstrate a key role for Sp1 and Sp3 in mediating HDACi-induced ATF3 expression, multiple other stimuli which can induce ATF3 expression, which have been previously explored. These include p53-mediated induction in response to UV radiation [395], MAPK signalling-mediated induction in response to cisplatin treatment [396] and ATF4-mediated induction subsequent to activation of the ER stress / unfolded protein response pathway [387]. These findings raise the possibility that combining HDACi with agents which stimulate ATF3 expression

through these alternate pathways may result in additive or synergistic induction of ATF3, and further enhance apoptosis. We directly investigate this in Chapter 5 of this thesis. Collectively, the results from this study demonstrate that HDACi-induced apoptosis is dependent on the induction of ATF3, and that this effect is independent of tumour cell type.

Chapter 4: HDACi-induction of ATF3 drives apoptosis through repression of the pro-survival protein BCL-X_L

4.1 Introduction

HDACi-induced apoptosis has been linked to activation of both the intrinsic (mitochondrial) and extrinsic (death receptor) apoptotic pathways, however, the majority of studies implicate activation of the intrinsic pathway as the major driver of HDACi-induced apoptosis [199-202]. HDACi have been shown to activate the intrinsic apoptotic pathway by changing the balance in expression of pro- and anti-apoptotic genes in favour of pro-apoptotic factors. Pro-apoptotic genes induced by HDACi include *TRAIL, DR5, BAX, BAK, BIM* and *APAF1* while anti-apoptotic genes including *BCL-2, BCL-X_L* and *MCL-1* have been shown to be repressed by these agents [43, 163, 198, 203]. However, studies investigating the altered expression of these genes have often been in single cell lines or tumour types, and their altered expression has not been consistently linked to the magnitude of HDACi-induced apoptosis across tumour types. Furthermore, the transcriptional mechanisms by which HDACi regulate changes in expression of these factors has not been systematically investigated.

Having established in the previous chapter that induction of the *ATF3* transcription factor is critical for HDACi-induced apoptosis across tumour types, we next sought to determine if induction of ATF3 mediates apoptosis through altering the expression of pro-and or anti-apoptotic components of the intrinsic apoptotic pathway. Specifically, we investigated if HDACi-induction of ATF3 in turn regulated expression of components of the intrinsic apoptotic pathway including the multi-domain proteins BAX and BAK, which alter membrane permeability, the BH3-only proteins BAD, BIK, BID, BIM, BMF, PUMA, and NOXA, which act as sensors of cellular stress leading to alterations in membrane insertion and remodelling, and anti-apoptotic members such as *BCL2, BCL-X_L(BCL2L1), BCL-W (BCL2L2) and MCL-1*. To ensure this was a comprehensive analysis, *APAF-1*, which forms the apoptosis inducing complex with BAX and BAK, was also included in the analysis along with Caspase 9, the initiating caspase in the apoptotic cascade.

4.2 Results

4.2.1 HDACi-induced ATF3 expression and apoptosis inversely correlates with BCL-X_L expression

Work previously undertaken in my laboratory investigated the change in mRNA expression of all components of the intrinsic apoptotic pathway following 24-hour treatment with the HDACi sodium butyrate in 15 cell lines derived from a range of tumour types with a range of sensitivities to HDACi treatment by qRT-PCR (Chueh, Tse et al, under revision in Clinical Cancer Research). We utilized these data to determine if the magnitude of change in expression of any of these genes correlated with the magnitude of induction of ATF3 across 15 cell lines. This analysis identified a significant inverse correlation between the magnitude of induction of ATF3 and the magnitude of repression of the pro-survival gene BCL-X_L (R= -0.553, P= 0.033) (Figure 4.1A). As expected, the magnitude of repression of BCL-X_L also correlated inversely with the magnitude of HDACi-induced apoptosis across the 15 cell lines (R= -0.697, P= 0.004) (Figure 4.1B). These findings identified BCL-X_L as a candidate ATF3 target gene and potential determinant of HDACi-induced apoptosis.

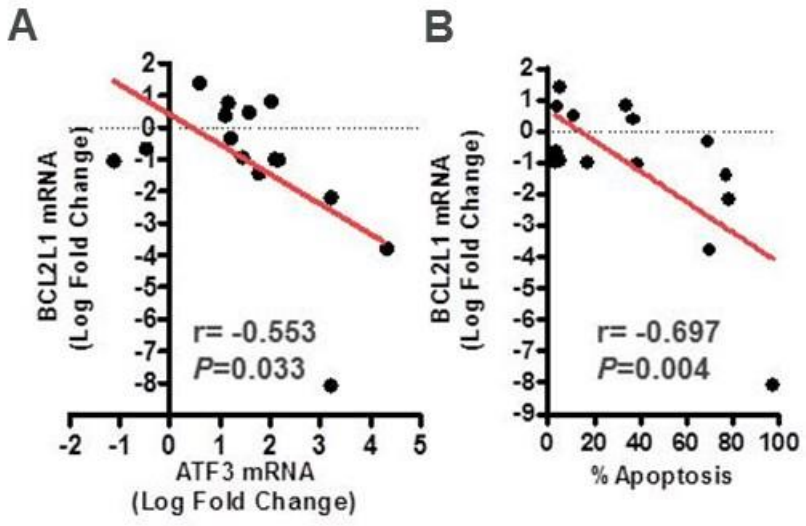

Figure 4.1. (A) Pearson's correlation showing that the magnitude of HDACi-induction of ATF3 mRNA correlates inversely with the magnitude of repression of BCL-X_L mRNA across 15 representative cell lines. (B) Pearson's correlation showing that the magnitude of HDACi-induced apoptosis correlates inversely with the magnitude of repression of BCL-X_L mRNA expression across the 15 representative cancer cell lines.

To confirm the preferential repression of BCL-X_L in HDACi-sensitive cell lines, we examined the effect of Vorinostat on BCL-X_L protein expression in the 3 representative HDACi-sensitive cell lines (A549, AGS and HCT116) and resistant (PANC1, PC3 and U87) cell lines, derived from different tumour types that were investigated in the previous chapter (Figure 4.2A). Consistent with the mRNA expression, BCL-X_L protein was downregulated to a greater extent following Vorinostat treatment in the 3 HDACI-sensitive cell lines compared to the resistant lines. Densitometry analysis revealed this to be a statistically significant difference (P= 0.043) (Figure 4.1B).

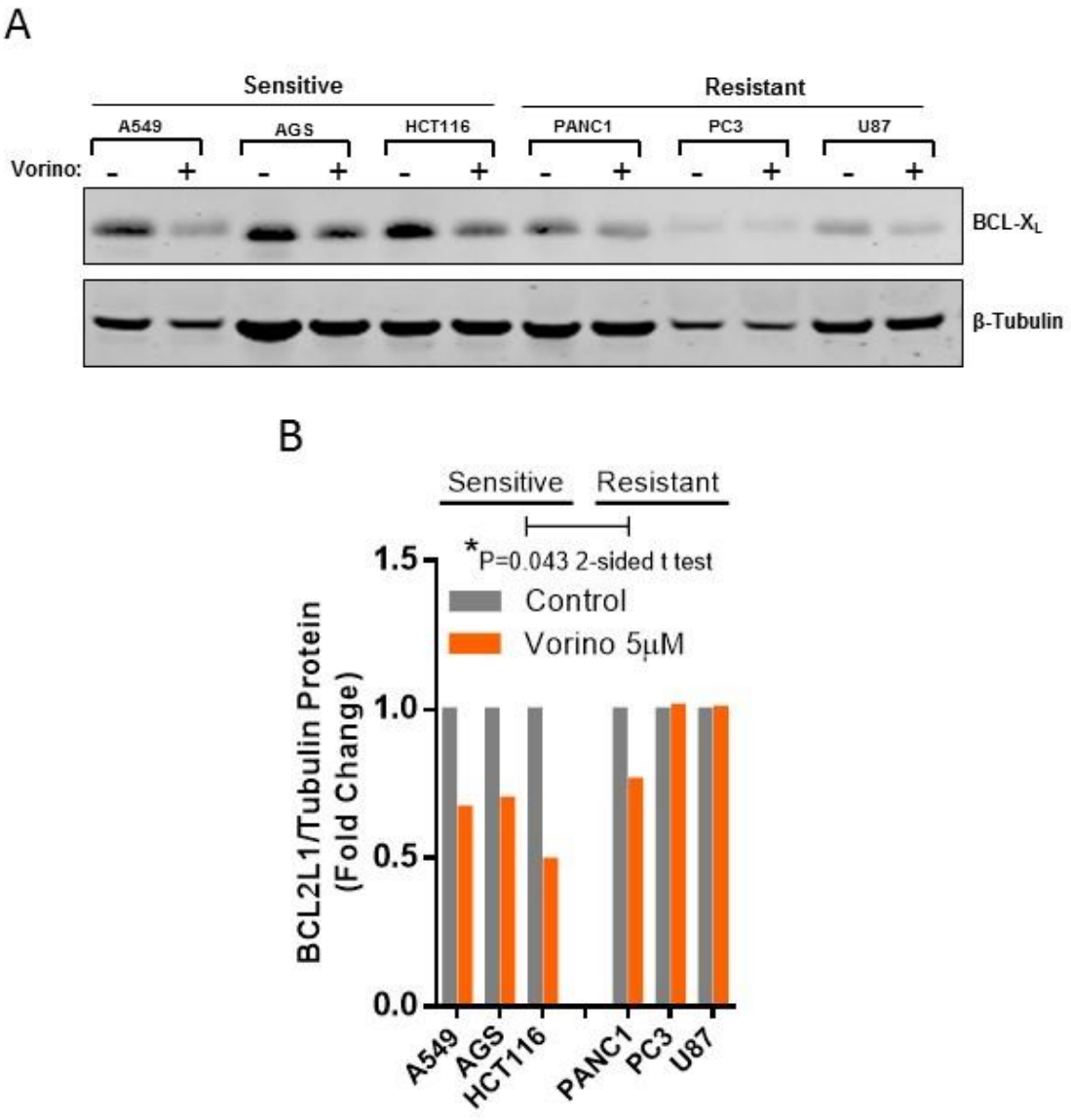

Figure 4.2 (A) Assessment of BCL-X_L protein expression following 24 hours Vorinostat (Vorino) treatment in 3 representative HDACi-sensitive (A549, AGS and HCT116) and resistant (PANC1, PC3 and U87) cell lines. (B) Densitometric analysis of the ratio of expression of BCL-X_L/β-tubulin.

4.2.2 *Knockdown of BCL-X_L promotes HDACi-induced apoptosis in resistant cells*

To determine whether the failure to downregulate BCL-X_L contributes to resistance to HDACi-induced apoptosis, we examined the effect of siRNA-mediated knockdown of BCL-X_L on HDACi-induced apoptosis in the 3 HDACi resistant cell lines PANC1, PC3 and U87. Knockdown efficiency of BCL-X_L-targeting siRNAs was assessed by Western blot and shown to result in a significant downregulation BCL-X_L protein expression in all 3 resistant cell lines (Figure 4.3). As previously observed, HDACi treatment alone resulted in minimal repression of BCL-X_L in the 3 resistant lines.

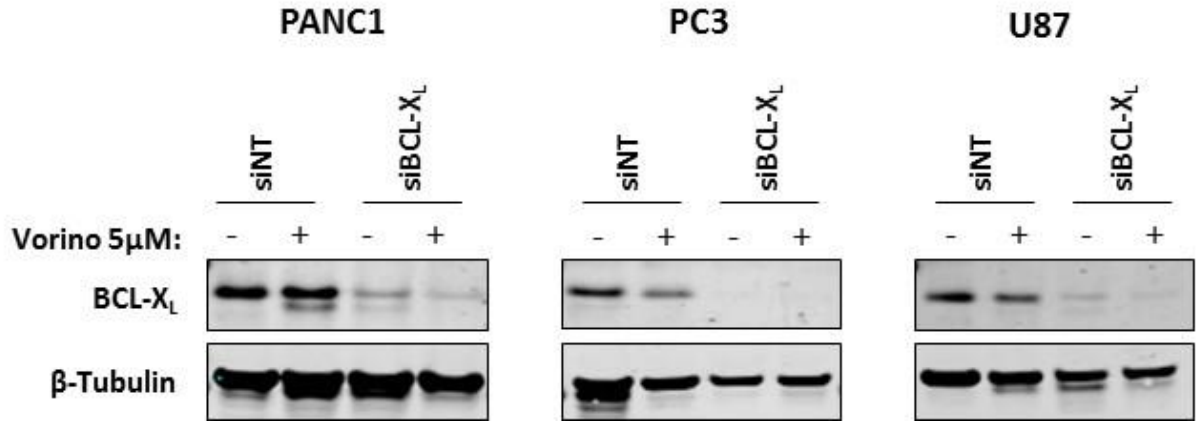

Figure 4.3: Knockdown efficiency of BCL-X_L in PANC-1 (pancreatic), PC3 (prostate) and U87 (glioblastoma) determined by western blot. Cells were transiently transfected with a non-targeting siRNA pool or a pool of 4 BCL-X_L targeting siRNAs for 48 hours. The following day cells were treated with or without Vorinostat (Vorino) for 24 hours.

The effect of BCL-X_L knockdown on basal as well as HDACi-induced apoptosis was subsequently investigated. Knockdown of BCL-X_L alone induced a modest increase in apoptosis compared to cells transiently transfected with a non-targeting siRNA in all 3 resistant cell lines (Figure 4.4). Comparatively, BCL-X_L knockdown enhanced HDACi-induced apoptosis 3 to 8-fold depending on the cell lines. In PANC1 cells, Vorinostat-induced apoptosis increased from 5.52±0.77% to 31.10±0.70% in cells transfected with a non-targeting or BCL-X_L-targeting siRNA respectively. Similarly, Vorinostat-induced apoptosis increased from 6.28±0.57% to 19.23±0.82% in PC3 cells and from

2.68±0.39% to 9.81±0.15% in U87 cells transfected with non-targeting and BCL-X_L siRNAs respectively (Figure 4.4). These findings demonstrate that downregulation of BCL-X_L is a key event in determining the extent of the apoptotic response to HDACi treatment.

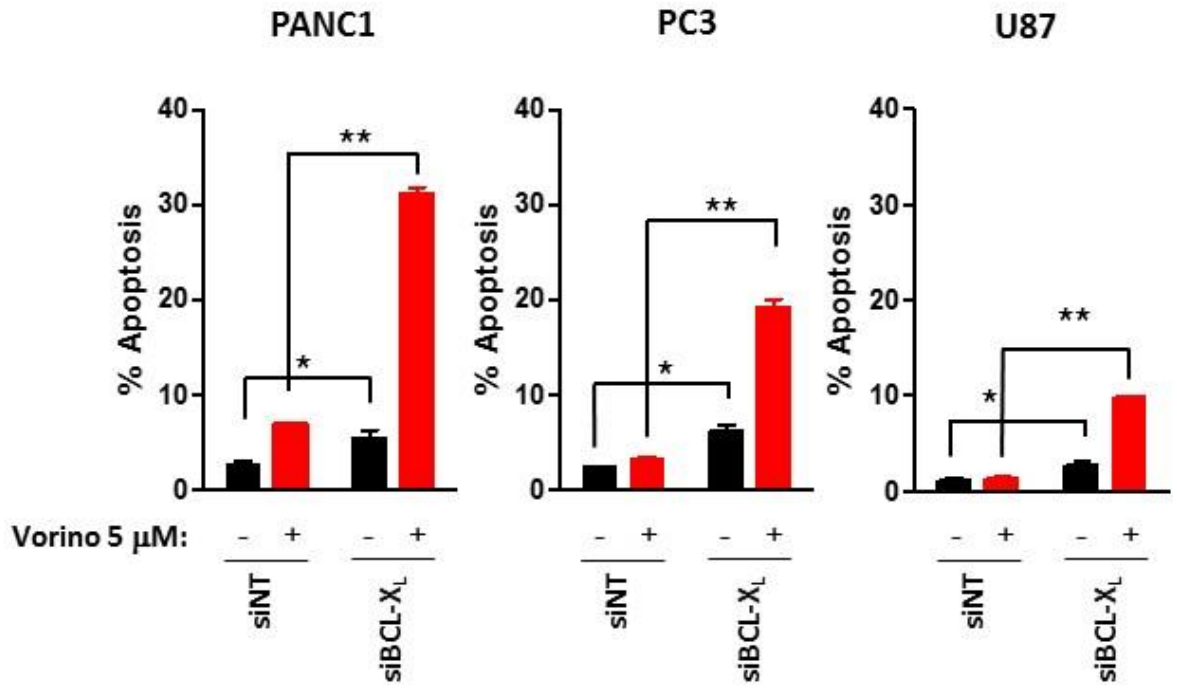

Figure 4.4: Effect of BCL-X_L knockdown on Vorinostat (Vorino)-induced apoptosis. PANC1, PC3 and U87 cells were transiently transfected with a non-targeting siRNA or BCL-X_L-targeting siRNAs for 48 hours. The following day cells were left untreated or treated with Vorinostat for 24 hours. Apoptosis was determined by PI staining and FACS analysis. Values shown are mean ± SEM from 2 independent experiments, each performed in triplicate. *P<0.05 and **P<0.01, Student's t-test.

We further confirmed the importance of BCL-XL in HDACi-induced apoptosis using MEFs in which BCL-X_L was overexpressed. The overexpression of BCL-X_L was first confirmed by Western blot analysis of Flag-Tagged human BCL-X_L (hBCL-XL) protein expression, which was only observed in the overexpressing cell line. Assessment of Vorinostat-induced apoptosis in WT and BCL-X_L overexpressing cells demonstrated that Vorinostat-induced apoptosis was significantly reduced from 49.40±1.50% in WT MEFs to 32.87±0.15% in hBCL-X_L overexpressing MEFs (Figure 4.5). These results

further demonstrate that the repression of BCL-X_L is a key mediator of HDACi-induced apoptosis.

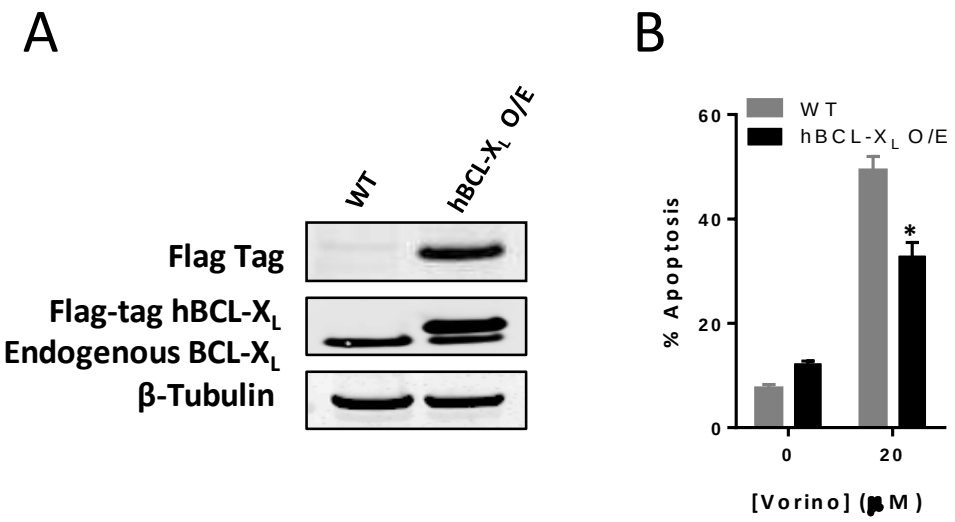

Figure 4.5: Effect of BCL-X_L overexpression on Vorinostat (Vorino)-induced apoptosis. (A) Assessment of BCL-X_L overexpression. (B) Effect on Vorinostat-induced apoptosis in WT and BCL-X_L overexpressing cell lines. Apoptosis was measured by treating cells with Vorinostat for 72 hours followed by PI staining and FACS analysis. Values shown are mean ± SEM from a representative experiment performed in triplicate. *P<0.05, Student's t-test.

