Molecular characterisation of paediatric ALL and new therapeutic strategies

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ABSTRACT

Despite high cure rates achieved in the last two decades, treatment resistance and therapy-associated toxicity is still observed in a significant proportion of children with ALL, warranting alternative therapeutic approaches. Results obtained in our laboratory suggest that transcriptional upregulation of prosurvival pathways plays a key role in paediatric B-precursor ALL chemoresistance, thus providing a strong rationale for the inhibition of prosurvival gene transcription. In this study, I investigated the role and feasibility of the epigenetics-regulating BET family of proteins as therapeutic targets. I showed that BRD4 is ubiquitously expressed in paediatric ALL tumours and that treatment with the BET protein inhibitor JQ1 led to potent *in vitro* sensitisation of ALL tumour cells regardless of cellular phenotype, as well as *in vivo* sensitisation using xenograft models of ALL. I observed strong *in vitro* synergy in ALL cell lines and primary tumours treated with a combination of JQ1 and dexamethasone. Using microarray technology I confirmed BET protein inhibition involves transcriptional downregulation of prosurvival pathways and identified potential biomarkers predictive of JQ1 sensitivity. I characterised the cellular effects of BET protein inhibition in pre-B ALL and showed this was associated with inhibition of cell cycle progression, downregulation of c-Myc protein, direct inhibition of DNA replication and induction of caspase-dependent apoptosis that was independent of p53 activity. Thus, pharmacological inhibition of BET proteins provides an alternative strategy by which to downregulate prosurvival signaling and target B-precursor ALL. Finally, I explored PIM kinases that co-operate with BET proteins during the regulation of transcription as alternative therapeutic targets in B-precursor ALL. I was able to show this therapeutic approach holds promise, and warrants further study.

This thesis is dedicated to

Arminda Cabecas

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CONTENTS

LIST OF FIGURES

RESULTS II: THE PIM2 KINASE AS A CANDIDATE FOR FURTHER STUDY

APPENDICES

8.4. Appendix 4- Photograph of original blot showing BRD4 expression in ALL cell lines

8.5. Appendix 5- Photograph of original blot showing BRD4 expression in ALL primary tumours

8.6. Appendix 6- Schematic showing the possible structures that can be identified using the fluorescent DNA replication fibre technique

LIST OF TABLES

Table 4.3: GSEA pathways positively associated with PIM2 gene expression in primary ALL tumours

APPENDICES

8.1. Appendix 1- Clinical information for the AML patients used as positive controls in JQ1 cytotoxicity experiments

8.2. Appendix 2- Clinical information for the B-precursor ALL patients used in the PIM2 qRT-PCR expression screen

LIST OF ABBREVIATIONS

Ab antibody
AIF apoptosis inducing factor
ALL acute lymphoblastic leukaemia
APS ammonium persulphate
ASPP apoptosis-stimulating proteins of p53
A-T ataxia-telangiectasia
ATLD ataxia-telangiectasia-like disorder
ATM ataxia-telangiectasia mutated
ATR ATM and Rad3-related
ATRIP ATR interacting protein
Bak BCL2 antagonist killer
Bax BCL2-associated X
Bcl2 B-cell lymphoma 2
BER base excision repair
BM bone marrow
bp base pair
BRCA breast cancer susceptibility gene
BRD4 bromodomain-containing 4
BSA bovine serum albumin
cALL common ALL
CD cluster of differentiation
CDK cyclin-dependent kinase
cDNA complementary DNA
cDNA complementary DNA
CLL chronic lymphocytic leukaemia
CNS central nervous system
DBD DNA binding domain
DDR DNA damage response
DISC death-inducing signalling complex

DMSO dimethyl Sulphoxide
DNA deoxyribonucleic acid
DNA-PK DNA-dependent protein kinase
DSB double-strand break
dsDNA double-stranded DNA
ECL enhanced chemiluminescence
FACS fluorescence-activated cell sorting
FCS foetal calf serum
GADD45 growth arrest and DNA damage-inducible 45
H3 histone variant H3
H3 S10-P phosphorylated histone H3
HR homologous recombination
Ig immunoglobin
IgH Ig heavy chain
IgL Ig light chain
IL interleukin
IR ionising radiation
kDa kilodalton
MDM2 murine double minute 2
ml millilitre
MMR mismatch repair
MRD minimal residual disease
mRNA messenger RNA
NBS nijmegen breakage syndrome
NER nucleotide excision repair
NHEJ non-homologous end-joining
PARP poly(ADP-ribose) polymerase
PBS phosphate buffered solution
PCR polymerase chain reaction
PI propidium iodide
PI3K phosphoinositide 3-kinase
pRB retinoblastoma protein

PUMA p53 upregulated modulator of apoptosis

qRT-PCR quantitative reverse transcriptase polymerase chain reaction

RNA ribonucleic acid

rpm revolutions per minute

TNF tumour necrosis factor

TP53 tumour suppressor 53

µM micromolar

UV ultraviolet light

WCC white cell count

WT wildtype

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

1.1. Overview

Leukaemias are the most common malignancy of childhood with acute lymphoblastic leukaemia (ALL) accounting for approximately 25% of all paediatric tumours [1]. ALL is a disease that affects both children and adults of all ages, with prevalence peaking between the ages of 2 and 5 years [2]. This disease is characterised by deregulation of the B-cell development process that can result in a block at specific stages of differentiation and uncontrolled clonal proliferation of early lymphoid progenitor cells of either T- or B-cell lineage, which invade the bone marrow, peripheral blood, and organs such as the liver and spleen [2]. Although ALL can occur in either cell lineage, Pre-B ALL accounts for the greatest proportion of cases (80%) in comparison to pre-T cell ALL (15%) [3].

As recently as the 1960s ALL was largely untreatable with nearly all cases proving fatal [1]. However, major improvements in paediatric haematology and oncology, together with independent clinical and molecular risk-stratification over the past 40 years have successfully increased overall survival to over 80%. Nevertheless, ALL is still a major cause of morbidity and mortality in children around the world with over 15% of patients suffering from disease relapse following first line therapy[1]. This, together with the fact that current treatment options lead to significant toxic side-effects, provide the rationale for the

requirement of alternative therapies with far less toxic consequences, whilst remaining specific and effective in reducing the burden of leukaemia [1].

1.2. Clinical classification of paediatric ALL

1.2.1. B-cell differentiation

Since ALL represents the proliferation of lymphoid progenitor cells that are unable to fully differentiate, it is important to consider B-cell development within the context of ALL.

Haematopoietic stem cells (HSCs) are pluripotent progenitor cells that may differentiate into either lymphoid cells (B- and T- lymphocytes), or myeloid cells (red blood cells, granulocytes, megakaryocytes, and macrophages) depending on the cellular signals they receive [4]. Mature immunocompetent B-cells are required for the production of antibodies essential to the adaptive humoral immune response. The formation of mature B-cells is a highly complex and tightly regulated process, which begins with B-cell lineage commitment of HSCs in the bone marrow compartment, and ends with the production of mature B-cells in secondary lymphoid organs such as the liver, thymus, and spleen, which also maintain proliferation and differentiation of HSCs throughout most of embryonic development [4].

Several transcription factors play critical roles in determining the cell fate of HSCs. The commitment of HSCs to the lymphoid lineage is regulated by transcription factors such as E2A, Early B cell Factor (EBF) and Pax5 [5-7]. Such transcription factors are essential for maintenance of B cell progenitors through the various stages of B cell differentiation and ensuring normal B cell

lymphopoiesis takes place. Thus, deregulation of B cell development often leads to malignant transformation, including B-precursor ALL [4].

More specifically, B-precursor ALL in particular is characterised by lymphoid cells showing maturation arrest within the late pro-B, large pre-B, and small pre-B stages of differentiation, meaning this type of leukaemia may be subdivided into pro-B (null), common, and pre-B ALL, respectively [8].

1.2.1.1. The early stages of B cell development

In order for a common lymphoid progenitor cell to undergo full maturation into a mature B cell capable of producing antibody, it must go through several stages of differentiation. These stages include the common lymphoid progenitor, the early B cell, the pro-B, pre-BI, pre-BII cells, and the immature B cell, before ending with the mature B cell [8, 9]. Rearrangement of the variable, diversity, joining, and constant regions of the immunoglobulin heavy chain (IgH) takes place at specific stages of the maturation process, together with the expression of a number of surface markers that allow for the identification and classification of different subtypes of leukaemia [8].

1.2.1.2. Immune system gene rearrangements

The development and maturation of B cells is required for the generation and expression of unique B cell receptors (BCRs), which specifically bind to new or recurrent antigens encountered by the human body [10]. The BCR is an immunoglobulin (Ig) expressed on the B cell membrane and consists of a heterodimer of matching pairs of Ig heavy chains (IgH) and two Ig light chains (IgL).