4.2.3 HDACi-mediated repression of BCL-X_L is dependent on ATF3 induction

Effect of Vorinostat on BCL-X_L promoter activity

Having established a significant correlation between the magnitude of induction of the ATF3 transcription factor and repression of BCL-X_L in response to HDACi-treatment we next sought to determine whether these events were directly associated. To address this, we first investigated the effect of Vorinostat on reporter activity of a 1261 bp BCL-X_L promoter reporter construct in the 3 HDACi sensitive cell lines, A549, AGS and HCT116. Vorinostat repressed BCL-X_L promoter activity by 17.10±0.02%, 82.80±0.03% and 64.00±0.04% in A549, AGS and HCT116 cells respectively (Figure 4.6).

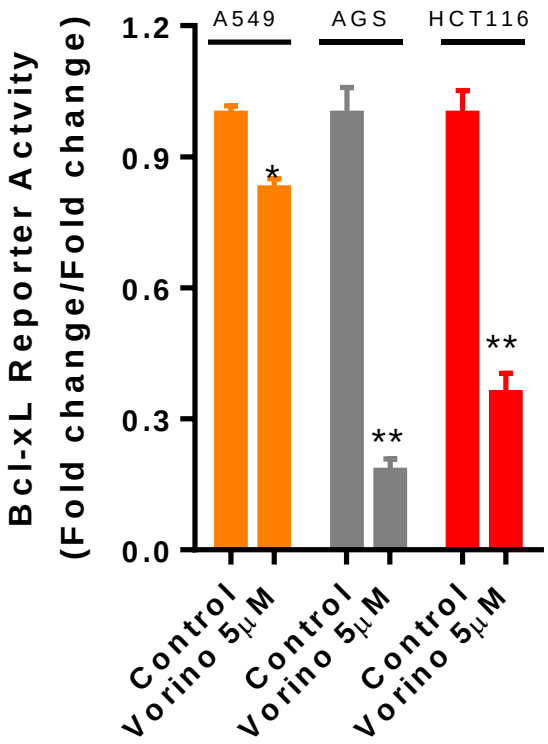

Figure 4.6: Effect of Vorinostat (Vorino) on BCL-X_L promoter activity. A549, AGS and HCT116 cells were transiently transfected with a 1261 bp BCL-X_L promoter-reporter construct and treated with Vorinostat for 24 hours. Reporter activity was determined by assessment of luciferase activity and corrected for total cellular protein. Values shown are mean ± SEM from 3 biological experiments, each performed in triplicate. *P<0.05 and **P <0.01, Student's t-test.

Effect of ATF3 overexpression on BCL-X_L promoter activity

We next determined the effect of ATF3 overexpression alone on BCL-xL promoter activity in the same 3 cell lines. ATF3 overexpression repressed BCL-X_L promoter activity by >80% in all 3 cell lines (Figure 4.7).

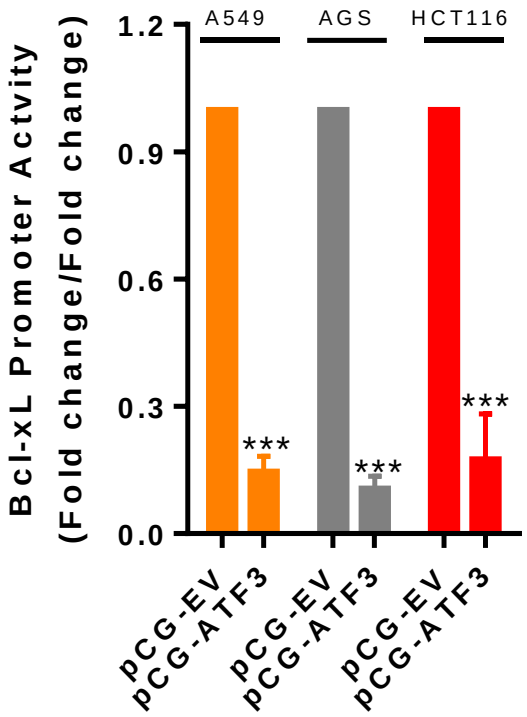

Figure 4.7: Effect of ATF3 overexpression on BCL-X_L promoter activity. A549, AGS and HCT116 cells were transiently transfected with a 1261 bp BCL-X_L promoter-reporter construct, an ATF3 expression vector and TK-Renilla for 24 hours. BCL-X_L reporter activity was determined by assessment of luciferase activity and corrected for TK-Renilla luciferase activity. Values shown are mean ± SEM from 3 independent experiments, each performed in triplicate. ***P<0.001, Student's t-test.

ATF3 is required for HDACi-mediated repression of BCL-X_L

Finally, to directly determine if HDACi-induction of ATF3 is required for subsequent BCL-X_L repression, we examined the effect of ATF3 knockdown on HDACi-mediated BCL-X_L repression in the 3 HDACi-sensitive cell lines. Samples used in these experiments were the same as those used in Chapter 3 (Figure 3.6), in which efficient knockdown of ATF3 was established. Knockdown of ATF3 resulted in marked attenuation of HDACi-mediated repression of BCL-X_L. These findings directly establish that ATF3 induction is required for HDACi-mediated repression of BCL-X_L in multiple cancer cell lines (Figure 4.8)

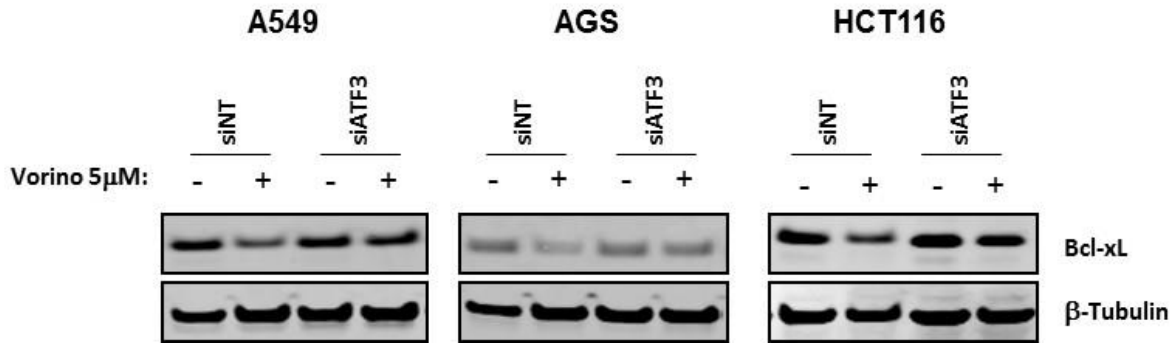

Figure 4.8: Effect of ATF3 knockdown on Vorinostat-mediated repression of BCL-X_L. A549, AGS and HCT116 cells were transiently transfected with a non-targeting or ATF3-targeting siRNAs overnight. The following day cells were treated with Vorinostat for 24 hours, and BCL-X_L protein expression determined by western blot.

4.2.4 Combination treatment with HDACi-inhibitors and BCL-X_L inhibitors overcomes inherent resistance to HDACi-induced apoptosis

Having demonstrated that siRNA-mediated knockdown of BCL-X_L can re-sensitise inherently resistant cell lines to HDACi-induced apoptosis, we next tested whether a similar effect could be induced pharmacologically, by combining HDACi with BCL-X_L inhibitors. To address this, the 3 HDACi resistant cell lines PANC1, PC3 and U87 were treated with Vorinostat alone and in combination with ABT-263 (Navitoclax), a BH3-mimetic which inhibits BCL-X_L, BCL-2 and BCL-w. Combination treatment of the 3 resistant cell lines with Vorinostat and ABT-263 resulted in significantly enhanced apoptosis compared to either agent alone (Figure 4.9). In the PANC1 cells, the combination induced 84.66±4.10% of apoptosis compared to 11.60±1.42% and

39.87±0.18% in cells treated with Vorinostat alone or ABT-263 alone, respectively. In PC3, combination treatment induced 82.47±0.99% apoptosis compared to 5.56±0.11% and 47.43±1.92% in cells treated with Vorinostat alone or ABT-263 alone, respectively. Finally, treatment of U87 cells produced a similar outcome whereby combination treatment induced 52.23±0.60% apoptosis compared to 11.97±0.49% and 19.90±0.31% in cells treated with Vorinostat alone or ABT-263 alone, respectively (Figure 4.9).

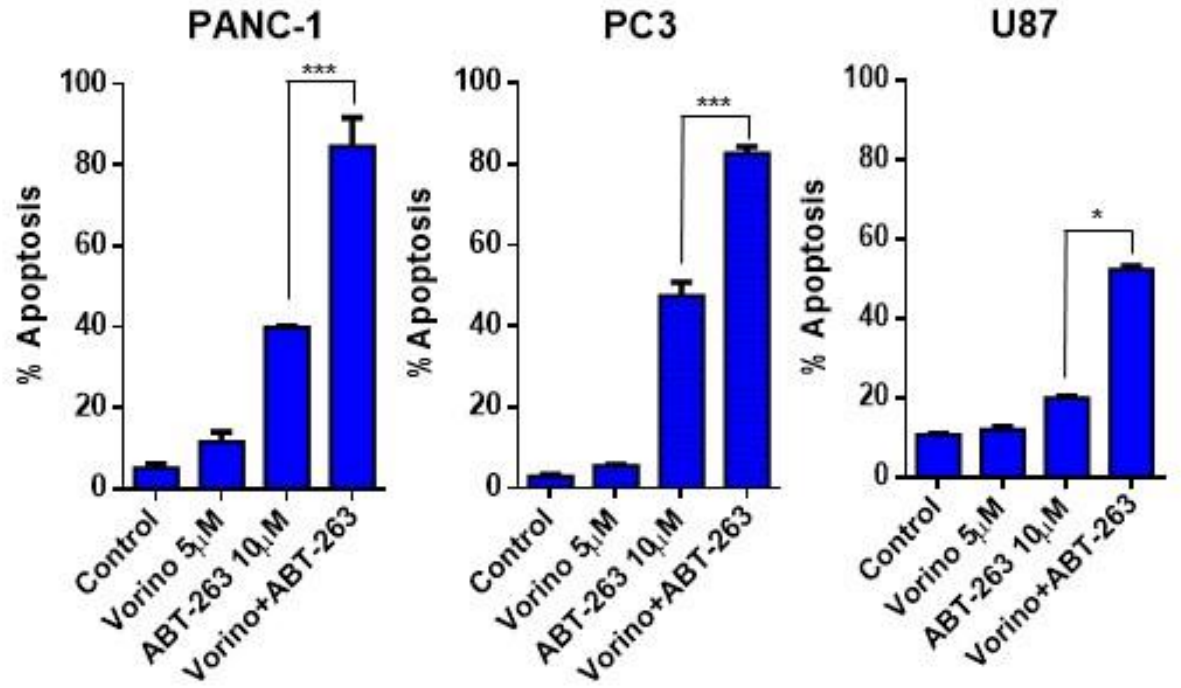

Figure 4.9: Effect of combination treatment with Vorinostat and ABT-263 on apoptosis induction in the HDACi resistant cell lines PANC-1, PC3 and U87. Cell lines were treated with both drugs alone or in combination for 24 hours, and apoptosis determined by PI staining and FACS analysis. Values shown are mean ± SEM from a representative experiment performed in triplicate. *P<0.05 and ***P<0.001, Student's t-test.

We next determined whether the combination of Vorinostat and ABT-263 could also provide an enhanced apoptotic response in HDACi sensitive cell lines, at significantly lower concentrations. To address this, we treated the HDACi-sensitive colorectal cancer cell line HCT116 with Vorinostat at 2.5 µM and ABT-263 at 0.1 µM, which are

doses 2-fold and 10-fold lower doses respectively than the doses used in the resistant cell lines (Vorinostat 5 µM and ABT-263 1 µM). Despite the lower doses used, the combination of Vorinostat and ABT-263 at significantly enhanced apoptosis induction compared to either agent alone, with the combination inducing 66.70±0.52% apoptosis, compared 23.47±1.72% apoptosis induced by Vorinostat alone and 9.11±1.02% by ABT-263 alone (Figure 4.10).

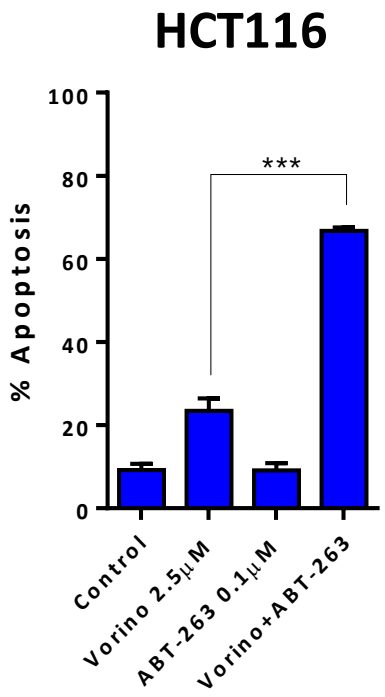

Figure 4.10: Effect of combination treatment with Vorinostat and ABT-263 on apoptosis induction in the HDACi sensitive colon cancer cell line HCT116. Cells were treated with both drugs alone or in combination for 24 hours, and apoptosis determined by PI staining and FACS analysis. Values shown are mean ± SEM from a representative experiment performed in triplicate. ***P<0.001, Student's t-test.

These experiments were repeated using ABT-737, the precursor compound to ABT-263 and pan BCL family-inhibitor. As observed for ABT-263, combination treatment of the 3 resistant cell lines with Vorinostat and ABT-737 induced significantly greater apoptosis than either agent alone (Figure 4.11). Furthermore, treatment of the HDACi-sensitive cell line HCT116 with a 2-fold lower concentration of Vorinostat (2.5 µM) and a 10-fold lower concentration of ABT-737 (1 µM) to that used in the resistant cell lines was sufficient to induce >60% apoptosis (Figure 4.12).

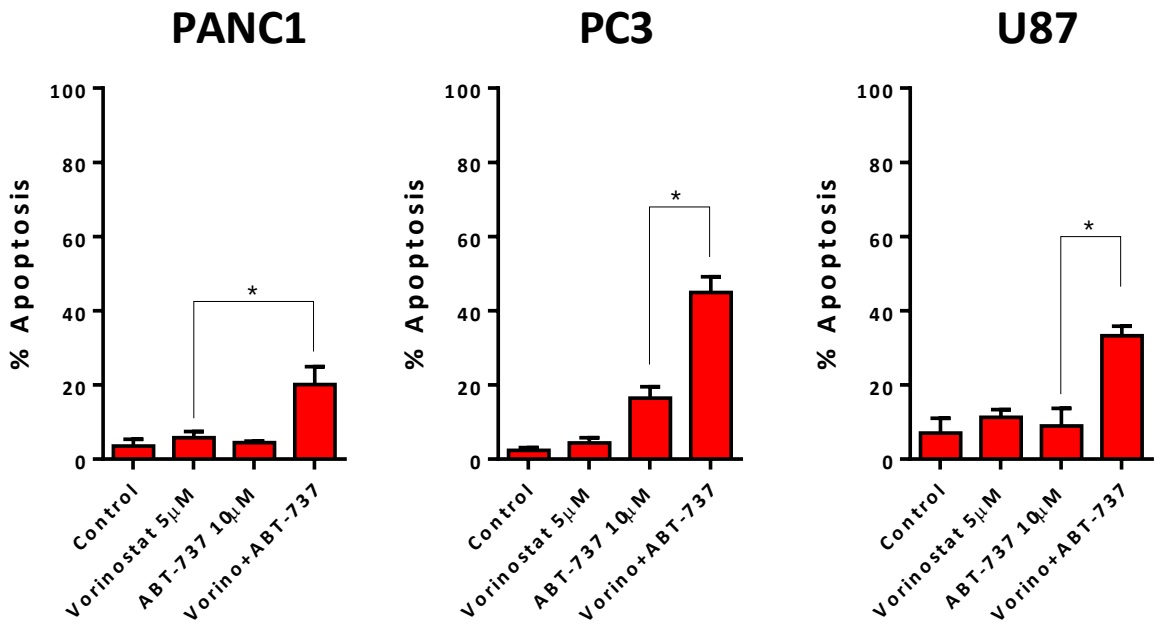

Figure 4.11: Effect of combination treatment with Vorinostat and ABT-737 on apoptosis induction of HDACi resistant cell lines PANC-1, PC3 and U87. Cell lines were treated with both drugs alone or in combination for 24 hours, and apoptosis determined by PI staining and FACS analysis. Values shown are mean ± SEM from 2 independent experiments, each performed in triplicate. *P< 0.05, Student's t-test.

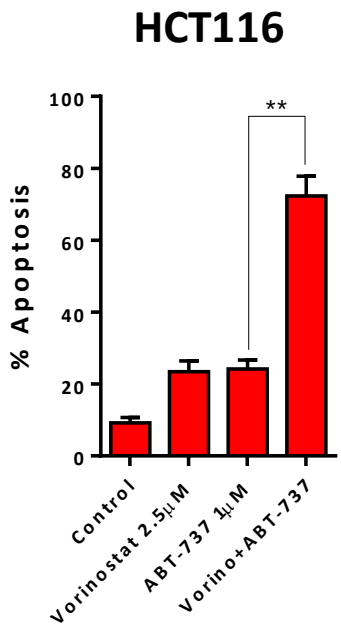

Figure 4.12: Effect of combination treatment with Vorinostat and ABT-737 on apoptosis induction in the HDACi sensitive colon cancer cell line HCT116. Cells were treated with both drugs alone or in combination for 24 hours, and apoptosis determined by PI staining and FACS analysis. Values shown are mean ± SEM from 2 independent experiments. each performed in triplicate. **P< 0.01, Student's t-test.

Effect of combination treatment with Vorinostat and the BCL-X_L-specific inhibitor A1331852 on apoptosis.

ABT-263 and ABT-737 are pan-BCL inhibitors which inhibit BCL-2, BCL-X_L and BCL-w. Therefore, to determine if the enhanced apoptosis induced by combining these agents with HDACi was due to inhibition of BCL-X_L, we determined the effect of combining Vorinostat with the BCL-X_L-specific inhibitor, A1331852. Similar to the effects observed when Vorinostat was combined with ABT-263 or ABT-737, combining Vorinostat with A1331852 significantly enhanced apoptosis induction compared to either agent alone, in all 3 HDACi resistant cell lines (Figure 4.13). In the PANC1 cells, the combination induced 36.24±1.44% apoptosis compared to 8.94±1.79% and 5.67±0.78% apoptosis in cells treated with Vorinostat alone or A1331852 alone, respectively. In PC3, the combination induced 40.44±3.57% apoptosis compared to 8.56±2.41% and 9.23±1.67% apoptosis in cells treated with Vorinostat or A1331852 alone, respectively. A similar result was observed in U87 cells in which the combination treatment induced 24.34±1.43% apoptosis compared to 4.44±0.49% and

10.66±0.80% apoptosis in cells treated with Vorinostat or A1331852 alone, respectively. Furthermore, treatment of the HDACi-sensitive colorectal cancer cell line HCT116 with a 2-fold lower concentration of Vorinostat (2.5 µM) and a 10-fold lower concentration of A1331852 (1 µM) to that used in resistant cell lines was sufficient to induce 79.83%±2.17% apoptosis compared to 13.25±0.51% and 6.62±1.39% apoptosis in cells treated with Vorinostat or A1331852 alone, respectively (Figure 4.14).