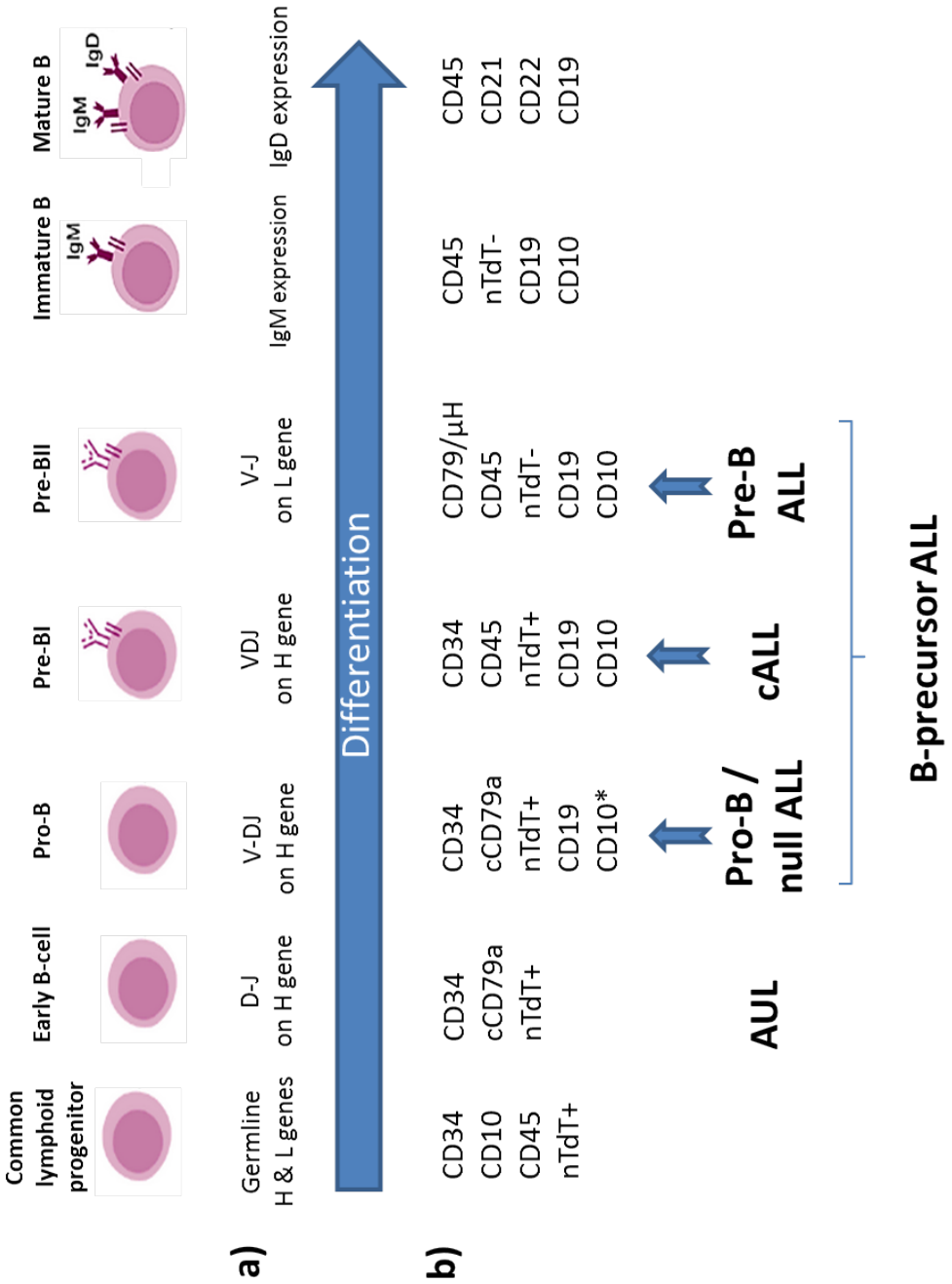


Figure 1.1: Stages of B-cell development

a) Rearrangement of the immunoglobulin heavy and light genes (H and L, respectively) during B-cell development.

b) Expression of markers that are characteristic of specific stages of B-cell differentiation and are used to identify leukaemia subtypes.

(n, nuclear; c, cytoplasmic; *, not expressed in all cells of this type; μH, μ heavy chain surface expression; V, variable; D, diversity; J, joining; AUL, acute undifferentiated leukaemia; cALL, common ALL)

These portions of the BCR are peptides with a C terminal region that exists in a limited number of forms, and which determines the class and effector function of the respective antibody molecule. Five classes of antibody exist, namely α (IgA), δ (IgD), ε (IgE), γ (IgG), and μ (IgM), with each class being specialised for the particular method by which it binds, neutralises and removes antigens [10]. However, the IgH and IgL heterodimer is itself unable to activate biological responses following the binding of a specific antigen. It is the disulphide-coupled heterodimer of Igα (CD79a) and Igβ (CD79b) located towards the C-terminal region of the IgH and IgL peptide chains that transduces the binding of specific antigens into alterations in intracellular signalling pathways. The IgH and IgL heterodimer contains the variable amino acid sequence (antibody variable region) that give rise to the binding specificity required for antigen recognition, and it is the ordered expression and assembly of these components of the BCR that allow for the definition and characterisation of each stage of B cell development [11].

1.2.1.3. VDJ recombination

In order for clonal diversity to be achieved, the genes encoding the IgH and IgL chains contain constant and variable regions that made up of segments know as V (variable), D (diversity), and J (joining), and are arranged and put together by a tightly regulated, site-specific recombination process known as VDJ recombination [4]. This process is essential for the adaptive immune function of the lymphocyte, and consists of rearrangement of the D and J fragments, after which the V fragment is joined to the DJ fragment. This occurs first in the heavy chain loci, then in the light chain loci, and is a process regulated by enzymes

encoded by recombination activating gene (RAG)-1 and RAG-2 [12, 13]. The fact that there are over 200 variable (VH), 30 diversity (DH), and 6 joining (JH) IgH chain genes means that a very large number of rearrangement combinations are possible [14-16]. Enzymes involved in DNA double strand (DSB) repair, such as DNA-PK, XRCC4, Artemis and DNA ligase IV, are all involved in the re-ligation of cleaved segments of V, D and J genes [17]. Further diversity is also attained by imprecise relegation of gene segments, as well as the random insertion of nucleotides between segments by the enzyme terminal deoxynucleotidyl transferase (TdT) [18]. The pro-B stage of B development is the first stage that exhibits B cell lineage commitment and is characterised by rearrangement of IgH. The pro-B stage may be further divided into sub-phases according to the extent of heavy chain recombination. When the heavy chain is in the germ line state, this is described in humans as early pro-B. The point at which D and J segments are recombined is known as pro-B. Finally, the point at which V-DJ recombination has occurred is known as pre-B I [4].

In the pro-B stage, chaperone proteins such as calnexin associate with Igα and Igβ, which are expressed on the cell surface [19]. Once the IgH chain is recombined, it is assembled together with Igα and Igβ, as well as IgL chain components, and expressed as an immature pre-BCR on the surface of the B cell membrane. Expression of this receptor marks entry into the pre-B stage of development [20].

Any errors during the VDJ recombination process or assembly of the pre-BCR components, prevents any further B cell development due to a block in signal transduction from the immature receptor, preventing pro-B cells from entering

the pre-B stage and instead leading to apoptosis. Cells in the pre-B stage undergo recombination of the IgL chain V and J fragments, allowing for the assembly of the BCR and marking the entry of cells into the pre-B I step [21-24].

The proliferative state and expression of surface markers also allow for the identification of pro-B and pre-B stages of B cell differentiation. Pro-B and pre-B stages are characterised by waves of VDJ recombination followed by waves of proliferation that do not occur simultaneously. These waves of activity are initiated and maintained by tightly regulated expression of recombinase associated genes 1 and 2 (RAG1 and RAG2) during G0, which encode two enzymes that are key to VDJ rearrangement and are then degraded before cells enter into S phase [25]. Keeping these two waves separate is important since VDJ rearrangement is associated with non-homologous recombination and would increase the mutation rate during DNA replication. The proliferative pro-B and pre-B stages of differentiation are often found to be deregulated in B cell ALL and so it is suggested that failure in keeping DNA replication and VDJ recombination separate during B cell development could be a factor in blocking differentiation and driving leukaemogenesis [4].

1.2.1.4. Immunophenotypic classification

Previous studies investigating immunological markers of lymphoid leukaemias provided invaluable information regarding cell lineage and stages of differentiation of leukaemia cells, which consequently greatly improved the precision with which ALL is diagnosed [26].

The maturation of HSCs into fully developed B cells is associated with changes in gene expression that modulate the presence of a variety of surface markers. Examples of such markers are the receptor and ligand proteins known as cluster of differentiation (CD) molecules. Studies that began including the assessment of immunologic markers revealed that immunophenotypically distinct subpopulations of ALL correlated with specific molecular and karyotypic abnormalities, and also provided further insight into the mechanisms of lymphocyte signal transduction [26]. Furthermore, antigen expression studies led to the notion that malignant lymphoblasts were a result of normal lymphoid progenitor cells arrested at various stages of differentiation [27, 28]. It is therefore clear to see how important the recognition of lymphocyte differentiation antigens and specific immunophenotypes are within the context of this disease.

B cells derived from common lymphoid progenitors are known to express CD34, CD10, and CD45 [29]. However, the pan B cell marker CD19 is not expressed and it is uncommon for surface CD127 (IL-7R) and nuclear TdT to be expressed [30].

Common lymphoid progenitor cells that enter the early B cell stage of differentiation begin to express B-lineage specific genes such as the Vpre-B and cytoplasmic CD79a (cCD79a) genes, which encode components of the surrogate light chain [31, 32]. Cells at the pro-B stage of B cell differentiation express CD34 and CD19, and sometimes CD10 [33].

Cells that are at the pre-B-I stage express surface markers CD34, CD45, CD10, and CD19. Nuclear TdT is also expressed at pre-B-I [34]. Further maturation into the pre-B-II stage is characterised by loss of nuclear TdT expression, along with CD34 [8]. At this point CD79 may be expressed, which coincides with expression of surface μ heavy chains [35].

Immature B cells express CD10, CD19, IgM, and increasing levels of CD45. Further development to mature B cells is characterised by loss of CD10 and surface IgD expression [8, 34].

Generally, B cell ALL expresses an immunophenotype that is similar to normal B cell progenitors and is characterised by the expression of CD34, CD19, cytoplasmic CD79a and nuclear TdT. In addition to these surface markers CD10, CD20, CD22, and CD24 are sometimes also expressed [2].

Surface expression of CD66c has been reported in approximately 33% of B-ALL cases, compared to normal B cell progenitor cells where this molecule is absent [36]. Other more discrete examples of deregulated surface markers during B-cell differentiation include the expression of CD21 on CD19+CD34+ B-ALL cells, and not on healthy cells with a similar immunophenotype. In B-ALL, CD10, CD19, and CD34 are usually found to be overexpressed, whereas CD45 and CD38 are commonly underexpressed [36].

1.2.2. Morphological classification

Morphology was for many years a main criterion for primary diagnosis of ALL and may be used to differentiate this type of leukaemia from acute myeloid leukaemia. The diagnosis of ALL is defined as malignant lymphoblasts being in

excess of 5% of the total number of cells in the bone marrow, equating to at least 10^8 lymphoblasts in a 10^{10} total cell count.