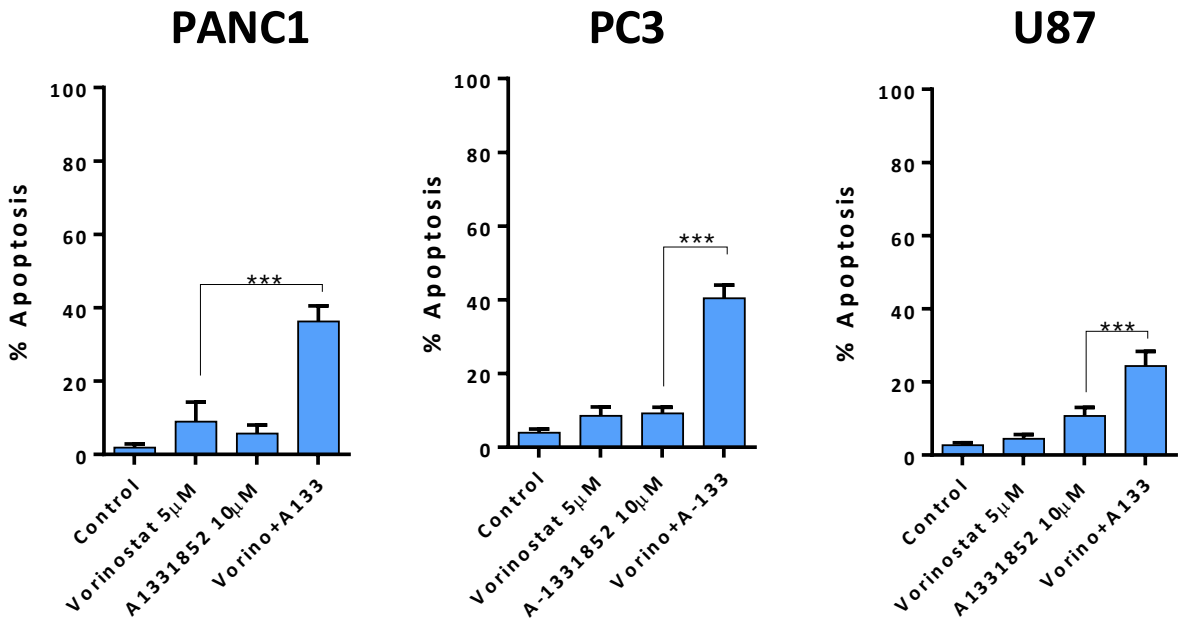

Figure 4.13: Effect of combination treatment with Vorinostat and A1331852 on apoptosis in the HDACi resistant cell lines PANC-1, PC3 and U87. Cell lines were treated with both drugs alone or in combination for 24 hours, and apoptosis determined by PI staining and FACS analysis. Values shown are mean ± SEM from 3 independent experiments, each performed in triplicate. ***P<0.001, Student's t-test.

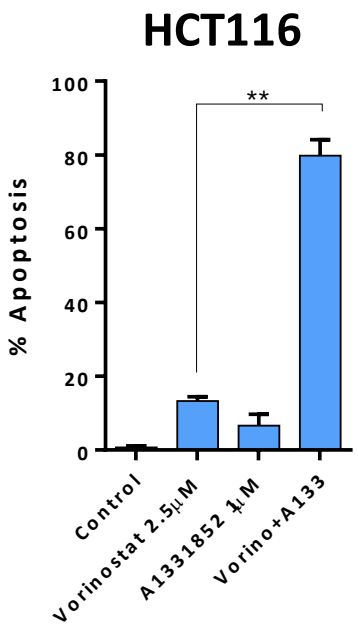

Figure 4.14: Effect of combination treatment with Vorinostat and ABT-737 on apoptosis induction in the HDACi sensitive colon cancer cell line HCT116. Cells were treated with both drugs alone or in combination for 24 hours, and apoptosis determined by PI staining and FACS analysis. Values shown are mean ± SEM from 3 independent experiments, each performed in triplicate. **P< 0.01, Student's t-test.

The BCL-2-specific inhibitor ABT-199 does not enhance HDACI-induced apoptosis.

Finally, we examined the effect of combining Vorinostat with the BCL-2-specific inhibitor, ABT-199 (Venetoclax). In contrast, to the effects observed with the BCL-X_L inhibitor, combining Vorinostat with ABT-199 resulted in minimal to no enhancement in apoptosis induction compared to either agent alone, in all 3 resistant cell lines (PANC1, PC3 and U87) (Figure 4.15). Similar results were obtained in the HDACi sensitive colorectal cancer cell line HCT116, of which lower doses of both agents were used at, to compare efficacy to the BCL-X_L inhibitors (Figure 4.16).

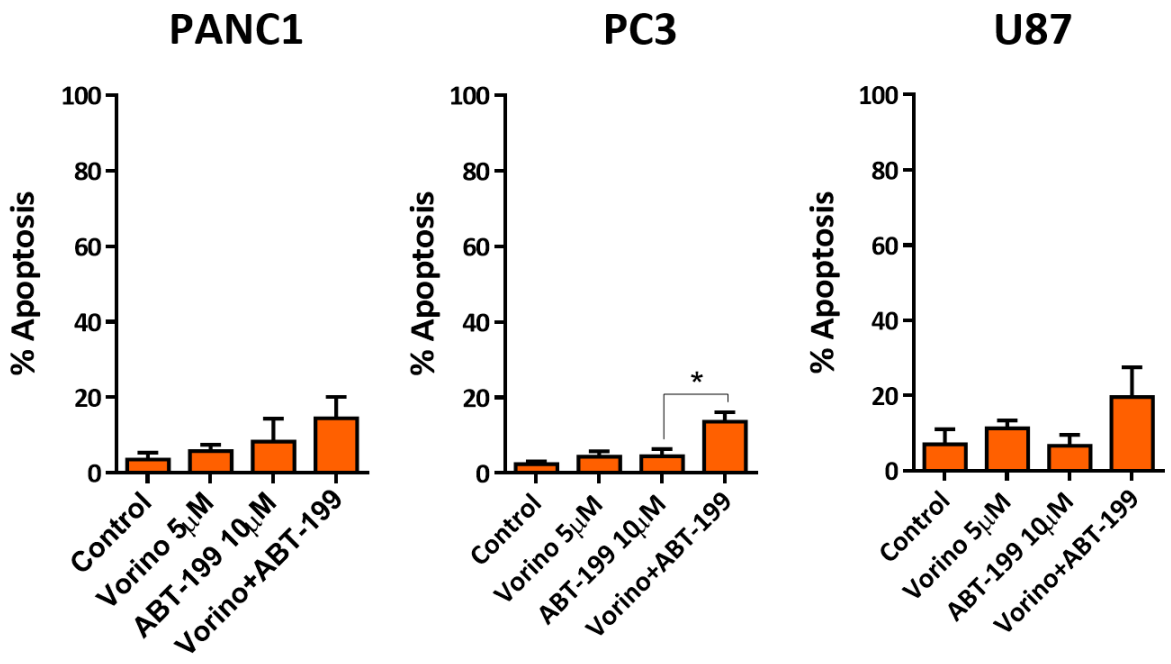

Figure 4.15: Effect of combination treatment with Vorinostat and ABT-199 on apoptosis induction in the HDACi resistant cell lines PANC-1, PC3 and U87. Cell lines were treated with both drugs alone or in combination for 24 hours, and apoptosis determined by PI staining and FACS analysis. Values shown are mean ± SEM from 2 independent experiments, each performed in triplicate. *P<0.05, Student's t-test.

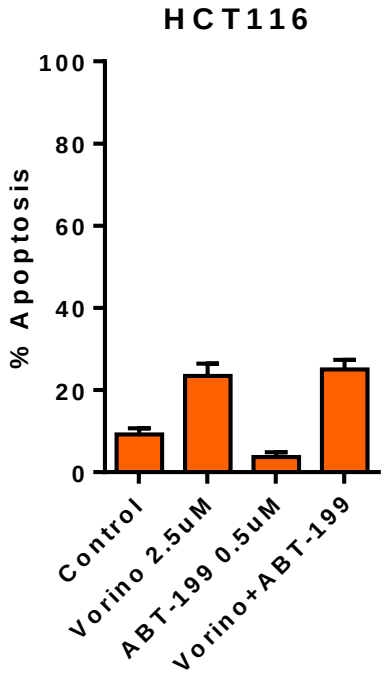

Figure 4.16: Effect of combination treatment with Vorinostat and ABT-199 on apoptosis induction in the HDACi sensitive colon cancer cell line HCT116. Cells were treated with both drugs alone or in combination for 24 hours, and apoptosis determined by PI staining and FACS analysis. Values shown are mean ± SEM from 2 independent experiments, each performed in triplicate.

4.2.5 Combination treatment with Vorinostat and ABT-263 inhibits growth of HDACi resistant tumour xenografts in-vivo

Having demonstrated that the combination of Vorinostat and ABT-263 significantly enhances apoptosis induction of HDACi-resistant tumour cells *in vitro,* we next sought to determine whether this combination could also inhibit tumour growth *in vivo*. To address this, HDACi resistant U87 cells were engrafted into the right and left flank of *Balb/c nu/nu* mice and treatments commenced once the tumours were of a palpable size, which was 4 days after initial engraftment. Mice were treated daily for 5 days, followed by 2 days break, for a total of 19 days. Consistent with the findings *in vitro*, Vorinostat or ABT-263 treatment alone had minimal effect on tumour growth (Figure 4.17A-C). Comparatively, the combination significantly inhibited tumour volume by >50% when assessed by calliper measurements (Combination 56.32±4.16 mm^3 vs Control 131.25±14.52 mm^3), or final tumour weights (Combination 0.010±0.01g vs Control 0.024±0.01g in control). No loss in body weight was observed during the course of the study in any of the groups, with the exception of the first day after IP drug administration where all mice lost weight. Notably, all mice regained their lost body weight over the following 24 hours (Figure 4.18).

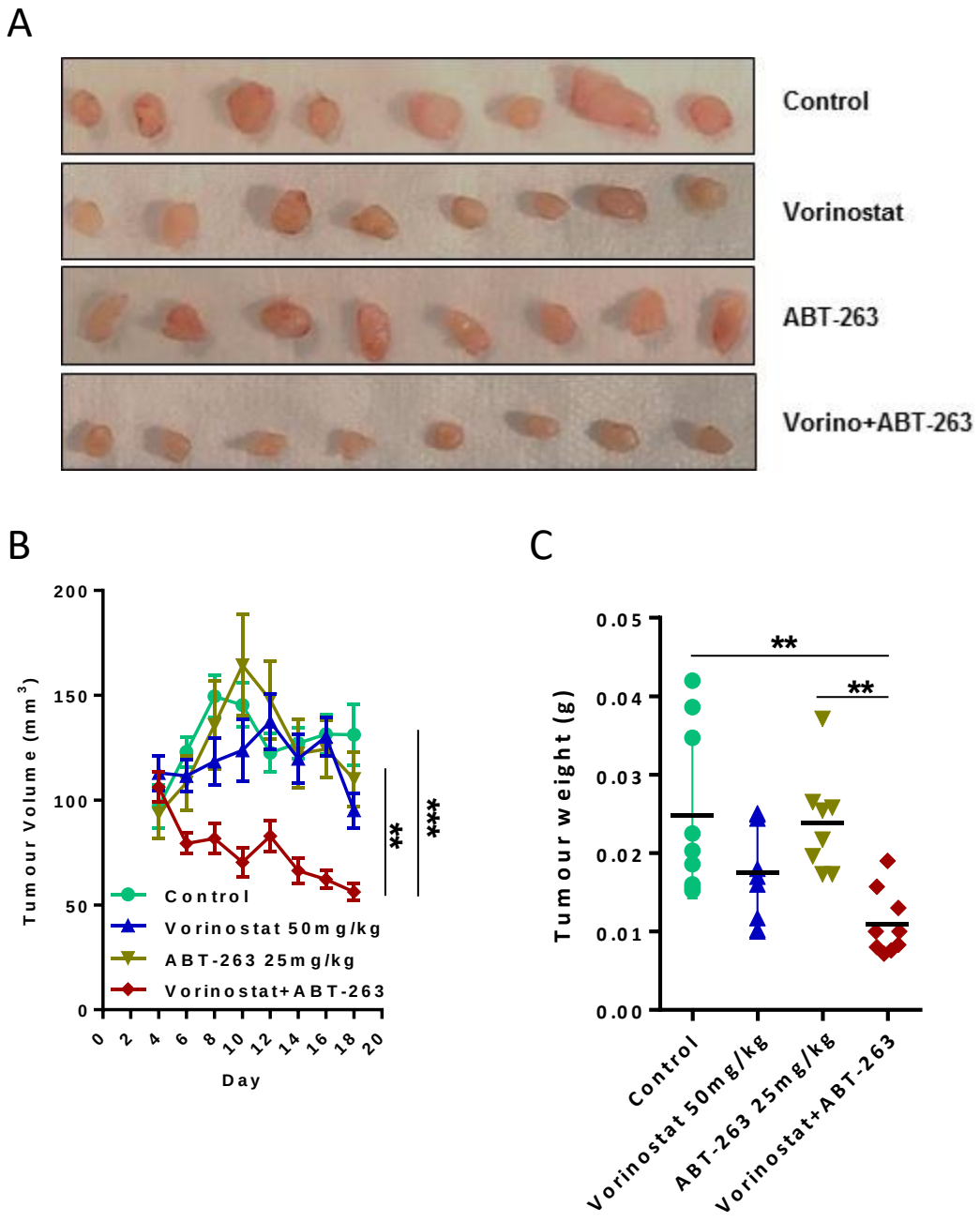

Figure 4.17: Effect of Vorinostat and ABT-263 on tumour growth *in vivo.* HDACi-resistant U87 cells were injected into the right and left flank of *Balb/c nu/nu* mice (day 0). On day 4, mice were randomised to receive vehicle, Vorinostat (50 mg/kg), ABT-263 (25mg/kg), alone or in combination. Mice were treated daily for 5 days followed by a 2-day break for a total of 19 days. (A) Extracted tumours at study completion. (B) Tumour volume measured using calipers. Data represented as mean ± SEM and analysed using Two-way ANOVA with Tukey's post hoc test. (C) Tumour weight at study completion. Data represented are mean ± SEM and analysed using One-way ANOVA with Tukey's post hoc test. **P<0.01 and ***P < 0.005.

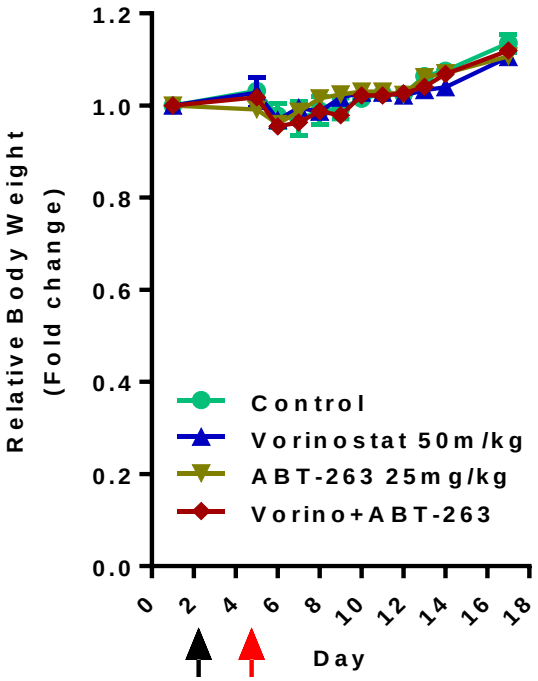

Figure 4.18: Effect of Vorinostat and ABT-263 treatment on change of body weight relative to starting weight, over the study period. Data presented are the mean ± SEM. Black arrow indicates day of tumour injection and red arrow indicates the start of drug treatments.

4.3 Discussion

In the previous chapter, we established a key role for induction of the ATF3 transcription factor in driving HDACi-induced apoptosis. However, the precise mechanism by which ATF3 induction regulates apoptosis remains unknown. A number of previous studies have demonstrated HDACi-induced apoptosis involves the altered expression of various regulatory components of the intrinsic apoptotic pathway across a range of tumour cell lines [199-202], including induction of the pro-apoptotic factors BIM, BMF, BAX and BIK, and repression of the anti-apoptotic factors BCL-2, BCL-X_L and MCL-1 [43, 163, 198, 203].

Prior work undertaken in my laboratory therefore sought to establish a link between the magnitude of HDACi-induction of ATF3 and corresponding change in expression of pro and or anti-apoptotic components of the intrinsic apoptotic pathway. This analysis identified a statistically significant correlation between the magnitude of HDACi-induction of ATF3 mRNA expression and the corresponding repression of expression of BCL-X_L in 15 cancer cell lines representing a range of HDACi sensitivities and tumour types. Importantly, this analysis also demonstrated a significant inverse correlation between the magnitude of repression of BCL-X_L and apoptotic sensitivity of to these agents. The aim of this chapter was to determine if the repression of BCL-X_L was required for HDACi-induced apoptosis, and if the repression of BCL-X_L was directly mediated by ATF3.

First, I extended the previous findings of my laboratory and demonstrated that BCL-X_L protein expression was also preferentially repressed in HDACi sensitive cell lines. Next, through promoter reporter assays we demonstrated that ATF3 overexpression repressed activity of a BCL-X_L promoter construct. Importantly, investigation of the sequence of the BCL-X_L promoter demonstrates the existence of several putative AP-1 binding sites capable of binding ATF3. Indeed, ChIP experiments undertaken by other members of my laboratory has confirmed direct binding of ATF3 to these sites following HDACi treatment (Chueh *et al*, Clinical Cancer Research). Notably, in these ChIP experiments we also identified regions in the BCL-X_L promoter which did not possess AP-1 binding sites, but also displayed high ATF3 binding and significant repression of BCL-X_L promoter activity. Within these regions we identified several

potential NF-κB binding sites, raising the possibility that ATF3 may interact with factors such as NF-κB to repress BCL-X_L. As previously discussed, the activation and accumulation of NF-κB has been previously demonstrated to be regulated by HDACi [397], therefore, such a model could be plausible and is worthy of further investigation.

The repressive effect of ATF3 on BCL-X_L promoter activity most likely also involves the recruitment of co-repressor complexes to induce epigenetic changes associated with transcriptional repression. The identity of these factors is currently unknown but may include histone deacetylase and histone methyltransferase containing complexes.

Collectively, these results demonstrate that HDACi treatment induces ATF3 expression which in turn represses expression of the pro-survival gene BCL-X_L. We demonstrate that this occurs preferentially in HDACi sensitive cells, providing new mechanistic insights into the basis for differential sensitivity of cancer cell lines to HDACi-induced apoptosis. The findings of this chapter also demonstrate that knockdown of BCL-X_L can sensitise HDACi-resistant cancer cell lines to HDACi-induced apoptosis, providing direct evidence for a key role for BCL-X_L repression in HDACi-induced apoptosis. This finding is consistent with the findings of Hamed *et al* who demonstrated that overexpression of BCL-X_L can attenuate apoptosis induced by the combination of Sorafenib and HDACi [398].

Having identified the repression of BCL-X_L as a critical mediator of HDACi-induced apoptosis, we went on to test whether combining HDACi and a BCL-X_L inhibitor may represent a means of overcoming inherent resistance to HDACi. We first demonstrated that combining HDACi with the pan-BCL family inhibitors, ABT-263 or ABT-737, significantly enhanced apoptosis compared to either agent alone. We demonstrated this in multiple HDACi resistant cell lines *in vitro* and in the HDACi-resistant U87 cell line *in vivo*. We subsequently demonstrated that the enhanced apoptotic response was specifically driven by the co-suppression of BCL-X_L as treatment of resistant cell lines with HDACi and the BCL-XL-specific inhibitor A-1338352, but not the BCL-2-specific inhibitor, ABT-199, significantly enhanced HDACi-induced apoptosis. We also demonstrated that a BCL-X_L inhibitor could enhance HDACi-induced apoptosis in a HDACi sensitive cell line, when used at lower

concentrations. Whether high dose HDACi treatment or combination treatment with lower doses of an HDACi and a BCL-XL inhibitor represents a more effective therapy for HDACi sensitive cells will need to be assessed *in vivo*, where anti-tumour activity can be assessed in parallel with toxicity. In this regard, a well-established side effect of ABT-263 treatment is thrombocytopenia as haemostatic platelet function is heavily reliant on BCL-X_L [399, 400], and several studies have demonstrated that which ABT-263 can enhance the anti-tumour activity of drugs such as Rituximab, Gemcitabine and Carboplatin/Paclitaxel, thrombocytopenia is a consistent dose-limiting side effect [400-405].

However, during the course of these studies, we also noted that the molecular or pharmacological inhibition of BCL-X_L alone in resistant cell lines did not induce apoptosis to the same extent to that observed during HDACi-mediated inhibition of BCL-X_L, suggesting HDACi-induced apoptosis likely requires additional molecular changes. To investigate this, members of my laboratory have investigated other pro and anti-apoptotic components of the intrinsic apoptotic pathway which are altered in expression following HDACi treatment, and identified consistent induction of the pro-apoptotic BH3-only genes BIM, BIK, BMF and NOXA (Chueh, Tse et al, under revision in Clinical Cancer Research). Notably, a number of these genes have previously been shown to be required for HDACi-induced apoptosis [406, 407]. It is important to emphasise however, that in contrast to the pattern of regulation of BCL-X_L which was preferentially repressed in HDACi sensitive cell lines, the magnitude of induction of these genes was similar in sensitive and resistant cell lines, indicating their induction alone is not sufficient to induce apoptosis. Therefore, we propose a model whereby HDACi-induced apoptosis involves both the induction of pro-apoptotic genes such as BIM, BIK, BMF and NOXA and the ATF3-dependent repression of the pro-survival factor BCL-X_L. Of these events, ATF3-dependent repression of BCL-X_L only occurs in some lines and is therefore the key event which determines apoptotic response.

In conclusion, we demonstrate that the induction of ATF3 and subsequent repression of BCL-X_L is a key mediator of HDACi-induced apoptosis. Furthermore, we utilize this information to test and demonstrate that combining HDACi with a BCL-X_L inhibitor can overcome inherent resistance to HDACi regardless of tumour type, and provides a strategy for broadening the use of HDACi as a cancer therapeutic.

Chapter 5: Histone deacetylase and proteasome inhibitors synergistically induce apoptosis through induction of ATF3

5.1 Introduction

HDAC inhibitors and proteasome inhibitors synergistically induce apoptosis in cancer cell lines.

Combination treatment with HDAC-inhibitors (HDACi) and proteasome inhibitors have been shown to synergistically induce apoptosis in multiple cancer cell lines, including cell lines derived from multiple myeloma [408-410], leukemia [411-415] and colorectal cancer [416, 417]. Combinatorial treatment of the HDACi, Vorinostat and Panobinostat, with the proteasome inhibitor, Bortezomib, have also been recently shown to confer additional clinical benefit for the treatment of multiple myeloma in two large randomized Phase III studies, VANTAGE 088 [418] and PANORAMA [121, 147], respectively. In the VANTAGE 088 trial, combination of Vorinostat and Bortezomib provided a modest increase in progression free survival of 0.8 months compared to Bortezomib alone. However, a statistical difference in overall survival was not observed. In comparison, the subsequent PANORAMA trial which tested the combination of Panobinostat with Bortezomib and Dexamethasone, increased progression free survival (PFS) by 4.8 months compared to Bortezomib and Dexamethasone alone and was approved for the treatment of relapsed multiple myeloma in 2015.

Proteasome inhibitors

Ubiquitin-Proteasome cascade of protein degradation

The degradation of un-wanted cellular proteins is a complex and tightly regulated process which aids in the regulation of cellular function and maintenance of homeostasis. The ubiquitin-proteasome pathway is one of the major pathways in which intra-cellular proteins are degraded and it is responsible for the degradation of proteins involved in a wide variety of cellular responses such as DNA repair, transcription, cell cycle and apoptosis. The degradation of un-wanted proteins occurs by a multi-catalytic process in which the protein to be degraded is tagged by a covalently linked polyubiquitin chain through the coordinate action of three enzymes: E1 (a ubiquitin-activating enzyme), E2 (a ubiquitin-conjugating enzyme) and E3 (a ubiquitin ligase) (Figure 5.1) [378, 419, 420]. This process is initiated by the activation

of a ubiquitin molecule through formation of a thiol ester bond between the ubiquitin and an E1 enzyme [421]. The activated ubiquitin is then transferred to the active site within an E2 enzyme which together with an E3 enzyme transfers the ubiquitin to a lysine residue on the target protein. This process occurs in multiple rounds, resulting in the formation of a polyubiquitin chain on the protein. The poly-ubiquitinated proteins are then shuttled to the 26S proteasome where they are degraded in the central portion (20S subunit) of the 26S proteasome complex [422].

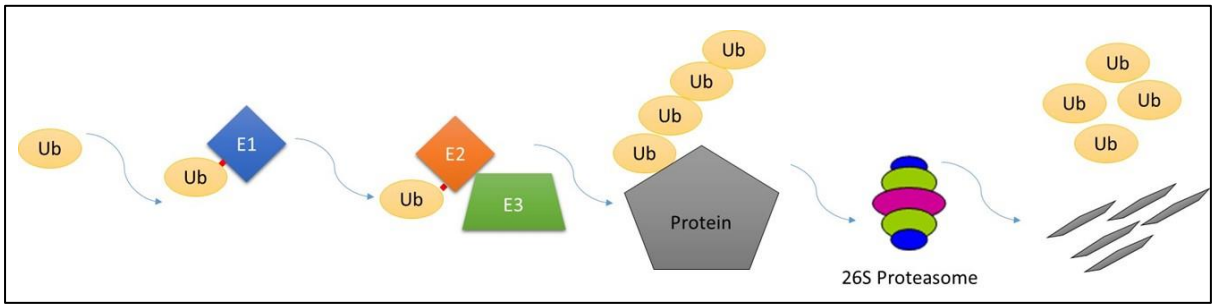

Figure 5.1 The ubiquitin-proteasome pathway. The addition of poly-ubiquitinated tails to specific lysine residue on proteins targets them for proteasomal degradation. Three enzymes are involved in protein ubiquitination: E1 (a ubiquitin-activating enzyme), E2 (a ubiquitin-conjugating enzyme) and E3 (a ubiquitin ligase). Proteins which have been ubiquitinated are subsequently degraded by the 26S proteasome.

Proteasome inhibitors as cancer therapeutics

Proteasome inhibitors were initially synthesised as a chemical compound to assist in the investigation of the proteasome's catalytic function and to identify proteins which were heavily dependent on 26S proteasomal degradation. However, early *in vitro* studies also demonstrated that proteasome inhibitors induced apoptosis in leukemic cell lines [423, 424] and provided anti-tumour activity in an *in vivo* model of Burkitt's lymphoma [425].

Several mechanisms for apoptotic sensitivity of tumour cells to proteasome inhibitors have been proposed. First, tumour cells have a greater dependence on protein turnover due to their higher proliferation rates [426]. A study by Crawford *et al* supported this hypothesis by demonstrating that increased proteasomal activity, which is indicative of higher protein turnover rates, correlated with an increased sensitivity of

leukemic cells to proteasome inhibitors [427]. Second, a number of tumours are dependent on the NF-κB transcription factor for their survival, and the proteasome plays a critical role in NF-κB activation by catalysing the ubiquitination and subsequent degradation of the inhibitory partner of NF-κB, IκB [428]. IκB binds to NF-κB preventing its nuclear localisation and maintaining it in an inactive state in the cytoplasm [429-431].

The proteasome inhibitor Bortezomib (Velcade) was initially chosen from a panel of boronic acid analogues based on its cytotoxicity in a screen of the National Cancer Institute's panel of 60 cancer cell lines [432], and its cytotoxicity was subsequently confirmed in a number of pre-clinical tumour models. The efficacy of Bortezomib was then further investigated in pre-clinical studies in various tumour types and displayed indications of activity in prostate cancer [433] and multiple myeloma cells [434]. In subsequent trials, Bortezomib demonstrated clinical activity in multiple myeloma [435-437], and the drug was FDA approved for the treatment of relapsed multiple myeloma in 2003 [438]. FDA approval of Bortezomib for multiple myeloma was based on a Phase II, open-label, single arm, multi-centre study of 202 heavily pre-treated patients. Of the 188 patients that were evaluable, complete responses were achieved in 5 patients (3%) and partial response in 47 patients (25%), with median duration of response being 365 days [438]. Bortezomib has also since been approved as a first line treatment option for mantle cell lymphoma in 2015 [439].

Several explanations as to why these tumour types are particularly sensitive to proteasome inhibitors have been proposed. One proposed mechanism is the ability of proteasome inhibitors to block NF-κB activity, a key transcription factor which promotes myeloma genesis [440, 441]. Inhibition of proteasome degradation would prevent the degradation of the inhibitory unit of NF-κB, IκB, and consequently in inhibition of NF-κB activity [442]. Second, in multiple myeloma cells unfolded and misfolded proteins often accumulate. Hence, multiple myeloma cells have a well-developed endoplasmic-reticulum-associated protein degradation (ERAD) system, in which misfolded proteins are excluded from the endoplasmic reticulum, poly-ubiquitinated and degraded by the proteasome. The inhibition of the proteasome would be expected to result in disruptions in the balance of the ERAD system, over-

stressing of the endoplasmic reticulum and induction of stress activated apoptosis in multiple myeloma cells [443].

Activation of the JNK and p38 stress response pathways

Numerous studies have demonstrated that the intracellular accumulation of unwanted proteins following proteasome inhibition results in activation of the JNK and p38 stress response pathways [320-322], which regulate a range of cellular responses, including cell proliferation, differentiation and apoptosis [444, 445]. The JNK and p38 pathways are typically activated by stress-inducing stimuli including osmotic stress, ionizing radiation [446], chemotherapy treatment and proteasome inhibition [320, 321]. Activation of these pathways in turn activates a number of transcription factors including ELK1, p53 and c-myc as well as the members of the AP-1 complex c-JUN, and AT3 [445].

The context by which activation of p38 induces ATF3 was investigated in a study by Lu *et al,* where they demonstrated p38 activation by Anisomycin, a well characterised JNK and p38 activator, was crucial for ATF3 mRNA induction and subsequent apoptosis in HeLa, COS-1 and HEK-293T cells. Analysis of the ATF3 promoter further found that CREB, which is a phosphorylation target of p38, was necessary for p38 induced expression of ATF3. However, the authors noted that their short-list of p38 phosphorylated transcription factors was not comprehensive, and that a range of other transcription factors may also have a role in p38 mediated induction of ATF3 [447]. Furthermore, the ATF3 promoter contains numerous binding sites including AP-1, CRE/ATF, E2F, p53 and NF-κB, for which other transcription factors can also bind and mediate ATF3 expression.

Proteasome inhibitor-induced apoptosis

As described for HDACi, a major mechanism by which proteasome inhibitors exert their anti-tumour activity is through the induction of apoptosis [448-450]. Mechanistically, this occurs through activation of either the intrinsic or extrinsic apoptotic pathways, although the majority of data indicate activation of the intrinsic apoptotic pathway. Various studies have demonstrated that proteasome inhibitor

treatment initiates apoptosis through the induction of pro-apoptotic proteins and repression of anti-apoptotic members. For example, Ling *et al* demonstrated that treatment of H460 (NSCLC) cells with Bortezomib leads to phosphorylation and cleavage of the pro-survival protein BCL-2 [448]. The phosphorylation and cleavage of BCL-2 has been shown to be dependent on JNK activation [320, 451]. Proteasome inhibitors also activate BAX and BAK, and up-regulate the pro-apoptotic factors NOXA, p53, p27, BIM, BAX, and SMAC [452-454]. Conversely, the inhibition of the proteasome has also been shown to cause an accumulation and increased half-life of the anti-apoptotic protein MCL-1. Tumour cells expressing elevated levels of MCL-1 are resistant to proteasome inhibitors and studies have combining proteasome inhibitors with MCL-1 inhibitors have demonstrated enhanced cell killing [455, 456].

In regards to the extrinsic pathway, proteasome inhibitor treatment results in the up-regulation of several death receptors such as Fas and DR5, and enhanced caspase 8 activity [449, 457]. Furthermore, the requirement of Fas for proteasome inhibitor-induced apoptosis was demonstrated by Tani *et al* where the attenuation of Fas with antibodies prevented proteasome inhibitor-induced apoptosis in glioblastoma cells [458].

Potential mechanisms for the enhanced anti-tumour activity induced by combining HDACi and proteasome inhibitors

While combination treatment with HDAC and proteasome inhibitors is clinically approved for refractory multiple myeloma, the mechanistic basis for the activity of this combination has not been clearly defined. One mechanism proposed is that this combination induces blockade of both the proteasome as well as the aggresome, resulting in inhibition of all routes of protein degradation. While the proteasome is the major pathway by which un-wanted proteins are degraded [378, 426], an alternate compensatory mechanism of protein degradation is via formation of aggresomes. When the proteasome is inhibited or overwhelmed, aggresomes are formed to facilitate the degradation of these proteins by lysosomes. Misfolded and un-wanted proteins aggregate to form aggresomal particles which are transported in a microtubule-dependent manner to the microtubule organising centre, where they are sequestered together to form the aggresome. Aggresome formation and function has

been demonstrated to require HDAC6 [459-463]. HDAC6 acetylates α-tubulin and associates with dynein to aid the transport of the aggregated protein particles through the cytosol to lysosomes for degradation [460]. Therefore, combination treatment with proteasome and HDAC inhibitors has been postulated to result in inhibition of both the 26S proteasome and the aggresome, resulting in greater accumulation of unwanted proteins, cell stress and ultimately apoptosis.

However, other studies which have demonstrated that combination treatment with proteasome inhibitors and HDACi which do not target HDAC6 can also synergistically induce apoptosis [464, 465]. For example, studies combining proteasome inhibitors with the Class I HDACi MS-275 (Entinostat) synergistically induced apoptosis in cholangiocarcinoma cells and nasopharyngeal carcinoma cells, without inhibiting aggresome function [465, 466]. These findings suggest that additional mechanisms may play a role in driving the synergistic induction of apoptosis by this drug combination.

Hypothesis:

As discussed previously, a number of studies have demonstrated the induction of ATF3 in response to proteasome inhibitor treatment subsequent to activation of the p38 and JNK stress response pathways [326, 445, 467, 468]. As we have demonstrated a key role for the induction of ATF3 in HDACi-induced apoptosis, and given that HDACi-induction of ATF3 is mediated through the Sp1 and Sp3 transcription factors [309], we postulated that combination treatment with an HDACi and a proteasome inhibitor would synergistically induce ATF3 expression. We hypothesise that the synergistic induction of ATF3 would subsequently drive the enhanced induction of apoptosis in response to this drug combination (Figure 5.2).

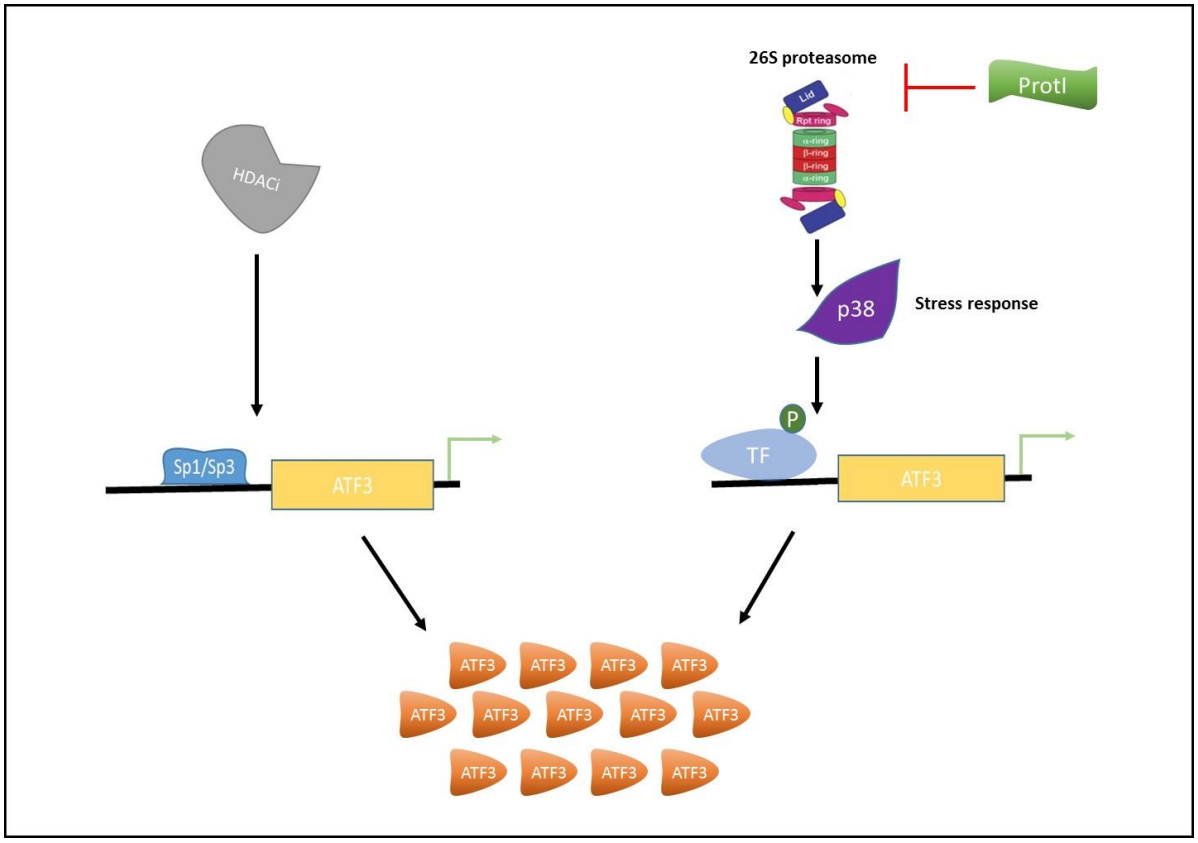

Figure 5.2 Proposed model by which HDACi and proteasome inhibitors may synergistically induce ATF3 expression. We propose that HDACi induce ATF3 in a Sp1/Sp3 dependent manner whereas proteasome inhibitors induce ATF3 expression via p38 activation and subsequent phosphorylation of transcription factors that mediates ATF3 induction.