Morphological classification of ALL first began in 1976 and followed guidelines devised by the French-American-British (FAB) Cooperative Group, which described three morphological subtypes: L1, L2 and L3 [37, 38]. The L1 subtype consisted of small lymphoid cells that contained hardly any cytoplasm but had regular nuclei exhibiting homogenous chromatin and no nucleoli. The incidence of this subtype in B-ALL is approximately 25-30%. The L2 subtype consists of larger heterogeneous cells with cytoplasm and a nucleus with an irregular shape that contains net-like chromatin and nucleoli. This particular subtype is observed in approximately 65-70% of cases. The third subtype L3, is used to describe homogenous cells with a basophilic cytoplasm containing vacuoles. A prominent nucleolus and chromatin with a speckled appearance is also descriptive of the L3 subtype, which is observed in 5-10% of cases [39].

Although cytochemistry is best used for distinguishing ALL from myeloid leukaemias, use of morphological examination by light microscopy together with cytochemistry often proves to be of limited use, since distinction between the subtypes can at times be subjective and difficult. B-ALL lymphocytes tend to exhibit a morphology resembling the L1 and L2 subtypes. ALL of the L3 subtype is reported to be very similar to Burkitt's lymphoma in terms of biological characteristics and in response to chemotherapy, highlighting the fact that the FAB classification system is on its own inadequate [40]. Indeed, following recommendation by the World Health Organisation (WHO), morphological examination is currently not routinely used for diagnostic

purposes. Instead, other more robust methods of diagnosis and risk stratification making use of immunophenotype, molecular features and cytogenetics have been subsequently adopted [41].

1.3. Aetiology of paediatric precursor B-cell ALL

1.3.1. Epidemiology

Approximately 30% of all cancers that are diagnosed under the age of 15 years are leukaemias [42]. Within the UK an estimated 370 children aged 0-14 years are diagnosed with ALL every year, whilst the incidence of ALL in Europe equates to 35 cases per million children every year [1]. ALL incidence peaks at 2-5 years of age [43], and is slightly higher in males compared to females, at a sex ratio of 1.3:1.0 [44]. A distinct difference in the occurrence of ALL among black and white children exists, where the incidence among white children is almost 2-fold greater. There is also substantial variation in annual international rates, where incidence for males is estimated to range from 9-47 per million males, and from 7-43 per million for females [45, 46]. A previous study showed that the incidence of lymphoid leukaemias in the UK and Europe between 1978 and 1997 was rising in children at a rate of 0.6% per year, and in adolescents at a rate of 1.9% per year [47].

1.3.2. Genetics of ALL

Very little evidence exists for ALL being a directly inheritable disease. Only a few sporadic cases exist, involving mothers that suffered from leukaemia whilst pregnant [48], and other cases where the offspring of individuals that were

treated for paediatric cancer also developed leukaemia themselves [49]. The exact pathogenic mechanism leading to ALL still remains unclear. However, years of research have led to many interesting observations and associations being made, indicating a predisposition towards the development of ALL (Table 1.1).

1.3.2.1. Predisposing syndromes

There are a number of heritable disorders that are known to predispose individuals to the development of ALL.

The incidence of Down's syndrome (DS) in new-born children within the UK is estimated to be 750 babies every year, which equates to approximately 1 in every 1000 babies. It is a chromosomal condition caused by the presence of all or part of an extra chromosome 21 (trisomy). The association of DS and an increased risk of leukaemia development in patients was first described in the late 1950s [50], generally occurring within the first 4 years of birth [51]. Although some variation in the reported relative risk for ALL exists, most studies have found that patients with DS are up to 20 times more likely to develop ALL [52]. The pathogenic events leading up to the development of ALL in DS patients remains largely unknown. Recent studies have provided evidence for the cooperation of multiple genetic events, such as chromosome aneuploidy, submicroscopic deletions of genes involved in cellular proliferation and differentiation, namely CDKN2A, ETV6, and PAX5, and also activating mutations of the JAK2 gene in the development of leukaemia in these cases [53]. The observation that these genetic alterations appear to be selective for

trisomic cells, suggest a multi-hit model for the development of ALL in DS patients [54].

Ataxia telangiectasia (AT) is a rare autosomal recessive disease that is commonly diagnosed in early childhood and is characterised by cerebella ataxia, immunodeficiency, increased sensitivity to ionising radiation, and a predisposition to developing lymphoid malignancies [55, 56], including pre B-cell ALL [57]. Over 100 mutations have been found among AT patients that span across the coding region of the ataxia telangiectasia mutated (*ATM*) gene [58-60], of which approximately 80% are believed to encode either a truncated protein product or no protein at all [58, 60, 61]. Modelling studies using *ATM -/-* mice have also provided much insight in to the pathogenesis of lymphoid tumour development in AT patients [62].

Nijmegen breakage syndrome (NBS) is another chromosomal instability syndrome, which differs from AT with respects to presenting clinical features, but is very similar in terms of radiosensitivity and predisposition to developing cancer [63]. In 1998, mutations in a gene involved in double-strand break (DSB) repair, *NBS1*, were identified and are thought to be responsible for many of the characteristic features of NBS [64-66]. Like with AT, NBS patients suffer from an increased incidence of induced chromosomal aberrations, which may give rise to tumour formation, with over 90% of NBS-associated cancers being lymphoid malignancies [63].

1.3.2.2. Inheritable germline polymorphisms

With respects to the B-lymphoid lineage, development of haematopoietic stem cells into healthy B-cells is dependent upon tight regulation of various stages of differentiation by multiple transcription factors that determine lineage commitment, repress signalling that promotes alternate lineage fates, and ultimately drive the maturation of B lymphocytes. Over 65% of patients suffering from B precursor ALL have genetic alterations that disrupt healthy B-cell development [67, 68].

Inherited genetic syndromes, such as AT and DS, constitute less than 5% of ALL cases [69]. The heritability of other predisposing factors that may influence ALL susceptibility are of great interest and still largely unknown. Data from large familial cancer registries indicate a heritable component to developing ALL [70]. However, until recently no clear susceptibility genes had been discovered, prompting the use of higher resolution genome-wide approaches to identify recurrent somatic alterations and gene polymorphisms. In recent years, an association between multiple low-risk gene variants, such as those involving the nicotinamide N-methyltransferase (*NNMT*) and reduced folate carrier (*RFC1)* genes, and an increased disease risk have become apparent [71].

1.3.2.3. Genetic alterations in B-cell development genes

The recent use of high-resolution, single-nucleotide polymorphism arrays and genomic DNA sequencing approaches have helped identify oncogenic lesions that disrupt pathways involved in the regulation of B-cell differentiation and contribute to B-cell ALL pathogenesis.

The transcription factor PAX5 is the only PAX gene of nine family members to be expressed in the haematopoietic system [72]. PAX5 is necessary for the commitment of lymphoid progenitor cells to the B-cell lineage, and does this in co-operation with the early B-cell factor EBF1 [73]. Absence of PAX5 activity results in blocked B cell development, since its main functions are to maintain the appropriate gene expression program required for B cell lineage specificity [7, 74]. PAX5 induces expression of B-cell specific genes such as *CD19*, *CD72*, *CD79A* and *BLNK*, and ensures repression of genes encoding Notch1 and the macrophage colony-stimulating factor receptor (Csflr), required for T-cell lineage commitment and myeloid cell development, respectively. Genome-wide analysis studies have revealed that copy number alterations of the *PAX5* gene are most common and occur in 29.7% of B cell ALL patients, with deletions occurring almost exclusively in a dominant leukaemic clone [67].

EBF1 is a transcription factor necessary for the normal development of B cells, co-regulating the expression programme required for B-cell lineage specificity together with TCF3, also known as E2A [67]. Mice lacking the *EBF1* gene have B-cells arrested at the pro-B cell stage of differentiation, whereas mice with one allele of the *EBF1* gene have a normal number of pro-B cells, but also 50% less mature B-cells, suggesting haploinsufficiency of this gene as a possible driving factor for leukaemogenesis [75]. A recent study also confirmed a synergistic initiation of ALL between *EBF1* haploinsufficiency and STAT5 in mice [76]. Mono-allelic deletions of *EBF1* occur in ALL at a frequency over approximately 3%, and have been shown to play a significant role in ALL pathogenesis [67].

The Ikaros family of transcription factors are essential for B-cell development and consist of the Ikaros, Aiolos, and Helios proteins, each expressed as multiple isoforms through alternative gene splicing, and share the capacity to heterodimerize [77]. Some of these isoforms confer dominant-negative activity when dimerized and so tight regulation of the splicing machinery is therefore required to avoid abnormalities that may otherwise lead to malignancy. Indeed, mouse modelling of the gene encoding Ikaros, Ikaros family zinc finger 1 (*IKZF1*), involving targeted deletion of exons 3 to 5, led to the splicing of a dominant-negative isoform and gene inactivation, resulting in a complete lack of lymphocytes [78]. The Ikaros transcription factor, activates *CD19* and down-regulates genes that are not B-cell lineage specific, such as *CD4* [79]. Genome-wide association studies have confirmed that 15% of pre-B ALL cases harbour focal deletions of the *IKZF1* gene. Although alterations of the *PAX5* gene occur more frequently in ALL, they do not correlate with poor treatment outcome [68], compared to cases with *IKZF1* gene alterations, which occur less frequently yet are strongly associated with a poor treatment prognosis in both high-risk BCR-ABL1 positive ALL [80, 81], and the novel subtype of 'BCR-ABL1-like' ALL [68, 82].

The *ARID5B* gene encodes a protein belonging to the ARID family of transcription factors, and is involved in cell growth, embryonic development, and regulation of gene expression [83]. Double-knockout mouse models exhibit disrupted B-cell development, suggesting that germline alterations of *ARID5B* may disrupt its function in lymphoid differentiation [84]. Consequently, genome-wide studies have confirmed that the presence of genetic polymorphisms within

the *ARID5B* gene predispose patients to the development of ALL. An interesting finding is that alterations of the *ARID5B* gene are enriched in cases of hyperdiploid ALL and are also associated with increased accumulation of methotrexate polyglutamate, resulting in better responses to treatment [85, 86].