5.2 Results

5.2.1 Combination treatment with HDAC and proteasome inhibitors synergistically induces apoptosis in colorectal cancer and multiple myeloma cell lines

To test our hypothesis that combination treatment with HDAC and proteasome inhibitors synergistically induces apoptosis through additive induction of ATF3, we first determined the effect of this drug combination on apoptosis induction in 2 colon cancer and 2 multiple myeloma cell lines. Combination treatment with Vorinostat and Bortezomib synergistically induced apoptosis in the 2 colorectal cancer cell lines, with the combination inducing >70% apoptosis in both cases (Figure 5.3A). In the HCT116 cell line, the combination induced 86.57±5.90% apoptosis compared to 13.17±3.09% and 41.60±3.06% apoptosis in cells treated with Vorinostat or Bortezomib alone, respectively. A similar result was obtained in SW948 where the combination induced 77.25±1.73% apoptosis compared to 17.50±3.25% and 17.37±1.14% apoptosis in cells treated with Vorinostat or Bortezomib alone, respectively.

Consistent with the clinical activity of Vorinostat and Bortezomib in haematological cancers, the combination also synergistically induced apoptosis in the 2 multiple myeloma cell lines, even when Vorinostat and Bortezomib were used at 5-fold and 10-fold lower concentrations respectively to the concentrations used in colorectal cancer cells (Figure 5.3B). In OPM2 the combination induced 34.78±2.78% apoptosis compared to 5.33±0.52% and 10.06±0.94% apoptosis in cells treated with Vorinostat or Bortezomib alone, respectively. Similarly, in U266 the combination induced 34.00±0.21% apoptosis compared to 11.47±2.06% and 10.0.4±1.91% apoptosis in cells treated with Vorinostat or Bortezomib alone, respectively. Synergy was statistically determined using the Chou & Talalay method (Figure 5.4 A & B).

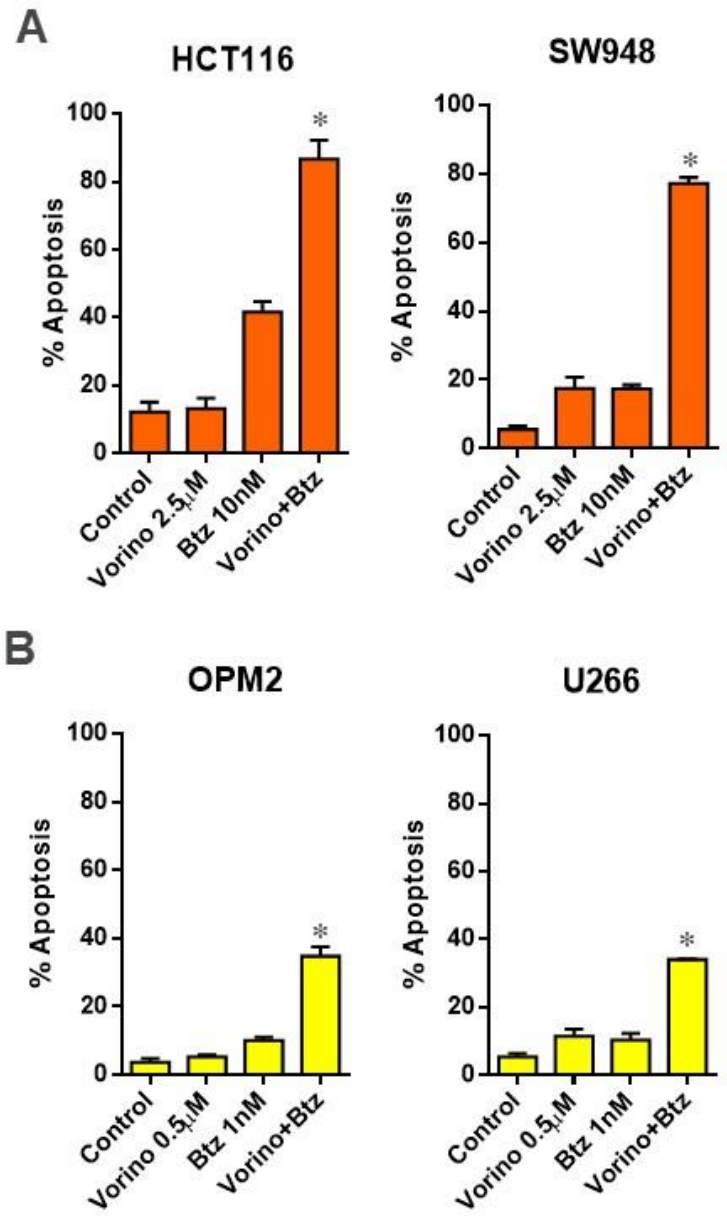

Figure 5.3 Effect of Vorinostat (Vorino) and Bortezomib (Btz) alone and in combination on apoptosis in (A) 2 colorectal cancer (HCT116 and SW948) and (B) 2 multiple myeloma (U266 and OPM2) cell lines. Cells were treated for 24 hours and apoptosis determined by FACS analysis of PI stained cells. Values shown are mean ± SEM from 3 independent experiments, each performed in triplicate. * Denotes synergy as determined by the Chou & Talalay method.

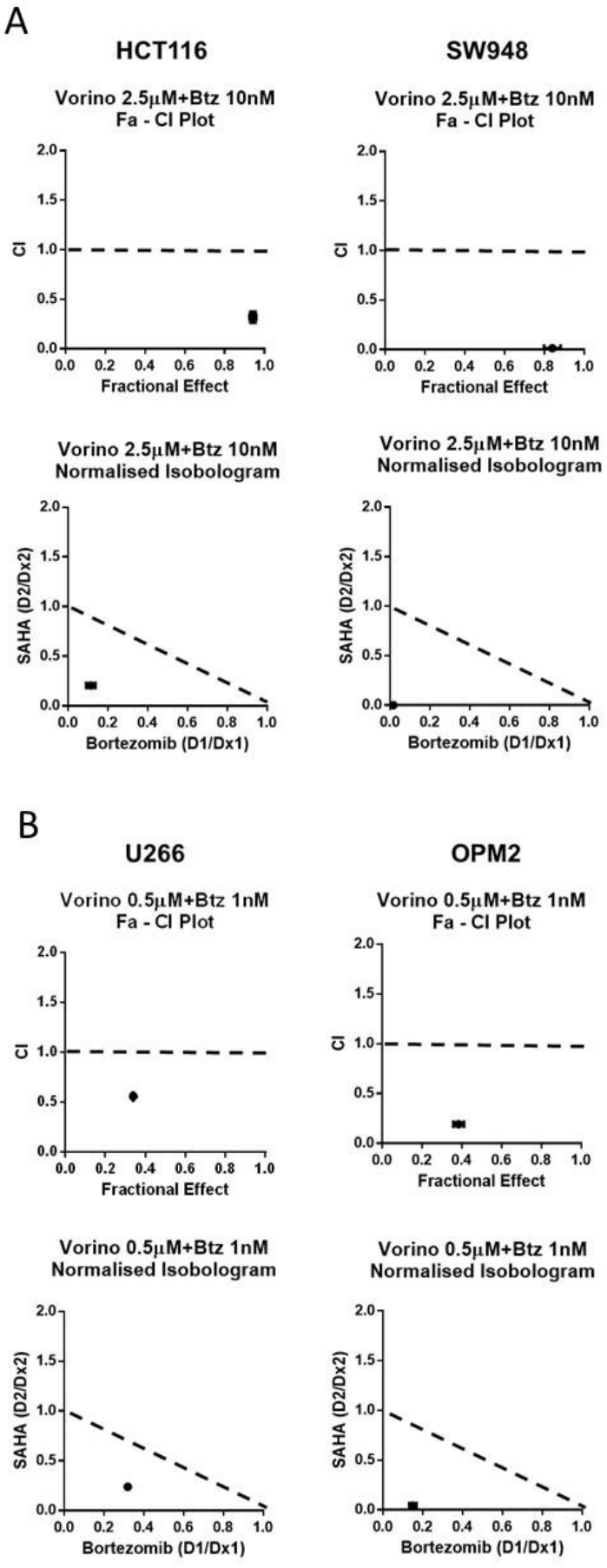
A
HCT116
SW948
Vorino 2.5μM+Btz 10nM
Fa - CI Plot
CI
Fractional Effect
Vorino 2.5μM+Btz 10nM
Normalised Isobologram
SAHA (D2/Dx2)
Bortezomib (D1/Dx1)
B
U266
OPM2
Vorino 0.5μM+Btz 1nM
Fa - CI Plot
Vorino 0.5μM+Btz 1nM
Normalised Isobologram

Figure 5.4 Combination index (CI) plots and Isobologram obtained from Chou & Talalay synergy calculation of Vorinostat and Bortezomib combination treatment in the (A) 2 colorectal cancer cell lines, and the (B) 2 multiple myeloma cell lines. Combination index values that fall below 1 and isobologram values that fall below the gradient of 1 are considered to by synergistic.

The synergistic induction of apoptosis was further demonstrated by measurement of cleaved caspase 3 which was markedly higher in the 2 colorectal (Figure 5.5A) and 2 multiple myeloma (Figure 5.5B) cell lines treated with the combination compared to either agent alone.

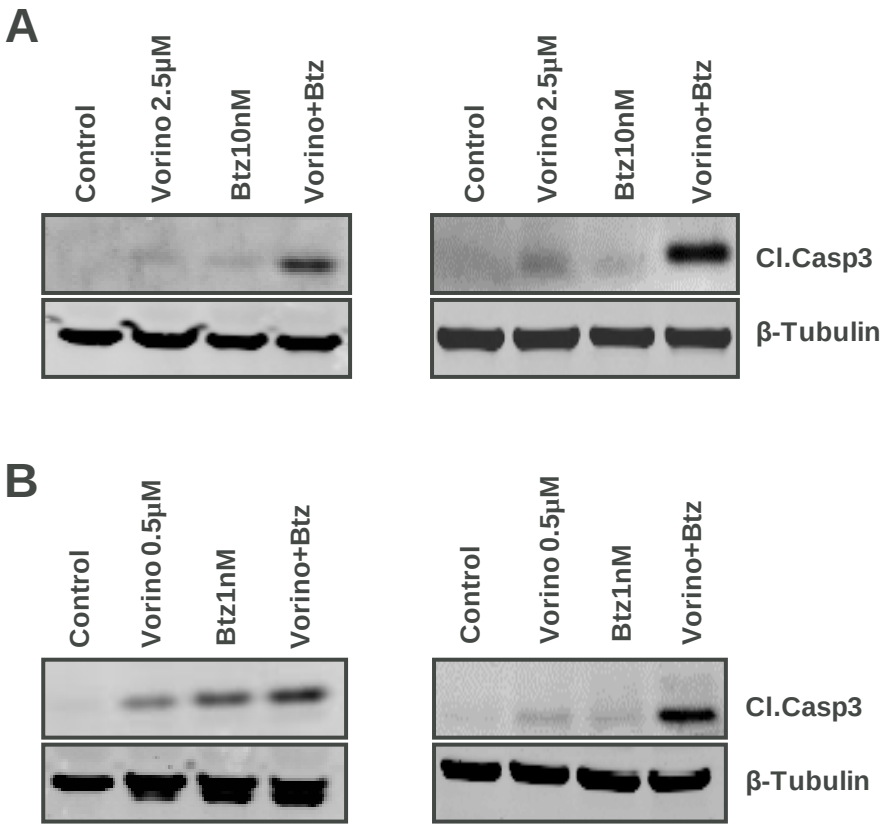

Figure 5.5 Effect of Vorinostat (Vorino) and Bortezomib (Btz) alone and in combination on apoptosis in (A) 2 colorectal cancer (HCT116 and SW948) and (B) 2 multiple myeloma (U266 and OPM2) cell lines. Cells were treated for 24 hours and cleaved caspase 3 induction determined by Western blot.

To determine whether combining other clinically relevant HDACi with Bortezomib also synergistically induced apoptosis, HCT116 and SW948 colorectal cancer cells were treated with Bortezomib and the other three FDA approved HDACi, Depsipeptide (Romedipsin), Belinostat (PXD101) and Panobinostat. All three HDACi induced an enhanced apoptotic when combined with Bortezomib in both colorectal cancer cell lines (Figure 5.6A-C).

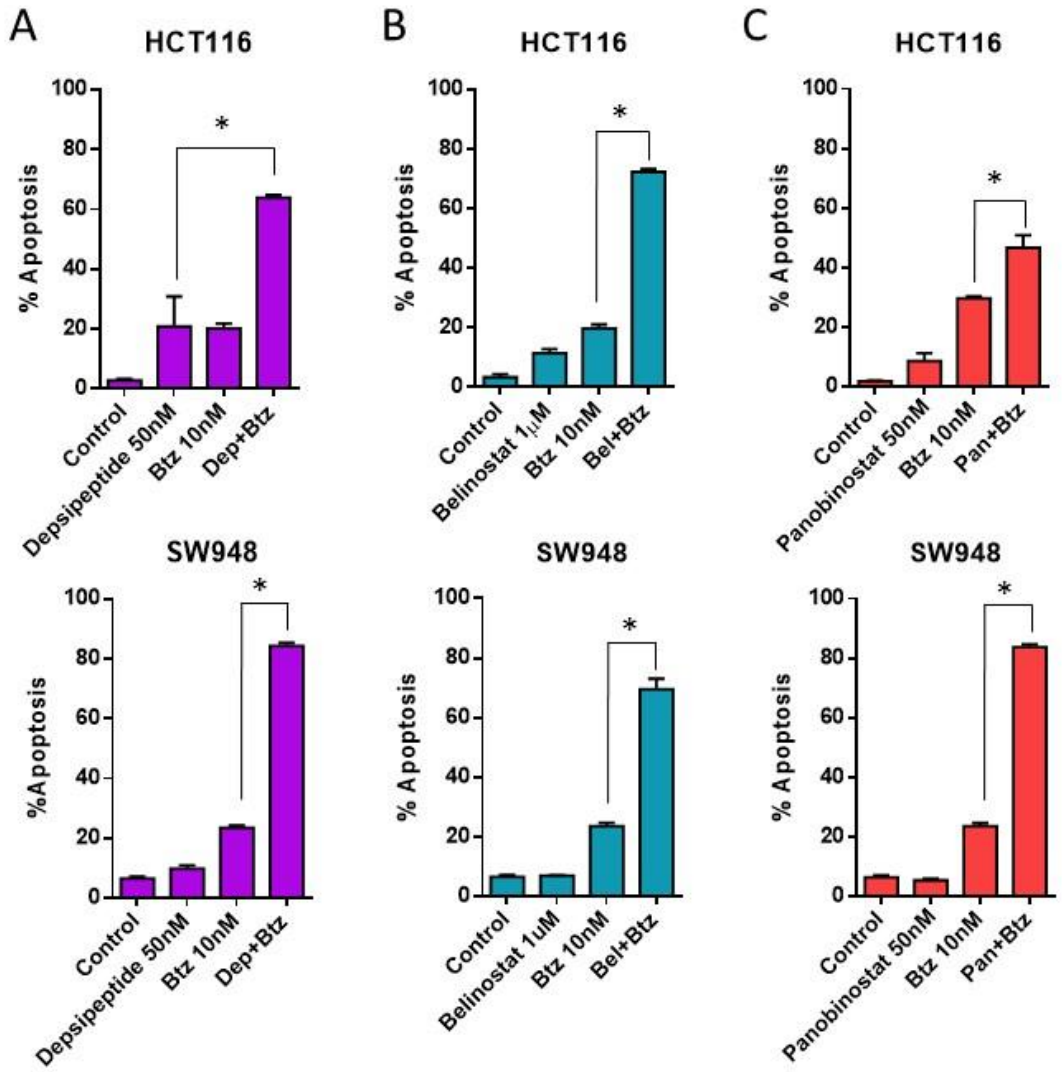

Figure 5.6 Effect of Bortezomib (Btz) and (A) Depsipeptide (Dep), or (B) Belinostat (Bel) or (C) Panobinostat (Pano) alone and in combination on apoptosis in 2 colorectal cancer (HCT116 and SW948) cell lines. Cells were treated for 24 hours and apoptosis determined by FACS analysis of PI stained cells. Values shown are mean ± SEM from a representative experiment from 2 independent experiments, each performed in triplicate. *$P < 0.05$, Student's t-test.

5.2.2 HDACi and proteasome inhibitors induce ATF3 mRNA and protein expression

To confirm the previous reports that proteasome inhibitor treatment induces expression of ATF3 and to extend this finding to multiple cell lines, we examined the effect of treating the 2 colorectal cancer cell lines (HCT116 & SW948) and 2 multiple myeloma cell lines (U266 & OPM2) with Bortezomib for 24 hours on ATF3 induction. For comparison, the same cell lines were also treated with Vorinostat. Bortezomib and Vorinostat both robustly induced ATF3 mRNA expression in all 4 cell lines (Figure 5.7 A-B). Notably, the magnitude of ATF3 mRNA induction in response to Bortezomib treatment was markedly higher in multiple myeloma cell lines compared to colorectal cancer cell lines.

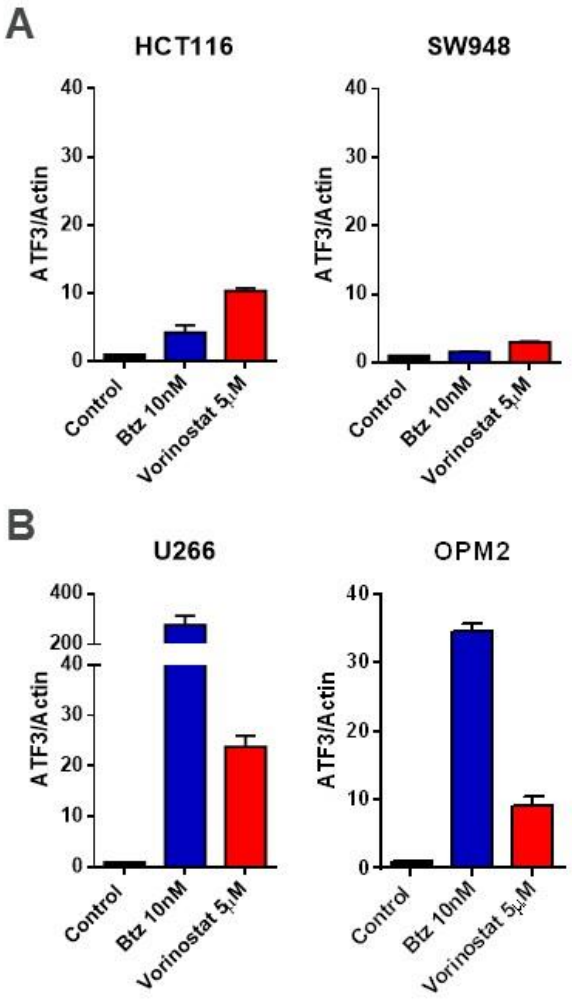

Figure 5.7 Effect of Vorinostat (Vorino) and Bortezomib (Btz) on *ATF3* mRNA induction in (A) colorectal cancer (HCT116 and SW918) and (B) multiple myeloma (U266 and OPM2) cell lines. Cells were treated for 24 hours and gene expression determined by q-RT-PCR. Values shown are mean ± SEM from 3 independent experiments, each performed in triplicate.

The induction of ATF3 by proteasome inhibitors and HDACi was also confirmed at the protein level with sustained induction of ATF3 observed over 24-48 hours in response to treatment with either Bortezomib or Vorinostat (Figure 5.8 A-B).