1.3.2.4. Cytogenetic abnormalities

Cytogenetic translocations that activate specific genes are a key characteristic of ALL. Apart from holding high prognostic value, they are largely responsible for the fact this disease is so biologically heterogeneous [2, 87]. Translocations tend to occur among genes that encode transcription factors that are critical to the regulation of B-cell differentiation [87]. The resulting fusion gene may then encode a fusion protein with oncogenic characteristics.

The t(12;21)(p13;q22) translocation is associated with younger age and low white cell count (WCC) is the most frequent translocation in pre-B ALL, occurring in approximately 25% of cases [39, 88]. This cytogenetic alteration results in the fusion of the ETV6 (TEL) and RUNX1 (AML1) genes. More specifically, the helix-loop-helix (HLH) domain found at the N-terminus of the *TEL* gene, along with the RUNT domain and the transactivation (TA) domain of the *AML1* gene located at the N-terminus and C-terminus of *AML1*, respectively, are retained and encode the chimeric fusion protein TEL/AML1 [89, 90]. The protein TEL is a member of the ETS-like family of transcription factors, and plays a key role in the development of haematopoietic stem cells within the bone marrow [91], whereas AML1 is a subunit of the Core Binding

Factor (CBF) transcription factor, essential for foetal liver haematopoiesis [92]. The TEL/AML1 fusion protein consists of the 336 amino-terminal region of TEL, fused to residues 21-480 of the transcription factor, AML1 [93-95]. The retained HLH and TA structures cause the TEL/AML1 fusion protein to have an increased affinity for co-repressor proteins such as mSin3 and N-CoR (a subunit of the nuclear receptor co-repressor complex that exhibits histone deacetylase activity), leading to leukaemogenic activity due to abnormal repression of TEL and AML1 target genes [89, 90].

The second most common translocation occurring at a frequency of between 3 to 6% of all cases of paediatric ALL and over 23% of all cases of pre-B ALL [96] is t(1;19)(q23;p13.3) E2A/PBX1 [97]. The E2A/PBX1 chimera is a fusion of the transactivation domains of E2A and the DNA-binding homeodomain region of the PBX1 gene [98]. The E2A gene encodes two different protein products known as E12 and E47 via alternative splicing [99]. Both E12 and E47 are members of the class I family of basic helix-loop-helix (bHLH) proteins and were found to bind to E box sequences within promoter and enhancer elements of genes encoding immunoglobulin (Ig) [99]. For this reason it was postulated that both E2A gene products regulate Ig locus activation and the development of B-cells. This was later confirmed by targeted disruption of the E2A locus in mice [100, 101], which led to the arrest of B-cell development at the early pro-B stage of differentiation, and before the start of Ig heavy chain rearrangement.

The Philadelphia chromosome was the first chromosomal translocation to be associated with malignancy and is caused by a t(9;22)(q34;q11) translocation [102], resulting in fusion of the 5’ region of the *BCR* gene to the 3’ region of the

ABL1 gene. When expressed, this fusion gene generates an oncogenic chimera protein known as BCR/ABL1, which acts as a constitutively active receptor tyrosine kinase. Multiple breakpoints in the BCR gene give rise to the expression of fusion products with molecular weights of 190, 210 and 230 kDa [103]. BCR/ABL1 is detected in B-cell ALL at a frequency of 3% in children and 25% in young adults [104]. The abnormally increased tyrosine kinase activity leads to the upregulation of prosurvival signalling pathways that can cause high resistance to treatment [105].

The *MLL* gene located at position 11q23 encodes a DNA-binding protein that methylates histone H3 K4 (H3K4) and is known to fuse with over 100 translocation partners in ALL and acute myeloid leukaemia [106, 107]. *MLL* is found rearranged in 5-8% of paediatric ALL cases, with rearrangements occurring in an estimated 80% of infants aged less than a year old at diagnosis [108, 109]. The 5' portion of the MLL gene is believed to translocate with the 3' portion of the partner gene, leading to leukaemogenic effects [107]. The most frequent MLL translocation is the t(4;11)(q21;q23)/MLL-AFF1, formerly known as AF4 [106, 107]. The next most frequent translocation is t(11;19)(q23;p13.3)/MLL-ENL. Fusions of MLL with AF6/6q27, AF9/9p211 and AF10/10p12-14 also occur, albeit very rarely and without any age-specificity [103]. Of all ALL cases involving translocations of the MLL gene, 70-80% are infants (less than 1 year of age), and of these infants approximately 50% harbour the t(4;11) translocation [110]. HOX genes are transcription factors involved in the regulation of the haematopoietic system [111]. MLL plays a key role in regulating HOX genes in order to maintain normal development, and it

has been shown that MLL fusion proteins lose their H3K4 methyltransferase activity, which greatly enhances transcriptional activity and the expression of Hox genes [112]. Consequently, this has been shown to cause disruption of normal stem cell growth and lineage commitment, and therefore drive leukaemogenesis [113, 114].

An estimated 3-5% of paediatric ALL cases have a structurally abnormal chromosome 21 containing inversions, deletions, and duplications along the long arm of chromosome 21, known as intrachromosomal amplification of chromosome 21 (iAMP21) [115, 116], which is commonly detected in children with ALL aged between 7 and 13 years old [117]. iAMP21 has been shown to be strongly associated with chromosomal instability of chromosome 21, which in turn is also associated with recurrent abnormalities observed in B-precursor ALL that affect the expression of genes essential for B-cell development such as *IKZF1* (22%) and *PAX5* (8%) [118]. A common feature among all cases is the amplification of a 5.1Mb region between 21q22.11 and 21q22.12, which includes the *RUNX1* gene encoding AML1, as well as a 1Mb deletion at chromosome ends found in approximately 80% of patients [118-120].

High hyperdiploidy is the most common genetic abnormality in ALL, occurring in approximately 30% of children with this disease. This type of chromosomal abnormality is most frequent in younger patients with a low WCC and an phenotype resembling B cell precursors [121]. This condition is defined as the gain of between 5 and 19 chromosomes, which would take the total number of chromosomes per leukaemic cell to between 51 and 65 chromosomes [122]. The majority of trisomies and tetrasomies occur from the gain of specific

recurring chromosomes, namely chromosomes 4, 6, 10, 14, 17, 18, 21 and X [123].

Hypodiploidy, defined as cells containing fewer than 44 to 45 chromosomes is observed in an estimated 6% of paediatric ALL cases and can fall into three different subgroups [124].

The first subgroup occurs in an estimated 1% of children suffering from ALL, which have a karyotype that is near-haploid as opposed to being diploid. In these children it is uncommon for them to only have between 25 and 28 chromosomes, meaning the majority of chromosomes only have one copy. Chromosomes 10, 14, 18, 21, X and Y are rarely lost [124].

The second hypodiploidy subgroup, known as low hypodiploidy, is defined by the presence of between 30 and 39 chromosomes, where karyotypes are commonly monosomic for chromosomes 3, 7, 15, 16 and 17, and disomic for chromosomes 1, 6, 11 and 18 [124, 125].

Although only observed at a very low frequency of 0.2% of ALL cases, strong evidence exists to suggest a third hypodiploidy subgroup known as high hypodiploidy also exists. This subgroup is defined as those ALL patients with between 40 and 45 chromosomes. Although there is debate regarding the upper limit of the number of chromosomes in high hypodiploid ALL, the exclusive presence of t(9;22), t(12;21), and t(1;19) chromosomal translocations in high hypodiploidy is a feature that helps to distinguish this subgroup from low hypodiploidy [124, 126].

1.3.3. Risk factors associated with ALL development

1.3.3.1. High birth weight

Studies have shown that birth weight is modulated by various genetic traits and exposures during the gestation period. Epidemiological studies have highlighted a positive association between high birth weight and the development of ALL [127, 128], especially in girls, where female babies weighing over 4000 g were almost twice as likely to develop ALL compared to female babies weighing less [129]. The biological mechanisms behind such observations remain speculative and unclear; however, it has been suggested that insulin-like growth factors (IGFs) may play a role in this association [130, 131]; supported by the fact IGFs influence both foetal growth [132], and the likelihood of developing cancer [133]. In addition to this, stem cell pool size has also been shown to be associated with birth weight, which may increase the risk of developing ALL since IGF along with other growth factors increase the total number of replicating cells and therefore also increase the risk for cells becoming tumorigenic [128].

1.3.3.2. Ionising radiation

The first observation that prenatal exposures could lead to the development of ALL in children was first made over 40 years ago by the Oxford Survey of Childhood Cancers. An association was made between mothers receiving diagnostic X-rays whilst pregnant and the development of ALL in their children [134, 135], which was supported by animal studies confirming that in utero exposures to ionising radiation and various chemical carcinogens did in fact increase the likelihood of cancer developing in the offspring [136]. Further

studies also revealed that ionising radiation affected foetuses and children more than adults, and more specifically that radiation dose and gestational age were of greatest importance [137, 138].

The idea that leukaemia risk is increased in offspring of individuals exposed to low doses of external ionising radiation has been subject to much controversy in the past, with many case-control studies conducted near nuclear power plants producing contradictory data and being unable to confirm this [139]. Hitherto, the lack of complete and comparable data from the exposed work forces involved in these studies, together with the fact that nowadays workers are exposed to greatly reduced doses of radiation, has made it difficult to draw additional human data. However, one particular study based on a substantial amount of good data was conducted in 1990 by Gardner et al [140]. Findings from this study suggested an association between paternal occupational exposure to ionising radiation at the Sellafield nuclear power plant and leukaemia incidence in their offspring. Fathers with a cumulative lifetime dose of 100 mSv or greater before conception, were over eight times more likely to have their children develop leukaemia.

1.3.3.3. Non-ionising radiation

Throughout the past few decades there have been huge increases in the generation of man-made power sources, with electromagnetic fields now being thousands of times greater than those which occur naturally. The potential role for residential exposures of extremely low-frequency electromagnetic fields (ELF-EMF) in the causation of childhood leukaemia was first brought to light in

1979, following data published by Wertheimer and Leeper that suggested an association [141]. Their findings have since raised substantial public concern and provoked further scientific investigations into this area.