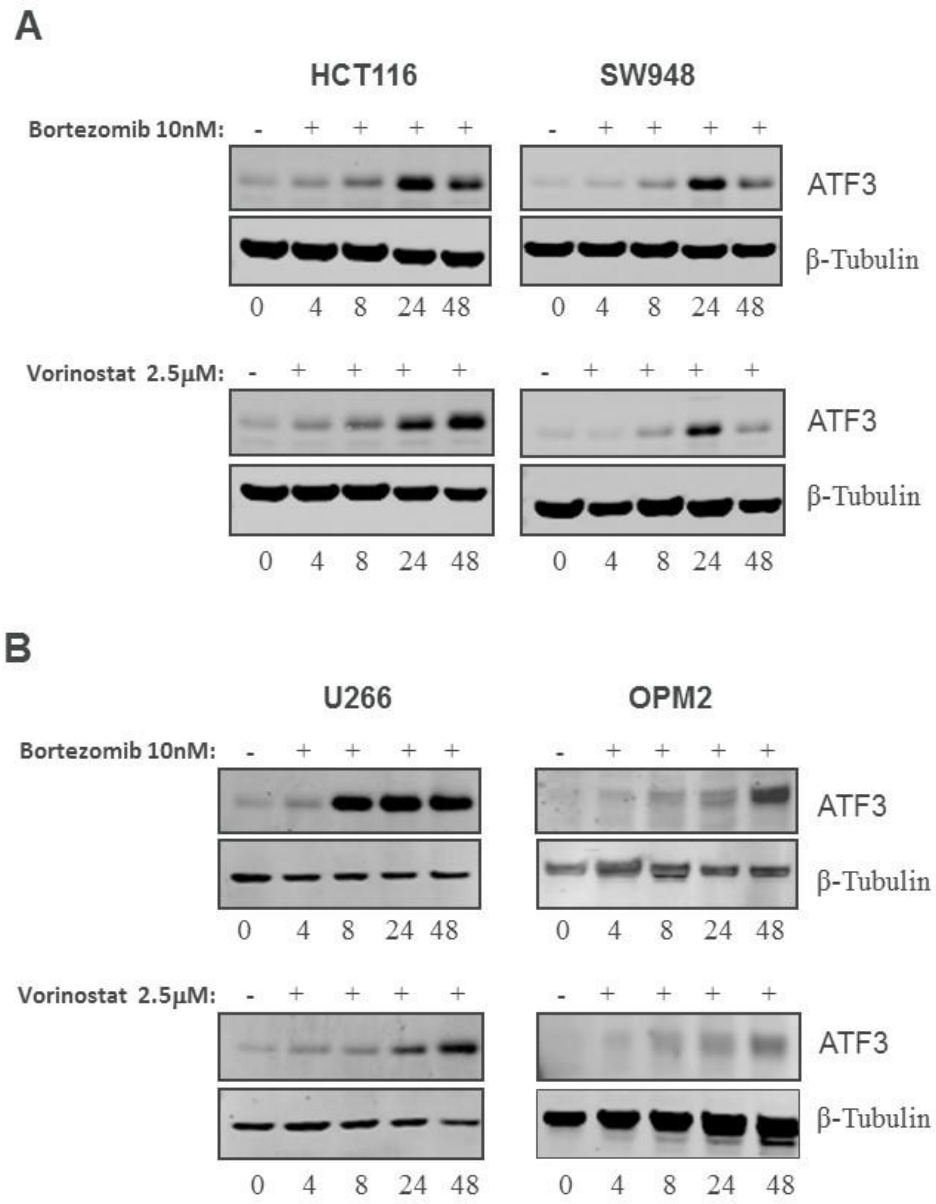

Figure 5.8 Effect of Bortezomib (Btz) and Vorinostat (Vorino) on ATF3 protein expression in (A) colorectal cancer (HCT116 and SW918) and (B) multiple myeloma (U266 and OPM2) cell lines determined by western blot. Cells were treated with Bortezomib or Vorinostat for 8-48hours.

5.2.3 Vorinostat but not Bortezomib-induced ATF3 expression is dependent on the Sp1 and Sp3 transcription factors

Wilson *et al* previously established that HDACi-induced expression of ATF3 was dependent on the Sp1 and Sp3 transcription factors, as combined knockdown of Sp1 and Sp3 attenuated HDACi-induced apoptosis [309]. To determine if Bortezomib induction of ATF3 was dependent or independent on Sp1 and Sp3, the effect of Sp1 and Sp3 knockdown on Bortezomib-induced ATF3 expression was investigated in SW948 colorectal cancer cells. The effect of Sp1 and Sp3 knockdown on Vorinostat-induced ATF3 expression was examined in parallel for comparison. Co-transfection with Sp1 and Sp3-targeting siRNAs partially inhibited Sp1 and Sp3 expression as assessed by Western blot. As previously demonstrated in HCT116 colorectal cancer cells, combinatorial knockdown of Sp1 and Sp3 attenuated induction of ATF3 expression following Vorinostat treatment. Comparatively, Sp1/Sp3 knockdown had minimal effect on Bortezomib-induced ATF3 protein expression (Figure 5.9).

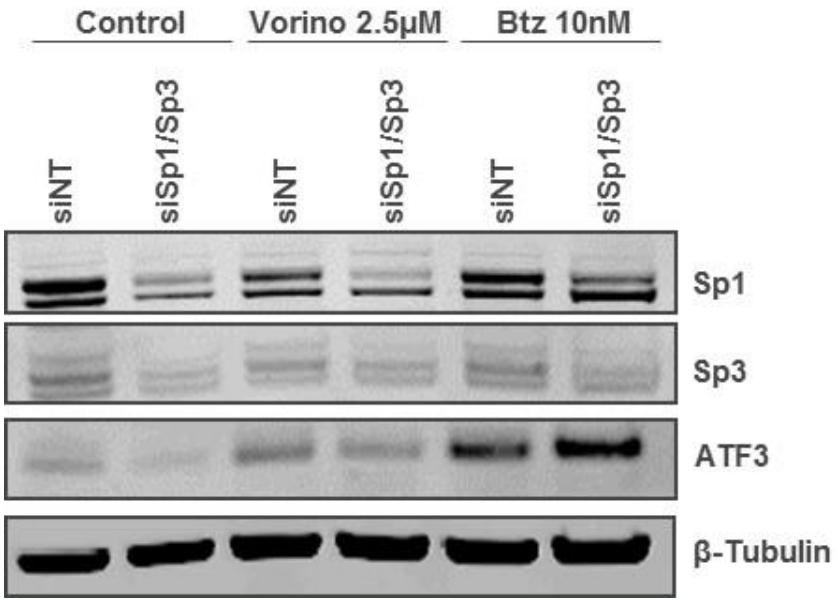

Figure 5.9 Effect of combinatorial knockdown of Sp1 and Sp3 on Vorinostat (Vorino) or Bortezomib (Btz)-mediated induction of ATF3 protein expression determined by Western blot. SW948 cells were transiently co-transfected with Sp1 and Sp3-targeting siRNAs overnight and the following day treated with Vorinostat or Bortezomib for 24 hours.

To confirm these findings, the effect of Mithramycin, a pharmacological inhibitor of Sp1/Sp3 binding to GC rich sequences, on Vorinostat and Bortezomib-induced expression of ATF3 mRNA was investigated. Mithramycin treatment significantly attenuated induction of ATF3 following Vorinostat treatment, with ATF3 mRNA expression induced 7.03±0.14-fold by Vorinostat alone compared to 1.60±0.99-fold, when treated with Vorinostat in combination with Mithramycin (Figure 5.10A). Unexpectedly, Mithramycin treatment alone induced ATF3 mRNA expression SW948 cells. In comparison, induction of ATF3 mRNA expression following Bortezomib treatment was not attenuated by Mithramycin, and instead an enhancement of ATF3 mRNA expression was observed (Figure 5.10B).

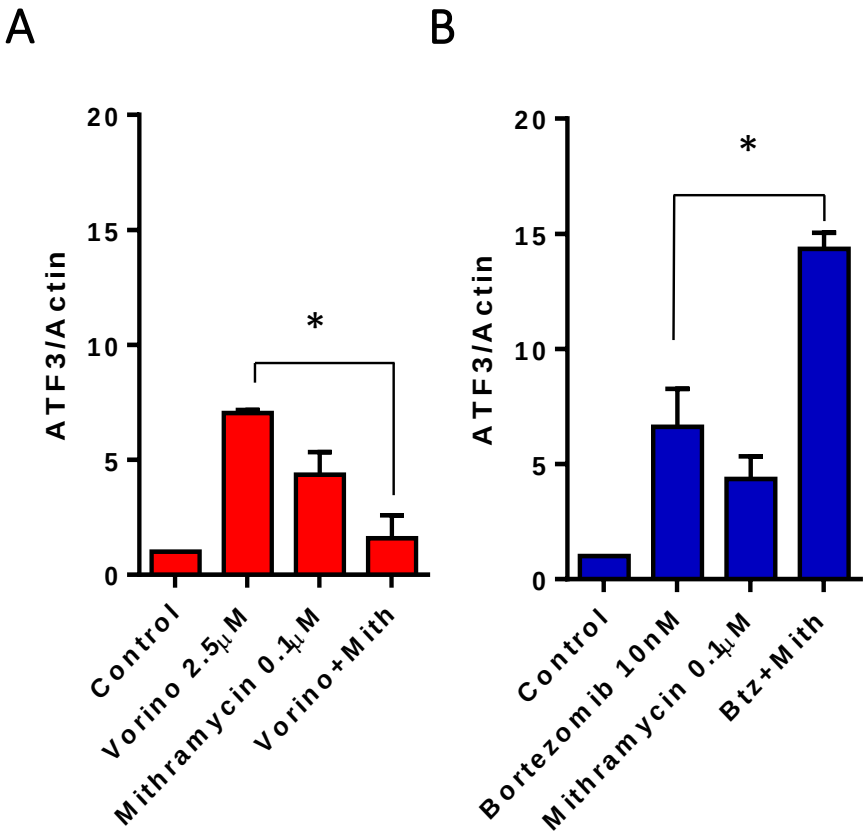

Figure 5.10 Effect of the Sp1/Sp3 inhibitor Mithramycin (Mith) on (A) Vorinostat (Vorino) or (B) Bortezomib (Btz)-mediated induction of ATF3 mRNA expression. SW948 cells were treated with Vorinostat or Bortezomib alone and in combination with Mithramycin for 24 hours, and ATF3 mRNA expression determined by qRT-PCR. Values shown are mean ± SEM from 2 independent experiments, each performed in triplicate. *$P < 0.05$, Student's t-test.

5.2.4 Vorinostat but not Bortezomib-induced apoptosis is dependent on the Sp1 and Sp3 transcription factors

Similarly, the importance of Sp1 and Sp3 on HDACi-induced ATF3 expression was also reflected on an apoptotic level, with Mithramycin inhibiting Vorinostat-induced apoptosis from 11.47±0.40% to 5.80±0.70% (Figure 5.11A). Conversely, Bortezomib-induced apoptosis was not attenuated but in fact enhanced when Mithramycin was combined, from 9.68±0.64% to 14.70±0.42% (Figure 5.11B).

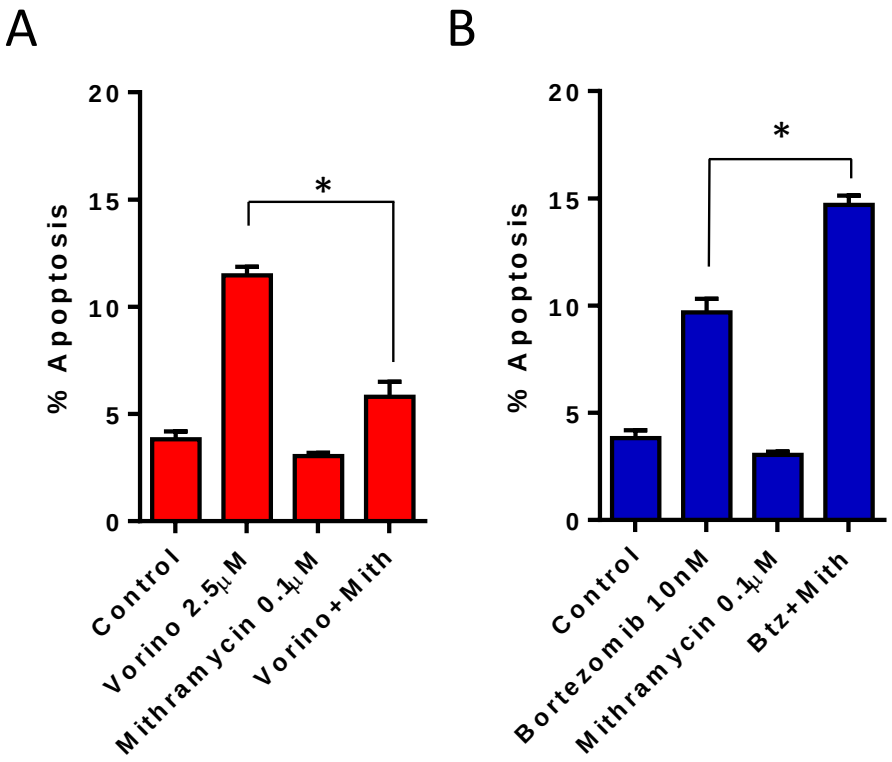

Figure 5.11 Effect of the Sp1/Sp3 inhibitor Mithramycin (Mith) on (A) Vorinostat (Vorino) or (B) Bortezomib (Btz)-mediated induction of apoptosis. SW948 cells were treated with Vorinostat or Bortezomib alone and in combination with Mithramycin for 24 hours, and induction of apoptosis determined by PI staining and FACS analysis. Values shown are mean ± SEM from a representative experiment performed in triplicate. *$P < 0.05$, Student's t-test.

5.2.5 Bortezomib but not Vorinostat-induced ATF3 expression is dependent on the p38 MAPK pathway

An established effect of proteasome inhibitor treatment is induction of cellular stress and consequent activation of the p38 and JNK stress response signalling pathways. Activation of these pathways culminates in changes in expression of a number of genes, including ATF3 [445, 447, 469]. To determine if proteasome inhibitor mediated induction of ATF3 is dependent on activation of the p38 stress response pathway, we determined the effects of p38 inhibition on ATF3 induction by Bortezomib. Consistent with previous reports, Bortezomib treatment of SW948 colorectal cancer cells strongly activated p38 signalling as determined by the induction of phosphorylated p38 (p-p38) (Figure 5.12). The p38 inhibitor SB203580, effectively inhibited Bortezomib-induction of p-p38 and in turn also attenuated Bortezomib-induced expression of ATF3 protein.

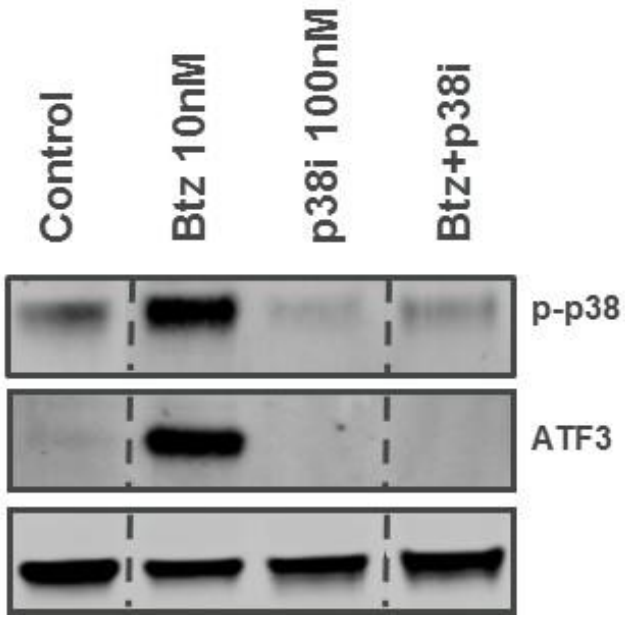

Figure 5.12 Effect of the p38 inhibitor (SB203580) on Bortezomib (Btz)-mediated induction of ATF3 protein expression determined by Western blot. SW948 cells were treated with Bortezomib alone and in combination with the p38 inhibitor for 24 hours. Dotted lines represent sections which were removed from the blot, as those samples were from other unrelated drug treatments.

Similar results were obtained at the mRNA level, where inhibition of p38 signalling attenuated Bortezomib-induced ATF3 expression from 11.55±0.73-fold to 3.84±2.34-fold (Figure 5.13A). Comparatively, inhibition of p38 signalling failed to attenuate Vorinostat-induced ATF3 mRNA expression, instead inducing a modest enhancement of ATF3 expression (Figure 5.13B).

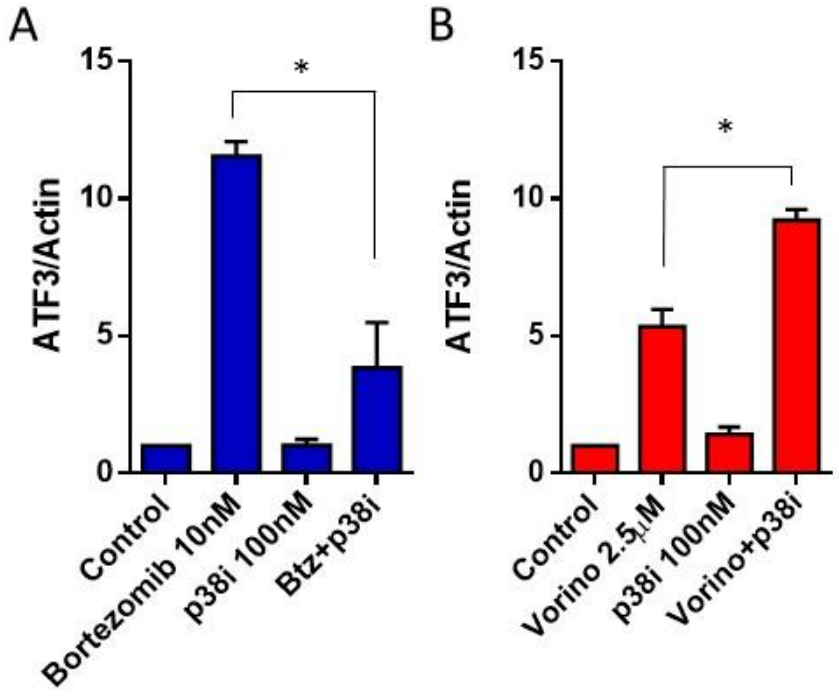

Figure 5.13 Effect of the p38 inhibitor (SB203580) on Bortezomib (Btz) or Vorinostat (Vorino)-mediated induction of ATF3 mRNA expression. SW948 cells were treated with (A) Bortezomib or (B) Vorinostat alone and in combination with the p38 inhibitor for 24 hours, and ATF3 mRNA expression determined by qRT-PCR. Values shown are mean ± SEM from 2 independent experiments, each performed in triplicate. *$P < 0.05$, Student's t-test.

5.2.6 Bortezomib but not Vorinostat-induced apoptosis is dependent on the p38 MAPK pathway

Finally, the importance of the p38 pathway on proteasome inhibitor-induced apoptosis was determined. Inhibition of p38 signalling significantly attenuated Bortezomib-induced apoptosis from 12.53±0.90% to 8.26±0.35% (Figure 5.14A). Conversely, p38 inhibition had minimal effect on Vorinostat-induced apoptosis (Figure 5.14B).