Studies involving the use of animals and cell line models have provided no evidence to support the idea that exposure to power frequency fields may lead to carcinogenesis. With respects to magnetic force field exposures, a study involving over 3000 cancer cases and 10000 control cases from North America and Europe reported that exposures to magnetic fields of up to 0.4 μT did not lead to increases in relative risks relative to the no-effect level [142]. Although inconclusive, the same study also reported a 2-fold increase in cancer incidence in 0.8% of cases exposed to magnetic fields above 0.4 μT. With regards to electrical field exposures, far fewer studies exist but have so far found no evidence to support the association of electrical fields with paediatric leukaemias in the UK.

1.3.3.4. Infection

It is well documented that certain infectious and parasitic agents, such as HTLV-1 in T-cell leukaemia and lymphoma, and Epstein-Barr virus (EBV) in Burkitts lymphoma, are able to cause cancer [143]. With respects to childhood ALL, there have been no reports of a specific virus being a causative agent. However, a series of observations have maintained the idea of infection playing a significant role in the development of ALL, and have led to the generation of three key hypotheses.

In 1988, Kinlen suggested that there was an above average incidence of leukaemia in young people living in the vicinity of two nuclear reprocessing plants in the UK and that this was due to exposure to new viruses in regions that were previously mainly rural and not so densely populated, which were transmitted from infected newcomers to susceptible inhabitants already present [144]. The reported increase in frequency of leukaemia in children aged 0 to 4 years in areas where substantial degree of population mixing existed supported this theory.

In 1988, Greaves first presented his two-hit hypothesis that the development of ALL was a consequence of at least two separate, spontaneously-occurring mutations [145]. He proposed that the first mutation occurs *in utero* or during early infancy and that this first hit is most likely to occur in B-cell precursors, since they are highly proliferative and susceptible to spontaneous mutation during early stages of development in the liver and bone marrow. This would give rise to a pre-leukaemic clone of B-cells. The second mutation occurs post-natally after an average of 3-4 years during the infant's first exposure to antigens and subsequent proliferation of antibody-producing cells, which then drives the leukaemogenic process.

Greaves also suggested that the timing of an individual's exposure to antigens in early infancy may play an important role in the development of childhood leukaemia [145]. Delays in exposure to antigens and infection might lead to an accumulation of pre-leukaemic lymphoid cells that were susceptible to acquiring the second spontaneous mutation and lead to overt leukaemia. Therefore a child's response to infection and the risk of developing leukaemia may be

affected by many factors such as the duration of breast-feeding, genetic background, socioeconomic status, and vaccinations, which all modulate the timing and level of positive feedback stimulation in lymphoid precursor cells [146]. Case-control studies have supported Greaves' hypothesis, with results showing that a statistically significant increase in the risk of developing ALL exists if there is a delay in an individual's exposure to infections, and other studies also reporting that exposure to infection early on in infancy confers a statistically significant protective effect [147, 148].

Maternal breast feeding has proved to be important in providing a means by which children are exposed to infection in early infancy and protected from developing leukaemia. A paper published by the UK Childhood Cancer Study (UKCCS), in which a total of fifteen case-control studies looking at the protective effects of breast feeding were reviewed, showed that overall leukaemia risk was decreased in children who were breast fed, and that longer duration of breast feeding was associated with an enhanced protective effect against the development of leukaemia [149]. It is postulated that the protective effect may be due to modulation of a child's immune system during early infancy to respond to infections effectively in later life [150].

Studies investigating the effects of different socio-economic status levels and their effect on leukaemia incidence have shown that richer families with higher status are associated with an increased incidence of childhood leukaemia, with as much as a two-fold difference in peak incidence observed between most affluent and least affluent countries [151].

- Age (peak incidence 2-5 years)
- Gender (males at greater risk)
- Ethnicity (white children at greater risk)
- High birth weight
- Genetics:
  - Inheritable syndromes:
    - Down's syndrome (DS)
    - Ataxia telangiectasia (AT)
    - Nijmegen breakage syndrome (NBS)
  - Germline polymorphisms (e.g. *NNMT* and *RFC1* genes)
  - Alteration of B-cell development genes (e.g. PAX5, EBF1, IKZF1, ARID5B)
  - Cytogenetic abnormalities:
    - t(12;21)ETV6-RUNX1 (TEL/AML1)
    - t(1;19) E2A-PBX1
    - t(9;22) BCR/ABL1
    - MLL gene rearrangements
    - iAMP21
    - Ploidy
- Environmental factors:
  - Ionising radiation (e.g. X-rays)
  - Non-ionising radiation (e.g. Extremely low-frequency electromagnetic fields)
  - Infection (i.e. delayed exposure)

Table 1.1: Aetiological risk factors for ALL

The above table summarises risk factors associated with the development of B-precursor ALL that have been described in section 1.3.

1.3.3.5. Two-hit model for ALL tumorigenesis

Many studies have shown evidence that support the idea of tumorigenesis being a multi-step process, first suggested in 1953 by Carl Nordling [152], which involves the accumulation of multiple genetic alterations that cause instability within the genome and promote the transformation of normal human cells into malignant tumour cells. Many diagnosed cancers demonstrate an age-dependent incidence, whereby an average of four to seven rate-limiting genetic alterations appear to be required for highly malignant tumours to form, following progression through multiple pre-malignant states into more aggressive cancers capable of metastasis; a process not very different to the principles of Darwinian evolution, whereby a distinct growth advantage is acquired [153]. Cells cultured *in vitro* require the introduction of multiple genetic alterations for transformation to occur, with rodent cells requiring at least two genetic alterations and human cells being slightly harder to efficiently transform [154]. Transgenic mouse models have also supported the idea that multiple rate-limiting steps are required for tumorigenesis. The ‘Knudson hypothesis’ derived from retinoblastoma studies in 1971 supports this concept and states that two mutational events are required to allow cancer to form. One of the mutations may be hereditary, or both may occur in somatic cells [153].

Monozygotic twin studies conducted by Mel Greaves investigating the natural history of leukaemia, closely relate to the two-step model proposed by Knudson. Using Guthrie cards with bloodspots taken from new-borns, Greaves showed that in most cases of ALL the TEL-AML1 translocation exists and occurs prenatally [155]. He also found that in ALL discordance rates amongst twins are

approximately 90%, suggesting that leukaemic clones initiated by TEL-AML1 fusions, for example, are insufficient to cause overt leukaemia and require a second postnatal ‘hit’ [156]. The peak in childhood ALL incidences at 2-5 years of age is therefore thought to be a reflection of the time taken for the second genetic alteration to take place following the first leukaemia-initiating lesion. The fact that the disruption of multiple cellular processes, such as proliferation, differentiation, and apoptosis, is commonly required for leukaemogenesis to occur, also provides support to the concept of at least two mutational events being needed for complete leukaemic transformation.

Despite the large number of cytogenetic abnormalities that exist in ALL, there is limited evidence for a single causal event in the aetiology of this disease, and so a “two-hit” model of leukaemogenesis is more applicable. Events which occur after the first hit and which are likely to drive the leukaemic transformation, may be derived from genetic, environmental, or other unknown factors.

1.4. Presentation and patient management

Presenting symptoms of ALL are mainly due to the expansion of lymphoblast populations within the bone marrow (commonly >90% blast cell infiltration), as well as the infiltration of other body organs. The most common symptom of patients with ALL is fever, occurring in approximately 55% of cases [157]. Many children also show presenting symptoms such as pallor, lethargy, recurrent infection (due to the suppression of normal haematopoiesis), swollen or tender lymph nodes, liver or spleen (due to leukaemia involvement and enlargement of

haemopoietic organs), as well as bone and joint pain [158, 159]. However, some clinical findings require special consideration, such as respiratory complications resulting from severe anaemia, mediastinal obstruction, or respiratory infection [157]. Although rare, complications also arise when white cell counts reach levels greater than $100x10^9$/L, which can lead to leukostasis and cause respiratory and neurological events. In severe cases, where counts reach greater than $400x10^9$/L, haemorrhages within the central nervous system may occur [160].

Painless enlargement of the testis in males indicates testicular involvement. Bone marrow aspirates and flow cytometry are used to identify the immunophenotype of the leukaemia in order to determine the lineage and stage of differentiation of the leukaemic cells, and allow for appropriate minimal residual disease monitoring by flow cytometry. Cytogenetic and molecular genetic techniques are then used to identify those patients that are at highest risk and to allow for correct treatment stratification and to consider use of molecularly targeted therapy if appropriate [69, 161-163].

1.5. Risk stratification

Risk stratification of patients is used to determine the relative risk of treatment failure and disease relapse. Stratification also ensures that the most aggressive forms of treatment are only given to those patients who are in the high risk category, thus sparing patients at lower risk from suffering unnecessary toxic side-effects. However, this does not imply that low-risk patients should not receive treatment intensification, since this is necessary in order to avoid

increased relapse rates [2]. Patients may be categorised into one of three risk groups: standard, intermediate, and high risk.

Advances in treatment and refinement of treatment protocols over recent years have led to patient survival rates now exceeding 80% [164, 165]. Studies showed that adolescents and young adults who were treated using adult protocols showed poor response, whereas patients from the same age-groups fared far better when treated with paediatric protocols instead [166-168]. Likewise, although national studies have shown that black children have a poorer outcome, independent institutions have reported high cure rates similar to those seen in white children [169]. Many clinical and biological variables that previously served as prognostic factors in ALL are today rendered obsolete, with treatment regimen now being the most important indicator of prognosis [163].

1.5.1. Prognostic factors

Age is a strong prognostic indicator of treatment outcome among patients with B cell ALL [170]. Studies conducted at St Jude Children's Research Hospital showed that children aged 1 to 9 years had a significantly better outcome than either infants or adolescents [171, 172]. Additionally, studies showed that 5-year event-free survival rates were estimated to be 88%, 73%, 69%, and 44% for children aged 1 to 9 years, adolescents aged 10 to 15 years, those aged over 15 years, and babies aged less than a year, respectively [173, 174].

An equally strong prognostic indicator of outcome is leucocyte count, with higher counts correlating with poorer outcomes [172, 175]. Some patients may suffer from extreme hyperleukocytosis, defined as a WCC greater than $400x10^9$/L, and are at an increased risk of complications such as CNS haemorrhage. Leucostasis may also occur, and lead to neurological and pulmonary events [160]. In order to compare responses to treatment in children with leukaemia, a risk-classification system was made which incorporated both age and WCC, and defines that children aged between 1 and 9 years with a WCC $<50x10^9$/L have a standard risk of suffering from relapse, and those in the same age group with a WCC $>50x10^9$/L are at high risk [171]. However, this method of risk assessment has its drawbacks since almost a third of standard risk patients may relapse and amongst high-risk patients it is difficult to differentiate between those at the highest risk requiring stem cell transplantation [171].

Gender has also been shown to influence patient prognosis in ALL. A study involving over 4000 cases of paediatric ALL treated on MRC UKALL trials showed that girls fare significantly better than boys, with 5-year event-free survival rates of 51-71% and 31-57%, respectively, independent of age and leucocyte count [176]. Furthermore, the MRC UKALL X trial provided data that showed girls stratified into the high-risk group with a diagnostic WCC $>100x10^9$/L, exhibited a similar prognosis to boys with a lower WCC. Consequently, a risk scoring system was derived, taking sex, age and WCC into consideration, and which demonstrated an improved discrimination between

standard and high-risk groups, with the latter consisting of 16% boys and 3% girls [176].

There are many frequently occurring chromosomal aberrations that act as prognostic markers in B cell ALL [117, 177-182] (Table 1.2).

1.5.2. Recognition of high-risk ALL

Relapsed ALL is very heterogeneous and still observed in over 15% of patients. Although many paediatric cases are successfully treated, a significant proportion of patients fail to regain stable remissions, with relapse rates of 2%, 23% and 75% being observed in standard-, intermediate-, and high-risk groups, respectively [1]. Attempts to elucidate the prognostic value in cytogenetics of either diagnostic or relapsed ALL have proven difficult. This is largely due to the lack of primary tumour sample availability and matched diagnostic-relapse samples, the lack of large clinical trials, and the heterogeneic nature of relapsed ALL. However, recent developments in genomic array technology have allowed for improvements in our understanding of the genetic events that lead to relapse [1].

Almost 75% of paediatric ALL cases exhibit aneuploidy or chromosomal rearrangements, many of which are associated with a favourable prognosis, for example TEL-AML1 and hyperdiploidy with >50 chromosomes. Previous studies using experimental models have shown that some alterations, such as TEL/AML1, are incapable of inducing leukaemia on their own, and support the notion that additional co-operating genetic alterations are needed to induce

overt leukaemia [183-187]. In contrast, a poor outcome is associated with MLL rearrangements, BCR-ABL1, and hypodiploidy.

A significant number of paediatric ALL samples from high-risk cases do not harbour any of the well-known chromosomal alterations observed in ALL [67]. As a result, great efforts have been made in using genomic approaches such as array-based comparative genomic hybridisation and single-nucleotide polymorphism (SNP) microarrays, candidate gene sequencing, next-generation sequencing, transcriptome sequencing, and whole-genome sequencing to identify novel genetic alterations not observed by cytogenetic analysis [67, 68, 188, 189] (See section 1.3.2.3 and 1.3.2.4).

ALL lymphoblasts acquire fewer large structural genetic alterations compared to solid tumours. However, they do contain approximately 50 chromosomal regions that are frequently subject to alterations such as focal deletions that target genes encoding proteins with essential roles in cellular signalling pathways. Such affected pathways include lymphoid development and differentiation (e.g., *IKZF1*, *PAX5* and *EBF1*), cell-cycle regulation and tumour suppression (e.g., *CDKN2A*, *CDKN2B* (*INK4/ARF*), *TP53*, *PTEN*), lymphoid signalling (*CD200* and *BTLA*), regulation of apoptosis (*BTG1*) [67]. Many of the genes in these pathways may be targeted for mutation by a number of different mechanisms, namely loss-of-function and dominant negative deletions, translocations, and sequence mutations [67]. Recent studies have reported the identification of genetic alterations associated with inferior outcome to treatment and that may be significantly associated with an existing subtype of ALL, or even define entirely novel subtypes with their own distinct gene expression

profiles. Such novel subtypes in ALL include those harbouring rearrangements of the *CRLF2* gene, focal deletions of *ERG*, and intrachromosomal amplification of chromosome 21 [118, 190-192].

BCR-ABL1 positive leukaemia

A common feature of chronic myeloid leukaemia (CML) and a subset of ALL is the expression of a constitutively active form of the tyrosine kinase BCR-ABL1, which confers an aggressive type of leukaemia associated with a poor response to therapy [193]. Interestingly, it has been shown that deletions and sequence mutations of the lymphoid transcription factor gene IKAROS (IKZF1) are associated with BCR-ABL1-positive leukaemia and are capable of inducing the onset of leukaemia in mouse models [194]. Furthermore, lymphoid leukaemias with alterations in the IKZF1 gene that do not express BCR-ABL1 are also associated with a poor outcome, indicating a key role for IKZF1 in treatment failure [68]. Since IKZF1 is involved in the regulation of normal lymphoid development, it is thought that perhaps the loss of IKZF1 activity may lead to leukaemia cells adopting a phenotype akin to that of stem cells, which are innately more resistant to therapy [68, 190]. These studies suggest that targeting IKZF1 activity and its downstream pathways may allow for improved outcomes in patients with this subtype of ALL.

Overall, detection of the BCR-ABL1 translocation in paediatric cases of B-precursor ALL requires treatment intensification and consideration of treating the patient with monoclonal antibodies (mAbs) or tyrosine kinase inhibitors (TKIs) that specifically target the BCR-ABL1 fusion protein [195, 196].

BCR-ABL-like ALL

In recent years, a novel ALL subgroup was discovered which had a gene expression profile very similar to that of BCR-ABL1 ALL, but which did not express the BCR-ABL1 oncogene [68, 192]. BCR-ABL-like ALL is associated with a poor outcome and typically harbours alterations of the IKZF1 gene, which is thought to be the genetic alteration to substitute BCR-ABL1 [68].

Studies have shown that BCR-ABL1-like leukaemias may be divided into two groups based on the genetic alterations, which cause constitutive activation of cytokine receptor and tyrosine kinase signalling [191]. Rearrangements of the cytokine receptor-like factor 2 (CRLF2) gene, located at the pseudoautosomal region of Xp/Yp, are observed in approximately half of all BCR-ABL1-like ALL cases [191, 192]. CLRF2 is capable of heterodimerising with interleukin receptor 7α (IL7R) to form the thymic stromal lymphopoietin receptor (TSLPR) [197]. Translocations, focal deletions, and point mutation of the CRLF2 gene result in deregulated expression of CRLF2 that may be detected by flow cytometric immunophenotyping at diagnosis [102, 190]. Almost 50% of ALL cases harbouring rearranged CRLF2 also have activating mutations in the Janus kinase genes [192]. Most mutations occur within the vicinity of the R683 codon of the pseudokinase domain of JAK2, but may also occur in the pseudokinase domain of JAK1, as well as the kinase domain of JAK1 and JAK2 [192]. The transforming capacity of CRLF2 mutations has been demonstrated in studies where coexpression of CRLF2 and JAK mutant alleles led to the transformation of cell line models [192, 198]. In addition to this, the JAK-STAT pathway is activated in human cells that harbour rearrangements of the CRLF2

gene, and is successfully inhibited with the use of selective pharmacological JAK inhibitors [199]. Interestingly, alterations of the IKZF1 gene have been shown to be associated with mutations of CRLF2 and JAK genes, and confer a poor treatment outcome [192, 200]. Consequently, much interest is going into the downregulation of the JAK-STAT pathway [201, 202], and phase I trials (COG ADVL1011 trial) are underway to determine whether the JAK inhibitor ruxolitinib could be introduced into current treatment protocols alongside conventional chemotherapeutic drugs currently being used to treat relapsed ALL [188].

The second subtype of BCR-ABL1-like ALL cases is characterised by the expression of wild-type CRLF2 together with a number of genetic abnormalities such as the expression of fusion proteins that encode constitutively active tyrosine kinases (EBF1-PDGFRB, STRN3-JAK2, and BCR-JAK2), activating mutations in the transmembrane domain of IL7R, dysregulation of cytokine receptors (IGH@-EPOR), and deletions in the SH2B3 gene, encoding the adaptor protein Lnk that acts as a negative regulator of JAK2 [203]. Cell line models and primary tumours cells expressing the EBF1-PDGFRB fusion protein or IL7R mutant alleles are capable of inducing cellular transformation and activate the JAK-STAT signalling pathway [189]. Large clinical trials involving high-risk ALL patients have also confirmed that many of these genetic abnormalities occur frequently in BCR-ABL1-like ALL [188, 204].

Hypodiploid ALL

Hypodiploidy in ALL is associated with an unfavourable prognosis and until recently not much was known about the genetics of this particular ALL subtype [124]. Studies using large-scale genomic profiling techniques to investigate a large cohort of hypodiploidy ALL cases have confirmed that near-haploid ALL cases exhibit a high frequency of deletions and mutations capable of activating the Ras signalling pathway [188]. Near-haploid (24 to 31 chromosomes) and low-hypodiploid (32 to 44 chromosomes) cases of ALL exhibit inactivation of transcription factors essential for normal lymphocyte development; features distinct from hypodiploid ALL. Some of these alterations involve genes in the IKAROS gene family, as well as genes involved in Ras signalling, such as NF1, KRAS, and NRAS [188].

- Age (<1 and ≥ 10years of age)
- White cell count (>50x10^9/L)
- Gender (Males at greater risk of poor treatment response)
- Cytogenetics:
  - Hypodiploidy (≤44 chromosomes)
  - BCR-ABL1 gene rearrangements
  - Amplified *AML1* (iAMP21)
  - *MLL* gene rearrangements

Table 1.2: Prognostic markers of high-risk ALL at presentation

The above table summarises prognostic criteria for high-risk B-precursor ALL requiring more intense forms of therapy.