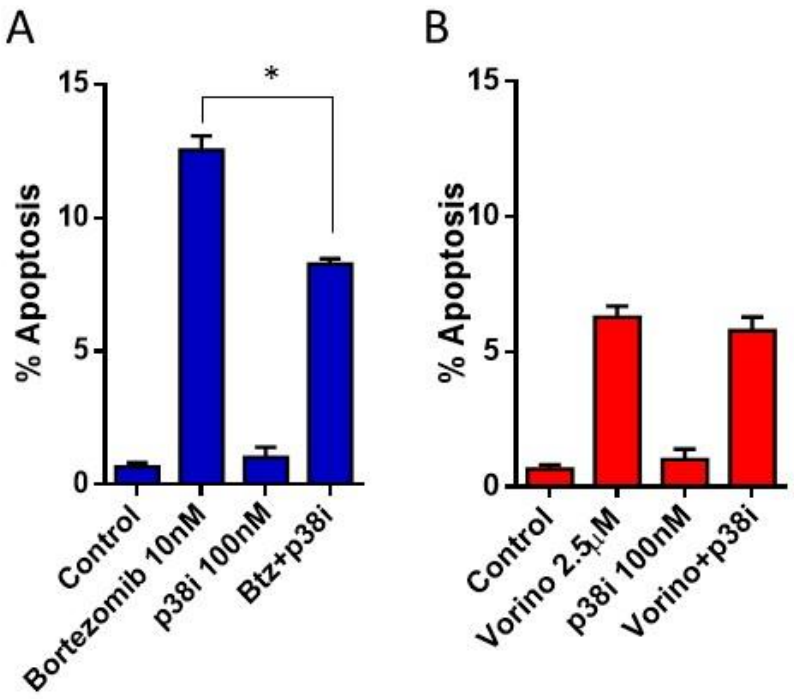

Figure 5.14 Effect of the p38 inhibitor (SB203580) on Bortezomib (Btz) or Vorinostat (Vorino)-induced apoptosis. SW948 cells were treated with Bortezomib or Vorinostat alone and in combination with the p38 inhibitor for 24 hours, and induction of apoptosis determined by PI staining and FACS analysis. PCR. Values shown are mean ± SEM from 2 independent experiments, each performed in triplicate. *P < 0.05, Student's t-test.

5.2.7 Combination treatment with HDACi and proteasome inhibitors markedly enhances induction of ATF3 expression

Having demonstrated that HDAC and proteasome inhibitors induce *ATF3* mRNA and protein expression via mechanistically distinct pathways, we next determined the effect of combination treatment on ATF3 gene expression in 2 colorectal cancer (HCT116 and SW948) and 2 multiple myeloma (OPM2 and U266)

cell lines. Combination treatment with Vorinostat and Bortezomib resulted in significant enhancement of ATF3 mRNA expression compared to either agent alone in all 4 cell lines. In HCT116 cells, the combination induced ATF3 mRNA expression by 19.86±1.27-fold, compared to 2.13±1.07-fold by Vorinostat alone, and 13.69±1.06-fold by Bortezomib alone (Figure 5.15A). Similar effects were observed in SW948 cells, where the combination induced ATF3 expression 24.60±2.0-fold, compared to a 3.29±0.56-fold by Vorinostat alone, and 1.58±0.14-fold by Bortezomib alone (Figure 5.15B).

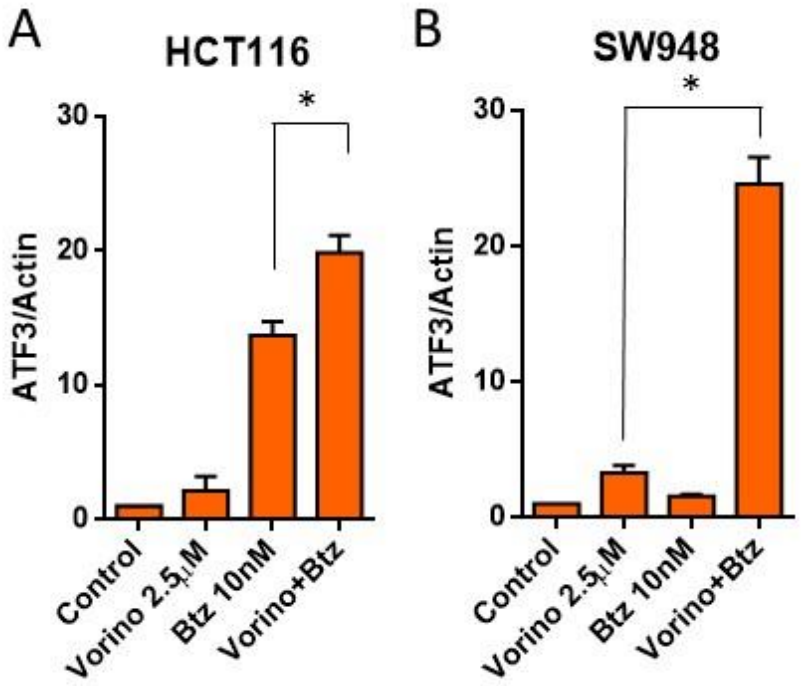

Figure 5.15 Effect of Vorinostat (Vorino) and Bortezomib (Btz) alone and in combination on ATF3 mRNA expression in 2 colorectal cancer cell lines (A) HCT116 and (B) SW948. Cell lines were treated with both drugs alone or in combination for 24 hours, and ATF3 mRNA expression determined by qRT-PCR. Values shown are mean ± SEM from 2 independent experiments, each performed in triplicate. *P < 0.05, Student's t-test.

The Vorinostat and Bortezomib single agent time course experiments performed in the 2 multiple myeloma cell lines (Figure 5.6) revealed these lines to be highly sensitive to single agent treatment. Therefore, for combination studies Vorinostat was used at a concentration of 0.5 □M and Bortezomib at a concentration of 1 nM. In the U266 multiple myeloma cell line combination treatment induced ATF3 expression 8.01±0.77-fold compared to a 3.58±0.31-fold and 1.49±0.31-fold induction when treated with Vorinostat or Bortezomib alone, respectively (Figure 5.16A). Finally, in the OPM2 multiple myeloma line, the combination induced ATF3 expression by 140.10±4.88-fold, compared to a 9.62±1.93-fold by Vorinostat alone, and 35.00±1.34-fold by Bortezomib alone (figure 5.16B).

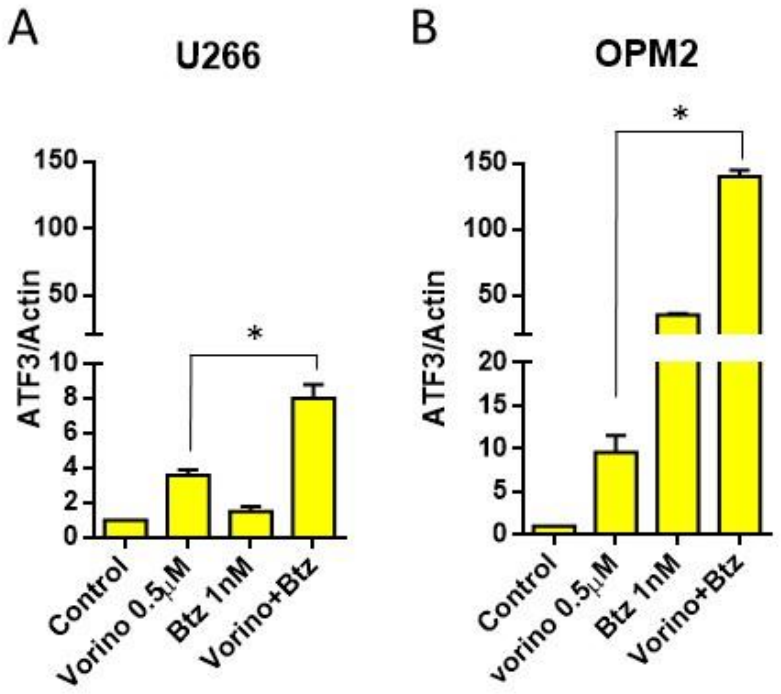

Figure 5.16 Effect of combination treatment with Vorinostat (Vorino) and Bortezomib (Btz) on *ATF3* mRNA expression in 2 multiple myeloma cell lines (A) U266 and (B) OPM2. Cell lines were treated with both drugs alone or in combination for 4 hours, and *ATF3* mRNA expression determined by qRT-PCR. Values shown are mean ± SEM from 2 independent experiments, each performed in triplicate. *P < 0.05, Student's t-test.

The enhanced induction of ATF3 by the combination was further demonstrated at the protein level where the combination enhanced ATF3 protein expression to a markedly greater extent compared to either agent alone in all 4 cell lines (Figure 5.17 A&B).

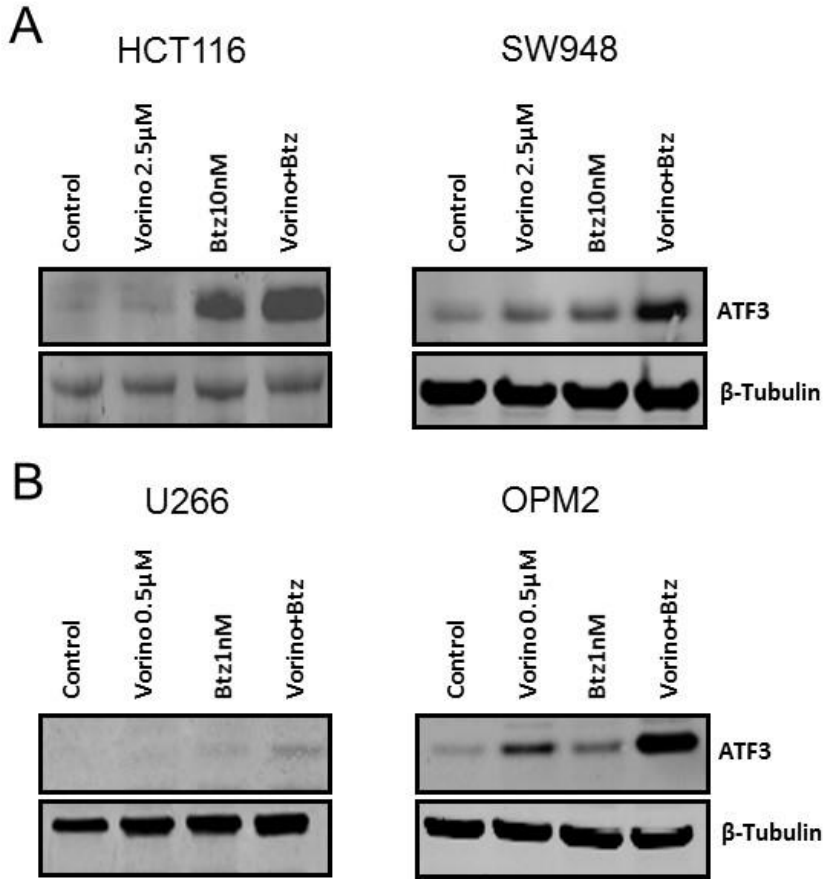

Figure 5.17 Effect of Vorinostat (Vorino) and Bortezomib (Btz) treatment alone and in combination, on ATF3 protein induction in (A) 2 colorectal cancer (HCT116 and SW948) and (B) 2 multiple myeloma (U266 and OPM2) cell lines determined by Western blot. Cells were treated with Vorinostat and Bortezomib alone and in combination for 24 hours.

5.2.7 ATF3 is required for the synergistic induction of apoptosis mediated by HDAC and proteasome inhibitor combination treatment

Our findings in Chapter 3, demonstrated a direct role for ATF3 in HDACi-induced apoptosis. Here, we demonstrate that the combination of HDAC and proteasome inhibitors further enhances ATF3 expression with a parallel induction of apoptosis. To determine if the additive induction of ATF3 drives this increase in apoptosis, ATF3 expression was downregulated in HCT116 and SW948 colorectal cancer cells using *ATF3*-targeting siRNAs, prior to drug treatment. ATF3-targeting siRNAs partially inhibited ATF3 induction by single agent and combination treatment in both cell lines (Figure 5.18). In parallel, the effect of ATF3 knockdown on the induction of apoptosis was determined by measuring levels of cleaved caspase 3. ATF3 knockdown markedly reduced levels of cleaved caspase 3 in cells treated with the combination, indicating a direct requirement for ATF3 in apoptosis induction.

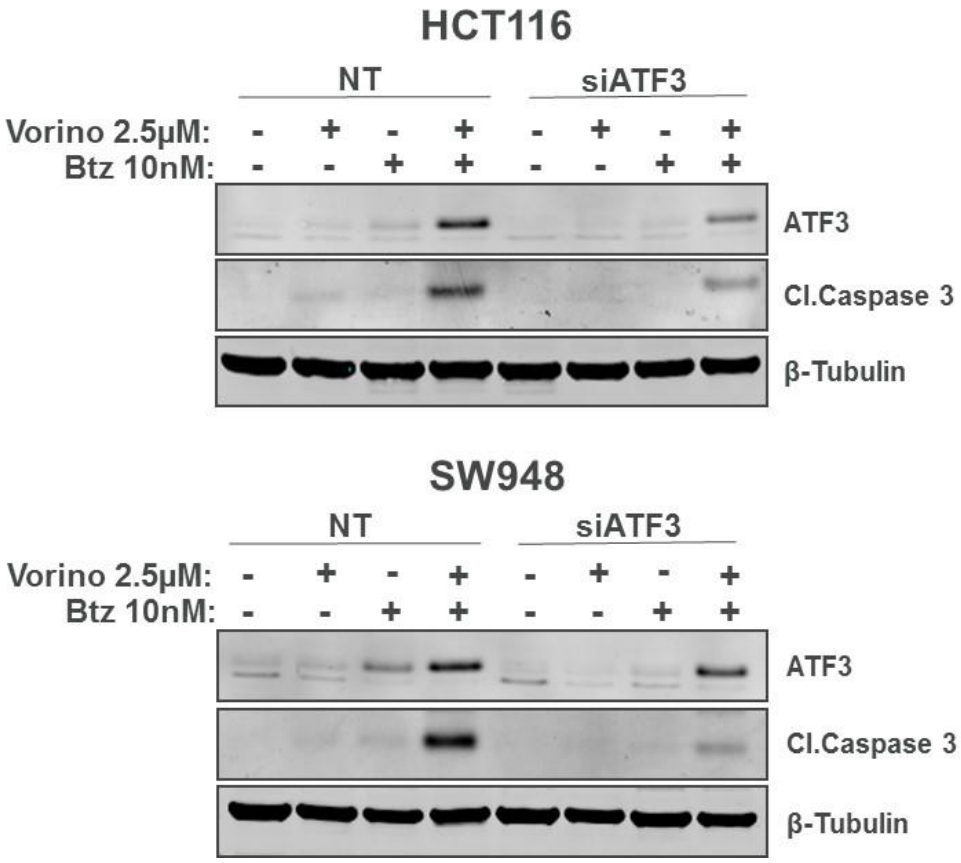

Figure 5.18 Effect of ATF3 knockdown on Vorinostat (Vorino) or Bortezomib (Btz)-mediated induction of apoptosis in 2 colorectal cancer (HCT116 and SW948) cell lines. Cells were treated for 24 hours and cleaved caspase 3 induction determined by Western blot.

These findings were also confirmed when apoptosis was assessed by PI staining and FACS analysis. In HCT116 cells, knockdown of ATF3 modestly attenuated Vorinostat and Bortezomib-induced apoptosis, with the greatest attenuation observed in cells treated with the combination, where ATF3 knockdown reduced apoptosis induction from 31.37±2.02% to 20.40±3.44%. Similarly, in SW948 cells the knockdown of ATF3 modestly attenuated Vorinostat and Bortezomib-induced apoptosis, with the greatest attenuation observed in cells treated with the combination, where apoptosis was reduced from 27.37±0.67% to 18.23±0.86% following ATF3 knockdown (Figure 5.19).

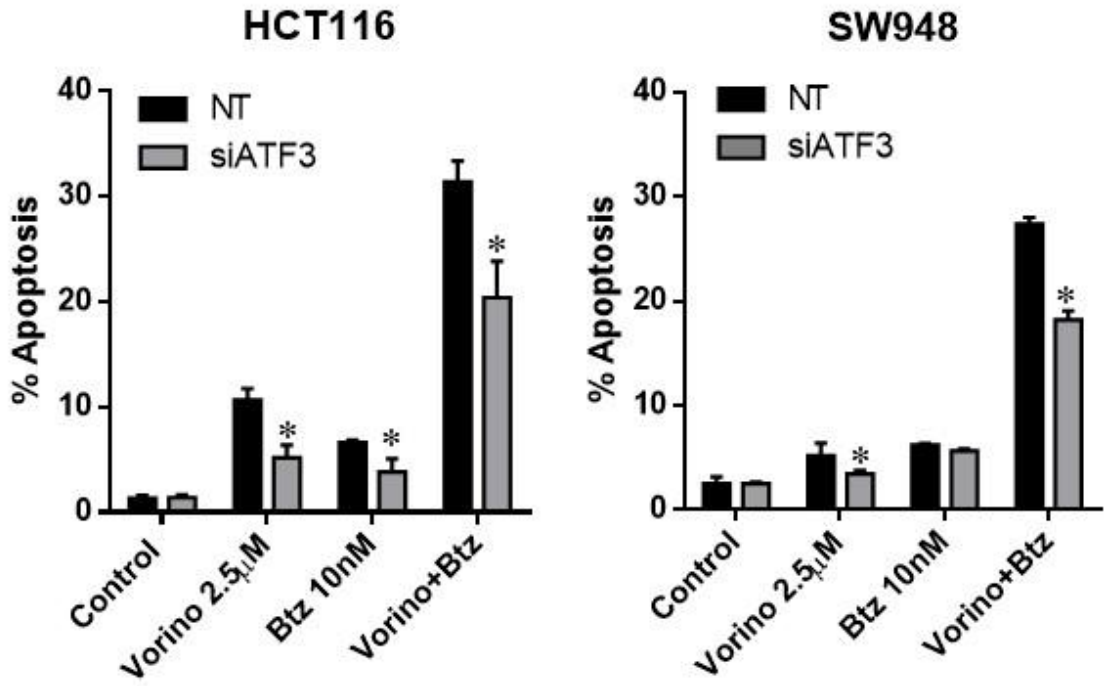

Figure 5.19 Effect of ATF3 knockdown on Vorinostat (Vorino) or Bortezomib (Btz)-mediated induction of apoptosis in 2 colorectal cancer (HCT116 and SW948) cell lines. Induction of apoptosis was determined by PI staining and FACS analysis. Values shown are mean ± SEM from a representative experiment performed in triplicate. *$P < 0.05$, Student's t-test.

5.2.8 *Enhanced ATF3 expression leads to an enhanced repression of BCL-X_L*

In Chapter 4 we demonstrated that HDACi-induced expression of ATF3 transcriptionally repressed BCL-X_L and that this was the mechanism by which HDACi induced apoptosis in HDACi-sensitive cancer cell lines. Therefore, we determined whether the enhanced induction of ATF3 by HDACi and proteasome inhibitor combination treatment also leads to an enhanced repression of BCL-X_L. Treatment of HCT116 cells with Vorinostat and Bortezomib in combination repressed BCL-XL mRNA to a greater extent compared to either agent alone (Figure 5.20).