1.6. Treatment of paediatric ALL

Treatment of paediatric B-cell ALL typically involves the administration of multiple chemotherapeutic drugs in three distinct phases: the remission-induction phase, an intensification/consolidation phase, and a continuation/maintenance phase. The first two phases together may last up to approximately 12 months, whereas the continuation phase typically lasts a further 2-3 years [196, 205]. Treatment is also directed towards the CNS as soon as possible to avoid relapses due to infiltration of leukaemic cells in this site [163].

1.6.1. Phases of chemotherapy treatment

1.6.1.1. Remission-induction phase

Current UK practice is such that in B-precursor ALL the first treatment phase is designed to reduce the initial leukaemia burden to below 1% and allow for normal haematopoiesis to recommence [163]. This involves the administration of a glucocorticoid (dexamethasone), vincristine, pegylated L-asparaginase, intrathecal methotrexate, and 6-mercaptopurine, typically over a period of 5 weeks [196, 205]. Cases of ALL in the standard-risk group generally respond well when treated with the three-drug induction regimen (Regimen A) if it is followed by intensified therapy involving intrathecal methotrexate and 6-mercaptopurine over a period of 3 weeks, whereas high-risk patients may receive four or more chemotherapy drugs [163].

Responses to therapy are monitored by measuring submicroscopic ALL present in the bone marrow and peripheral blood of remission patients, known as

minimal residual disease (MRD) [206-208]. Generally, MRD is monitored 2 weeks following the initiation of treatment. If residual lymphoblasts are above 1%, treatment is intensified. Remission-induction therapy is largely successful, with 96-99% of paediatric ALL cases reaching clinical remission [163]. With respect to steroid drugs used to treat ALL, studies have shown that dexamethasone confers superior efficacy than either prednisone or prednisolone, since it has a longer half-life and penetrates the CNS more readily [209, 210]. Despite these findings, other reports also show that increased doses of prednisolone may be just as effective as treatment with dexamethasone [211].

1.6.1.2. Consolidation/intensification phase

Once normal haematopoiesis is restored, the risk of relapse is reduced by intensifying treatment to eliminate the residual leukaemia cells that may reside in the bone marrow and other organs [163]. Treatment regimens in this phase follow on from the remission-induction phase and lasts 3 weeks. It includes the administration of intrathecal methotrexate on days 1, 8 and 15, the dose for which is dependent upon the patient's age, together with daily oral doses of 6-mercaptopurine [196, 205].

Intensification therapy has proven to be very effective in the treatment of ALL and is a key component of current treatment protocols [163]. Additionally, a randomised study showed that two courses of intensification improved the outcome of patients compared to those patients who received only a single course followed by additional pulses of the glucocorticoid prednisone and

vincristine [212], suggesting that increasing the dose-intensity of drugs used during intensification therapy was far more beneficial to patients.

The intensification phase incorporates an ‘interim maintenance I’ phase (I.M. I) that lasts 8 weeks and involves the administration of dexamethasone and 6-mercaptopurine to standard-risk patients. This is followed by a single ‘delayed intensification I’ phase (D.I. I), serving as reconsolidation therapy, and consisting of the administration of 6-mercaptopurine for the duration of 7 weeks. Standard-risk patients that are MRD-positive at day 28 after having received standard therapy as part of Regimen A are transferred to Regimen C and receive a second 8-week round of dexamethasone and 6-mercaptopurine as part of ‘interim maintenance II’ (I.M. II) therapy, followed by a second round of delayed intensification (D.I. II) [196, 205].

1.6.1.3. Continuation/maintenance phase

Patients with ALL require continuation treatment as a final phase of therapy to prevent relapses occurring, and so receive an additional phase of continuation therapy for up to 2.5 years as standard [213]. Generally, over 65% of paediatric ALL cases can be treated successfully within a year of therapy [214]. The key components of continuation therapy regimens include daily 6-mercaptopurine and weekly methotrexate, along with discontinuous doses of dexamethasone and vincristine, over a period of 112 weeks for girls and 164 weeks for boys.

1.6.2. Late complications and therapy-associated toxicity

Intensive intrathecal and systemic treatment involving chemotherapeutic agents such as methotrexate and the glucocorticoid dexamethasone can lead to various complications in patients. The use of glucocorticoids may lead to short-term complications including infection, myalgia, myopathy, hyperglycaemia, adrenal axis suppression and behavioural problems [215], with more severe and long-term side-effects of including osteonecrosis, joint collapse, and the requirement for complete replacement of the joint [216, 217]. Risk factors for developing treatment-related osteonecrosis include age between 10-20 years, white race, female sex, and a high body mass index [216-218]. Although the pathogenic events leading to glucocorticoid-induced osteonecrosis are not yet fully understood, it is believed that intravascular thrombotic occlusion, adipocyte hypertrophy, and bone marrow ischemia, as well as apoptosis of endothelial cells, osteoblasts, and osteoclasts may be responsible [215]. Intermittent administration of dexamethasone at days 1-7 and 15-21 of intensification treatment instead of continuous administration has been shown to reduce the toxic side-effects significantly [219]. However, studies are still on-going to determine whether or not this approach has a negative impact on maintaining lymphoblast counts below acceptable levels.

Anthracyclines, such as doxorubicin and daunorubicin, may lead to severe cardiomyopathy that may persist for many years after treatment, especially when administered to female patients and at high cumulative doses [220]. Dexrazoxane is a drug used to counteract the negative effects associated with the use of anthracyclines, and has been shown to reduce cardiotoxicity without

compromising antileukaemic activity [221, 222]. However, current trials still limit the use of anthracyclines even for high-risk patients, since some cases of cardiotoxicity are so severe [69].

1.6.2.1. Stem cell transplantation

Allogeneic haemopoietic stem-cell transplantation (SCT) is recommended for patients failing to respond to treatment, or that show a slow early response to remission-induction therapy indicated by the presence of more than 1% leukaemic blasts [69]. However, although SCT is a highly effective form of therapy, its use must be frequently reviewed as many new agents become available that may supersede this form of therapy in terms of efficacy, toxic side-effects and the ability to improve treatment outcome [223].

Previous studies have shown that transplantation does not improve the outcome of patients with hypodiploid ALL [181] or MLL-rearranged ALL [109, 182, 224], but is recommended for high-risk patients with BCR-ABL1-positive ALL that do not respond well to the remission-induction treatment that consists of intensive chemotherapy combined with a tyrosine kinase inhibitor such as imatinib mesylate [69].

1.6.3. Minimal residual disease

Responses to therapy are monitored by measuring submicroscopic ALL present in the bone marrow and peripheral blood of remission patients, known as minimal residual disease (MRD) [206-208]. MRD measurements are used in conjunction with common prognostic factors to be able to stratify patients according to risk, and give an indication as to whether treatment intensification

is required [225-227]. UK protocols assess treatment response by taking bone-marrow aspirations and performing morphological analysis at day 8, ± day 15, and day 28 following the start of induction therapy. MRD monitored at weeks 4 and 11 using quantitative polymerase chain reaction (PCR)-based methods is also used to assess the risk of relapse [1]. Many previous studies provide evidence that poor clearance of leukaemic blasts from the bone marrow or peripheral blood is associated with a poor prognosis in ALL and that MRD measurements are valuable even in low-risk cases of ALL if defined by clinical and biologic features alone [228, 229]. Conventional techniques based on cell morphology lack sensitivity and accuracy in detecting leukaemic blasts and can only do so when the total population of leukaemic blasts constitute greater than 5% of the total cell population (clinical remission is defined by a reduction of lymphoblasts below 5%) [162, 171]. The PCR technique is able to detect leukaemia cells with a 100-fold increase in sensitivity over conventional morphology-based techniques (1 lymphoblast in 10,000 healthy cells) and is the preferred method for measuring MRD at very low levels (<0.01%), allowing for a more accurate prediction of relapse in individual ALL cases [162, 163, 230, 231]. The detection of MRD by PCR involves the amplification of breakpoint fusion regions of chromosomal aberrations in B cell ALL, such as TEL/AML1, BCR-ABL, E2A-PBX1, and MLL fusion transcripts, which give this method its tumour-specificity [232]. However, some scepticism exists regarding the accuracy of using these markers of MRD to predict the risk of relapse, since it has been reported that clinical relapse did not occur for over 27 months in some cases that were still MRD-positive [233]. MRD may also be detected by PCR

amplification of antigen receptor gene rearrangement sequences in individual patients. The Ig and TCR gene loci contain many recombined V, D, and J gene segments, which when combined with random interstitial nucleotide insertions and deletions, provide unique tumour-specific sequences ideal for measuring MRD by PCR analysis [234]. Although, Ig and TCR gene rearrangements are ideal targets for MRD detection in individual patients, some tumours exhibit oligoclonality at diagnosis and may be subject to further rearrangements or deletions [234]. It is therefore current practice to amplify at least two sequences in order to avoid false negative results [39]. Over 95% of ALL patients harbour Ig and TCR rearrangements, namely *IGH* (>95%; predominantly VH-JH), *IGK* (~65%; predominantly Kde), *IGL* (15-20%), *TCRG* (~55%), *TCRD* (~40%), *TCRB* (~35%), and *Vδ2-Jα29* (40-45%) [235, 236].

A second method of detecting MRD is by identifying tumour-specific immunophenotypes associated with ALL using multiple-channel flow cytometry. Abnormal phenotypes that are indicative of ALL disease, are identified using antibodies with specificity against surface expression of antigens such as CD19, CD34, TdT, CD10, CD22, CD45, CD38, and CD45 [161, 237, 238]. Flow cytometry allows for direct quantification of MRD as opposed to extrapolation from a PCR product. This method has the potential to be more accurate than PCR [239]. However, it does not supersede PCR techniques in terms of sensitivity, since flow cytometry is unable to provide consistent results when detecting MRD as low as 1 lymphoblast among 10^5 normal healthy cells. A further limitation with this technique is that it is dependent upon quantifying differences in surface antigen expression and not simply the presence or

absence of leukaemia-specific markers as used in PCR-based MDR detection. Nonetheless, large studies such as the Total Therapy Study XIII program have shown that MRD detection by flow cytometry holds great predictive power, where detectable MRD (≥0.01%) was strongly associated with higher relapse rates, and found to be independent of other clinical and biologic predictors of treatment outcome [162, 237, 240].