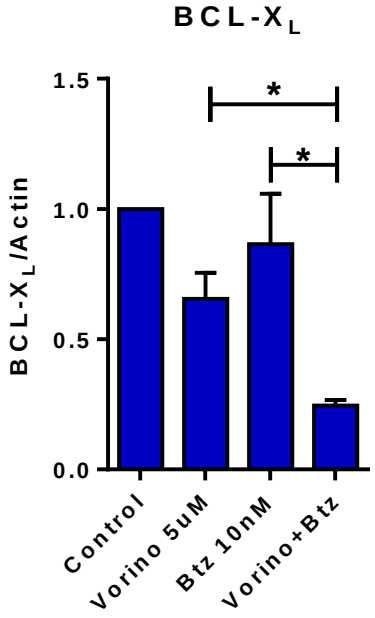

Figure 5.20 Effect of combination treatment with Vorinostat (Vorino) and Bortezomib (Btz) on *BCL-X_L* mRNA expression in HCT116. Cells were treated with both drugs alone or in combination for 24 hours, and *BCL-X_L* mRNA expression determined by qRT-PCR. Values shown are mean ± SEM from a representative experiment of 2 independent experiments, each performed in triplicate. *$P < 0.05$, Student's t-test.

5.3 Discussion

HDACi are a novel class of anti-cancer therapeutics which are approved for the treatment of CTCL and multiple myeloma [438, 470] and have displayed anti-tumour activity in a variety of other haematological malignancies and solid tumours [43, 122, 416, 471]. HDACi induce their anti-tumour effects via a diverse array of mechanisms, including induction of cell cycle arrest, differentiation and apoptosis. Induction of apoptosis has been investigated in the greatest detail, and been shown through studies in mouse models to be key event in mediating the anti-tumour activity of these agents [43, 194, 198]. HDACi-induced apoptosis is associated with altered expression of a number of pro and anti-apoptotic genes [43, 198], however these changes vary significantly both between cell types as well as within a particular tumour type [11]. In the Chapter 3 of this thesis, we demonstrated that induction ATF3 is the key determining factor of HDACi-induced apoptosis which transcends tumour type.

The proteasome inhibitor Bortezomib, is approved for the treatment of multiple myeloma [438] and mantle cell lymphoma [439]. Notably, a number of previous studies have demonstrated that combination treatment of HDAC inhibitors and proteasome inhibitors synergistically inhibits cell proliferation and induces apoptosis in several tumour types *in vitro* [416, 472, 473]. For example, Pitts *et al* demonstrated synergistic induction of apoptosis by combining HDAC inhibitors and proteasome inhibitors by measuring levels of active caspase-3 and -7, and levels of the pro-apoptotic regulator protein, BIM [416]. Based on these pre-clinical findings, a number of clinical trials of this combination were undertaken and the combination of Panobinostat and Bortezomib was recently approved for the treatment of multiple myeloma [121].

In this Chapter, we determined the mechanistic basis for the synergistic apoptotic activity of this drug combination. We first confirmed that combination treatment with Vorinostat and Bortezomib synergistically induced apoptosis in multiple cell lines. We also demonstrated similar effects when Vorinostat was replaced by 3 other HDACi, Depsipeptide, Belinostat or Panobinostat.

We specifically focused on the possibility that the synergistic apoptotic activity induced by this combination is due to enhanced induction of ATF3. This was based on the established observation that proteasome inhibitor treatment in cancer cells robustly induces the JNK and p38 stress response pathways, which are known to induce the expression of ATF3. We demonstrate that similar to HDACi, proteasome inhibitors induce the expression ATF3 in both colorectal cancer and multiple myeloma cell lines and that combination treatment with HDACi further enhanced the expression of ATF3.

Furthermore, our results indicate that the mechanistic basis for the enhanced induction of ATF3 expression by the combination was due to the 2 agents inducing ATF3 expression via independent mechanisms. First, as expected, we demonstrated that HDAC inhibitor, but not proteasome inhibitor mediated induction of ATF3 is dependent on the Sp1 and Sp3-transcription factors. The role of the Sp1 and Sp3 transcription in regulating HDACi-induced gene expression changes is consistent with a number of previous studies [309, 474, 475]. Conversely, we demonstrated that proteasome inhibitor mediated induction of ATF3 is dependent on the p38 pathway. Finally, through ATF3 knockdown experiments, we were we were able directly establish that ATF3 induction by the combination was required for the synergistic induction of apoptosis.

Having previously established that HDACi-induced apoptosis involves ATF3-mediated repression of BCL-X_L (Chapter 4) we hypothesized that the combination of HDACi and proteasome inhibitors would further repress BCL-X_L expression. As expected, the combination enhanced the repression of *BCL-X_L* mRNA. However, further experiments are required to determine if this effect is mediated through ATF3.

Our finding that the enhanced induction of ATF3 can synergistically induce apoptosis, suggests that other drug combinations which also induce similar effects. For example, the chemotherapeutic agent cisplatin has been shown to induce apoptosis via the MAPK- dependent induction of ATF3 [476]. Furthermore, a study by St Germain *et al* demonstrated that the enhanced induction of ATF3 was a key driver of the enhanced apoptotic response induced by the combination [377]. Analysis of the Broad Institute's Connectivity Map (CMap) could potentially identify a range of therapeutics which induce ATF3 which could be explored in therapeutic combinations.

In conclusion, these findings demonstrate that the synergistic induction of apoptosis by the combinatorial treatment of cancer cells with HDAC and proteasome inhibitors is driven by the enhanced induction of the ATF3 transcription factor. We demonstrate that the enhanced induction of ATF3 is due to HDAC and proteasome inhibitors inducing expression of ATF3 via different mechanisms. These findings provide mechanistic insight into the molecular basis for the clinical activity of this drug combination.

Chapter 6: General Discussion

6.1 Summary

HDACi have proven to be efficacious in the treatment of some haematological cancers, however their activity in solid tumours is limited. Elucidation of the mechanisms by which HDACi induce apoptosis could thus provide important biological insight into the molecular basis for the differential tumour responses to these agents, which in turn could aid in broadening the use of this approved anti-cancer therapeutic to solid tumours. Initial experiments performed in our laboratory that have been submitted for publication (Chueh, Tse *et al*, under revision in Clinical Cancer Research), demonstrated that the induction of c-FOS, c-JUN and ATF3 by HDACi were correlated with apoptotic sensitivity to HDACi. This thesis identifies an apoptosis inducing cascade wherein HDACi-induced expression of ATF3 directly represses BCL-X_L to promote HDACi-mediated apoptosis. These results led to the finding that HDACi-resistant cells could be re-sensitised by molecular or pharmacological inhibition of BCL-X_L. Lastly, we demonstrated that the synergistic apoptosis induced by combining HDACi and proteasome inhibitors, is a direct consequence of the enhanced induction of ATF3 induced by these agents.

6.2 Mechanism by which HDACi induce ATF3 expression

The finding that the magnitude of ATF3 induction by HDACi is a central determinant of apoptotic response to these agents, raises the question of why HDACi induce ATF3 expression in some cell lines more than others? In Chapter 3 we investigated this by determining whether differences in drug uptake or the ability of the drug to induce histone acetylation may account for the difference. However, analysis of acetylated histone H3 (AcH3) levels following HDACi treatment revealed no differences between sensitive and resistant lines, indicating the differential induction of ATF3 in these cell lines was not due to differences in drug uptake or a reflection of a global difference in substrate targeting.

HDACi-regulation of a number of genes has been previously shown to be mediated by the Sp1 and Sp3 transcription factors [224, 309, 474]. Likewise, the requirement of Sp1/Sp3 transcription factors in HDACi-mediated induction of ATF3 was specifically

investigated in the previous study from my laboratory in HDACi sensitive colorectal cancer cell lines [309]. This study demonstrated that the ATF3 promoter harbors multiple Sp1 and Sp3 binding sites to which Sp1 and Sp3 was shown to localise in ChIP analyses. Furthermore, knockdown of Sp1 and Sp3 with siRNA or the Sp1 and Sp3 inhibitor Mithramycin attenuated the induction of ATF3 by HDACi. In this thesis, these findings were confirmed and extended to additional tumour types by demonstrating that the inhibition of Sp1 and Sp3 attenuated HDACi-mediated induction ATF3 as well as apoptosis (Chapter 5, Figure 5.9). Collectively, there is now strong evidence to demonstrate the importance of the Sp1 and Sp3 transcription factors in mediating HDACi-induced expression of ATF3.

Why Sp1 and Sp3 are preferentially activated by HDACi in sensitive compared to resistant cells to cause the differential induction of ATF3 and subsequently HDACi-induced apoptosis remains to be determined, although some possibilities can be eliminated.

First, my laboratory previously examined whether the abundance of Sp1 and Sp3 may explain the differential induction of ATF3 and other genes in HDACi sensitive versus resistant colorectal cancer cells but failed to observe any difference. Simple differences in abundance of these factors therefore are unlikely to explain the differences in induction ATF3 gene expression. Second, while Sp1 and Sp3 are required for induction of several HDACi target genes, HDACi do not alter expression of these transcription factors, and furthermore HDACi treatment does not alter Sp1/Sp3 enrichment at the promoters of target genes [106, 309, 477, 478]. Therefore, differential induction of Sp1 or Sp3 following HDACi treatment or differential recruitment of these factors to target gene promoters is unlikely to explain the differential induction of target genes.

Instead, there is now extensive evidence that Sp1 and Sp3 are regulated by post-translational modification, and that these changes can regulate their transcriptional activity. For example, Sp1 and Sp3 are both regulated by acetylation, and HDACi-induced acetylation of Sp1 has been demonstrated in several cancer cell lines including pancreatic, colorectal cancer and cervical cancer lines [479-481]. The acetylation of Sp1 was also shown to induce transcriptional activation the Sp1 target

gene MMP28 [481]. Similarly, HDACi induces acetylation of Sp3 in breast and colorectal cancer cells [106, 482], and Ammanamanchi *et al* provided several lines of evidence to demonstrate that acetylated Sp3 acts as a transcriptional activator of the TGFβRII gene [106]. One possibility, therefore is that Sp1 and Sp3 may be differentially acetylated by HDACi in sensitive and resistant cell lines, which may explain the differential activation of these factors. To address this, we do plan to perform immunoprecipitation and mass-spectrometry analyses to determine whether HDACi do mediate post-translational modification of Sp1 and Sp3, and to determine whether these effects may vary between HDACi sensitive and resistant cell lines.

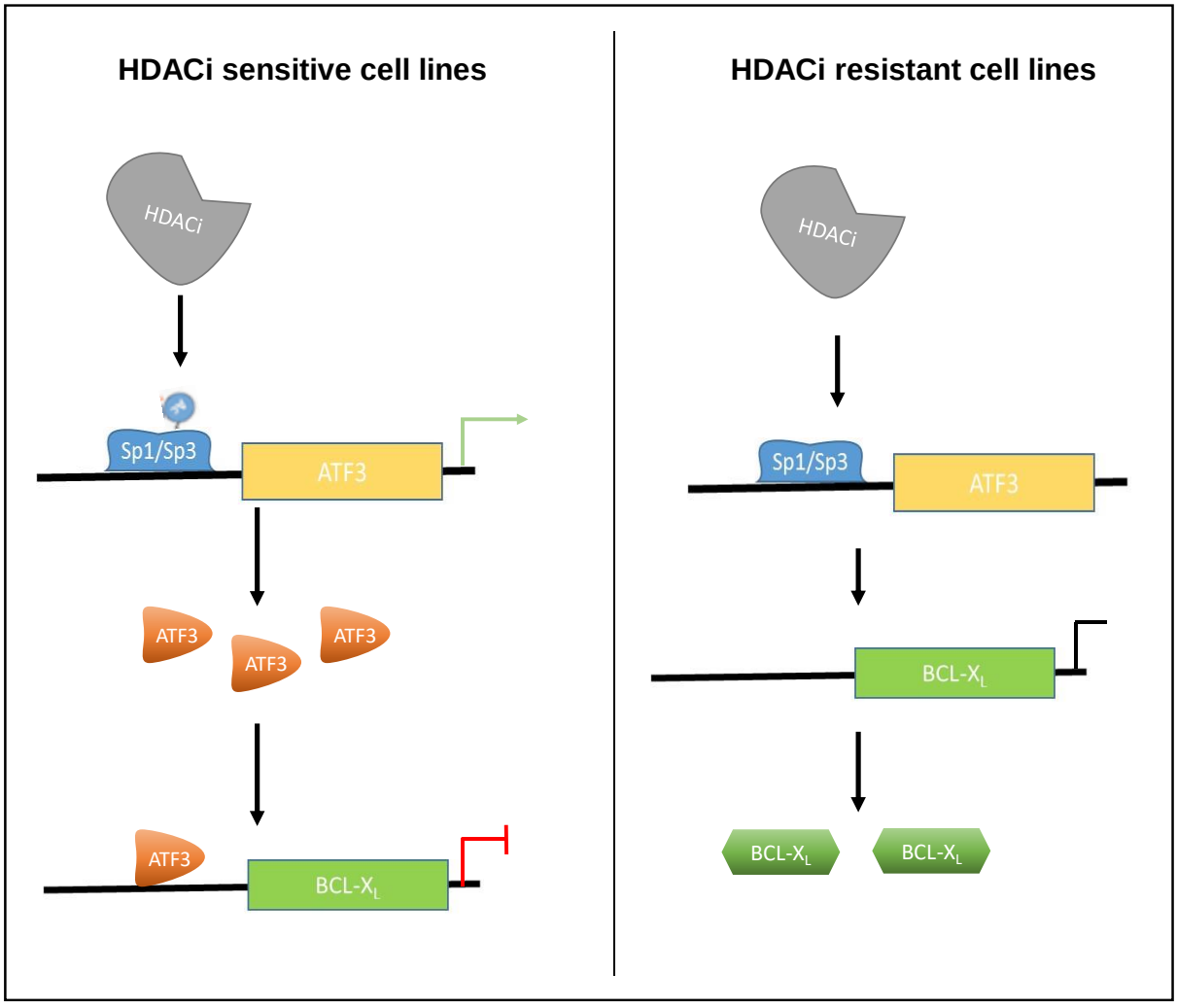

Figure 6.1 The postulated apoptotic cascade by which HDACi treatment induces apoptosis. Sp1 and Sp3 may be more likely subjected to HDACi-induced post-translational modifications such as acetylation in the sensitive cell lines compared to the resistant cell lines. Therefore, providing a mechanistic rationale for the differential induction of ATF3 between the cell lines. The induced ATF3 subsequently binds to the BCL-X_L promoter to repress the expression of BCL-X_L and as such induces apoptosis.

6.3 Predictive biomarkers of response to HDACi

Clinical responses of HDACi as single agents in solid tumours have demonstrated limited efficacy, however studies investigating rational combinations of HDACi with other anti-cancer therapeutics have demonstrated promising results [483]. Moreover, single agent therapy in CTCL patients only provides a response rate of approximately 30% [484]. Therefore, biomarkers to identify those patients likely to benefit from treatment with these agents and as well as strategies to broaden the use or improve the efficacy of HDACi are needed. Work described in this thesis supports the notion that ATF3 could serve as possible biomarkers of response to HDACi.

In Chapter 4 of this thesis, we demonstrated that HDACi preferentially induce expression of the immediate-early genes c-FOS, c-JUN and ATF3 in HDACi sensitive compared to resistant lines, and that this effect is independent of tumour type. Notably, induction of these genes is observed within 2-6 hours of HDACi treatment. This finding suggests that the induction of these genes following short-term HDACi treatment could serve as a biomarker of response to HDACi. Indeed, in our recently submitted paper (Chueh, Tse et al, submitted), we determined whether the induction of immediate early genes including ATF3 could be detected in CTCL patients receiving HDACi treatment. Analysis of peripheral blood mononuclear cells (PBMCs) isolated from 2 patients containing high levels of tumour cells before and 4 hours post HDACi treatment demonstrated that HDACi-induction of these immediate-early genes is indeed detectable in clinical samples (data not shown), demonstrating the feasibility of using immediate-early genes (including ATF3) induction as a potential biomarker of response to HDACi therapy. Unfortunately, the anti-tumour response elicited in these 2 patients is not known, and a prospective study involving sufficient patient numbers is required to address this question.

Interestingly, while investigating the effect of HDACi on repression of BCL-X_L we observed that basal levels of BCL-X_L were higher in the sensitive lines (Chapter 4, Figure 2A). While the number of cell lines investigated was small, it does raise the possibility that high basal levels of expression of BCL-X_L may also serve as a potential biomarker of sensitivity to HDACi, although this finding will need to be validated in a larger panel of cell lines and in patient samples.

Furthermore, we also noted that the HDACi sensitive cell lines with basally higher levels of BCL-X_L were also sensitive to single agent BCL-X_L inhibitor treatment (Figure 4.10, 4.12 and 4.14), raising the possibility that sensitivity to HDACi may be related to both the ability to induce ATF3 but also the co-incident reliance of at least a subset of these cell lines on BCL-X_L for their survival. Addition study is required to further investigate this association.

6.5 Clinical development of the HDACi and BCL-X_L combination

Consistent with our findings, several previous studies have also demonstrated synergistic anti-tumour activity when HDACi are combined with a BCL-X_L inhibitor. This has been demonstrated in cell lines derived from various tumour types including leukemia, melanoma and small cell lung carcinoma [227, 485, 486]. Mechanistically, this has been attributed to HDACi inducing pro-apoptotic genes such as BIM, but their sequestration by BCL-X_L limits their apoptosis inducing capacity. The addition of a BCL-X_L inhibitor has been proposed to free BIM enabling apoptosis induction. In agreement with this model, our screen did identify induction of several pro-apoptotic genes, including BIM, BIK, BMF and NOXA in response to HDACi treatment, although notably induction of these genes occurred in both sensitive and resistant cell lines. One possibility therefore is that in HDACi resistant cell lines, these factors are sequestered by BCL-X_L, due to the failure of HDACi to induce ATF3 and drive the repression of BCL-X_L in these cell lines. Consistent with this model, we demonstrated that the addition of a BCL-X_L inhibitor can re-sensitise these cells to HDACi-induced apoptosis, while notably, treatment with a BCL-X_L inhibitor alone, was insufficient to induce apoptosis. Conversely, in sensitive cell lines, the parallel induction of BIM, BMF, BIK and NOXA with repression of BCL-X_L, alters the balance of pro and anti-apoptotic factors in the cell in favour of pro-apoptotic proteins, resulting in apoptosis.

The consistent synergistic induction of apoptosis induced by this combination, coupled with the additional mechanistic insight obtained, suggests further clinical investigation of this combination may be warranted. However, one hurdle that needs to be

overcome are the side effects of BCL-X_L inhibitors, most notably thrombocytopenia [399, 400].

6.7 Concluding remarks

Collectively, the research presented in this thesis presents a novel mechanism by which HDACi-induced expression of ATF3 directly represses the expression of BCL-X_L, subsequently leading to apoptosis in multiple tumour types (Figure 6.1). Elucidation of this apoptotic cascade further led to the rational identification of the HDACi and BCL-X_L inhibitor combination as a strategy for overcoming inherent resistance to HDACi.

In addition, the identification of a key role for ATF3 in apoptosis induction by HDACi provided the basis for understanding the mechanism by which combining HDACi and proteasome inhibitors synergistically induces apoptosis.

Finally, these data provide the basis for the exploration of ATF3 induction and BCL-X_L repression as biomarkers of response to HDACi therapy. In summary, the work from this thesis may ultimately contribute to the broader use of HDACi therapy in solid tumours through the identification of potential biomarkers of response to HDACi and the identification of rational drug combinations.

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