In general, studies have provided evidence to show that the persistence of MRD levels of 1 lymphoblast in 10^4 healthy cells indicates a significantly greater risk of a patient suffering a relapse [237, 241, 242]. Other studies have shown that positivity for MRD in children in clinical remission following induction therapy have a 5-year incidence of relapse rate of approximately 43%, increasing to 68% if the patient continues to test positive for MRD beyond week 14 of maintenance therapy. This particular study also confirmed that those children who tested negative to MRD following induction therapy had a 5-year incidence of relapse rate of 10%, reducing further to 7% if those children were MRD negative at week 14 of maintenance therapy [240]. It is not uncommon for remission marrows to contain up to 10^{10} leukaemic blasts following induction therapy [243], which requires consequent consolidation and maintenance therapy as standard, with the intention of completely eliminating MRD.

1.7. Apoptosis as a factor determining treatment response

Defective regulation of apoptosis plays a fundamental role, not only in the development of tumours, but also their resistance to chemotherapy [244]. Cancer cells typically acquire multiple mutations that commonly lead to deregulation of signalling pathways and promote genomic instability, which in turn, increase the sensitivity of tumour cells to cellular stress such as that caused following DNA damage. Conventional chemotherapy and irradiation exploit this increase in sensitivity and are used with the intention of causing enough DNA damage to cause these tumour cells to undergo apoptosis, whilst attempting to minimise the damage to normal healthy cells [245]. However, tumour cells that acquire additional defects in the apoptotic pathways may become protected against cytotoxic agents and exhibit chemoresistance and radioresistance [245].

Apoptosis is a homeostatic process of programmed cellular death, which is activated during development and aging, and serves to maintain cell populations in the various tissues of the human body in a controlled manner [246]. Apoptosis may also be triggered by immune reactions or when irreparable damage is sustained to a cell through hypoxia, disease or exposure to harmful agents, thus acting as a defence mechanism. This prevents genetically damaged somatic or germ line cells from acquiring cancer-promoting mutations and continuing to proliferate [245].

Previous findings in our group have shown that a subset of primary ALL tumours demonstrate a defect in the induction of apoptosis following exposure

to IR, regardless of normal p53 upregulation [247]. It was also shown that an association exists between *in vitro* resistance to apoptosis following DNA damage and the clinical response *in vivo* determined by measuring MRD. Furthermore, primary ALL cells that were resistant to apoptosis exhibited deregulated transcription of genes involved in multiple prosurvival pathways. When these pathways were individually targeted by pharmacological inhibition, this led to widely heterogeneous responses *in vitro* [247, 248]. Thus, an alternative strategy is warranted in order to counteract apoptotic resistance observed in ALL.

1.7.1. Induction of apoptosis

1.7.1.1. The role of tumour suppressor p53

The p53 protein is critical to the regulation of cellular processes in response to a variety of intracellular and environmental stresses, such as chemotherapy, ultraviolet and ionising radiation, and hypoxia [249-251]. With p53 regulating multiple key cellular processes, it is clear to see why this protein is frequently inactivated by multiple mechanisms in human cancer. Mutation of the p53 gene is found in approximately 50% of all human cancers, and is the most common mechanism of inactivation [252]. Interestingly, an estimated 80% of p53 mutations still allow a full-length p53 protein monomer to be expressed, but which lacks the ability to bind to DNA [253].

Under normal conditions of cellular growth, p53 protein levels are kept low via interactions with its negative regulatory partner mouse double minute 2 (Mdm2).

The binding of Mdm2 to p53 prevents its transactivation functions and ubiquitinates p53, targeting it for proteosomal degradation [254]. Cellular stress in the form of direct DNA damage, oncogene activation, hypoxia and deprivation of nutrients all lead to activation and stabilisation of p53 following post-translational modifications. When a cell is under genotoxic stress, such as that caused by chemotherapeutic drugs used in the clinic, stabilisation and activation of p53 occurs through phosphorylation of Serine-15 at the N-terminal region by the damage response kinases ATM and ATR, whilst checkpoint kinase-2 (Chk2) and checkpoint kinase-1 (Chk1) phosphorylate p53 on Serine-20 [255]. Once activated, p53 accumulates in the nucleus and is then capable of regulating transcription of target genes involved in the response to DNA damage. Early response genes upregulated by p53 are mainly those encoding proteins with growth arrest and DNA repair functions, including p21, GADD45α, 14-3-3α and MDM2 [245, 256]. If DNA damage persists and is unsuccessfully repaired, p53 stabilisation is maintained leading to the expression of pro-apoptotic genes such as *Bax*, *PUMA/NOXA* and *Fas/DR2* [257, 258].

1.7.2. The intrinsic apoptotic pathway

A large number of non-receptor-mediated stimuli are involved in initiating apoptosis via the intrinsic pathway, which can be activated by intracellular signals in either a positive or negative way [245]. For example, negative signals involve the downregulation of specific growth factors, hormones and cytokines that inhibit programmed cell death programs and promote survival, thus triggering apoptosis. Other positively acting stimuli include radiation, hypoxia, toxins, viral infections, and free radicals [245]. Both of these types of stimuli

lead to changes in the inner mitochondrial membrane, which cause the opening of the mitochondrial permeability transition pore and a loss of the mitochondrial transmembrane potential. Once this occurs, two key groups of pro-apoptotic proteins that usually reside within the intermembrane space are released into the cytosol [259]. The first group of proteins include cytochrome *c*, Smac/DIABLO, and the serine protease HtrA2/Omi, which activate the caspase-dependent mitochondrial pathway [260-263]. Cytochrome *c* then binds and activates both Apaf-1 and procaspase-9 to form a caspase-activating complex known as the 'apoptosome.' The formation of this complex causes the activation of caspase-9, which in turn activates downstream effector caspases-7, -6, and -3 that cleave various substrates such as PARP1, cytokeratins, and proteins within the plasma membrane [256, 264]. The second group of pro-apoptotic proteins consists of AIF, endonuclease G, and CAD, which are released from the mitochondria at a later stage of apoptosis when a cell is committed to death and translocate to the nucleus where they fragment DNA into pieces approximately 50-300 kb in size, as well as condensing peripheral nuclear chromatin [265-267].

The previously described mitochondrial events are regulated by a group of proteins belonging to the B-cell lymphoma gene-2 (Bcl-2) family of proteins, and can be either pro-apoptotic (Bcl-10, Bax, Bak, Bid, Bad, Bim, Bik, and Blk) or anti-apoptotic (Bcl-2, Bcl-x, Bcl-XL, Bcl-XS, Bcl-w, BAG, and Mcl-1). The expression of these proteins determines whether or not a cell commits to apoptotic cell death via regulation of cytochrome *c* release from the mitochondria [245]. Phosphorylation of pro-apoptotic Bad keeps this protein

sequestered in the cytosol bound to 14-3-3. Once Bad is unphosphorylated, it translocates to the mitochondria and causes the release of cytochrome *c* [268]. Bad is also capable of binding and neutralising the anti-apoptotic effects of Bcl-XL and Bcl-2, thus promoting cell death [269]. When Bcl-2 and Bcl-XL are not sequestered by Bad, these proteins prevent the release of cytochrome *c*.

Two additional pro-apoptotic proteins of the Bcl-2 family, Puma and Noxa, also play a key role in p53-mediated apoptosis. Both these proteins are upregulated by p53 when a cell is subjected to oncogene activation or genotoxic stress [245]. Overexpression of Puma *in vitro* has been shown to cause concomitant upregulation of Bax expression [270], whereas Noxa is capable of translocating to the mitochondria and binding to anti-apoptotic Bcl-2 family members, which results in the release of cytochrome *c* and activation of caspase-9 [271].

1.7.3. The extrinsic apoptotic pathway

Apoptotic signalling by the extrinsic pathway is mediated via transmembrane receptors. These include death receptors belonging to the tumour necrosis factor (TNF) receptor gene superfamily [272], which contain cysteine-rich extracellular domains and a cytoplasmic domain known as the ‘death domain’ [273]. The death domain is essential for the transmission of apoptotic signals from the surface of the cell to intracellular signalling pathways. Many of the ligands and their corresponding receptors involved in the extrinsic apoptotic pathway are well characterised and include TNF-α/TNFR1 [273], FasL/FasR [274], Apo3L/DR3 [275], Apo2L/DR4 [276] and Apo2L/DR5 [277].

Following activation of the death receptors by its corresponding ligand, the receptors cluster and trimerise. Adaptor proteins located within the cytoplasm are then recruited and bind to the receptor death domains [245]. For example, the adapter protein FADD binds to the Fas receptor following activation by the Fas ligand, whilst binding of the adapter protein TRADD and consequent recruitment of FADD and RIP occurs once the TNF ligand binds to the TNF receptor [278]. FADD then binds with procaspase-8 to form the death-inducing signalling complex (DISC), which leads to the activation of procaspase-8 and triggering of apoptosis through activation of the downstream effector caspase-3 [245, 279].

1.7.4. Execution of apoptosis

Typically, cells undergoing apoptosis are subject to various biochemical modifications such as protein cleavage, cross-linking of proteins, DNA breakdown, and expression of surface markers for phagocytic recognition [280]. A group of highly conserved intracellular cysteine proteases known as cysteine aspartyl-specific proteases, or caspases, are ubiquitously expressed in most cell types and are key mediators of apoptosis. Caspases are expressed as an inactive proenzyme, which once activated, initiate a proteolytic cascade that serves to cleave other caspase enzymes and amplify the apoptotic signalling to aid rapid cell death [245]. Once activated, caspases are able to cleave protein at aspartic residues and cells become committed to apoptosis. There are ten major caspases that are grouped into three categories, namely upstream initiator (caspase-2, -8, -9, -10), downstream executioner (caspase-3, -6, -7), and inflammatory caspases (caspase-1, -4, -5) [281, 282]. The two main

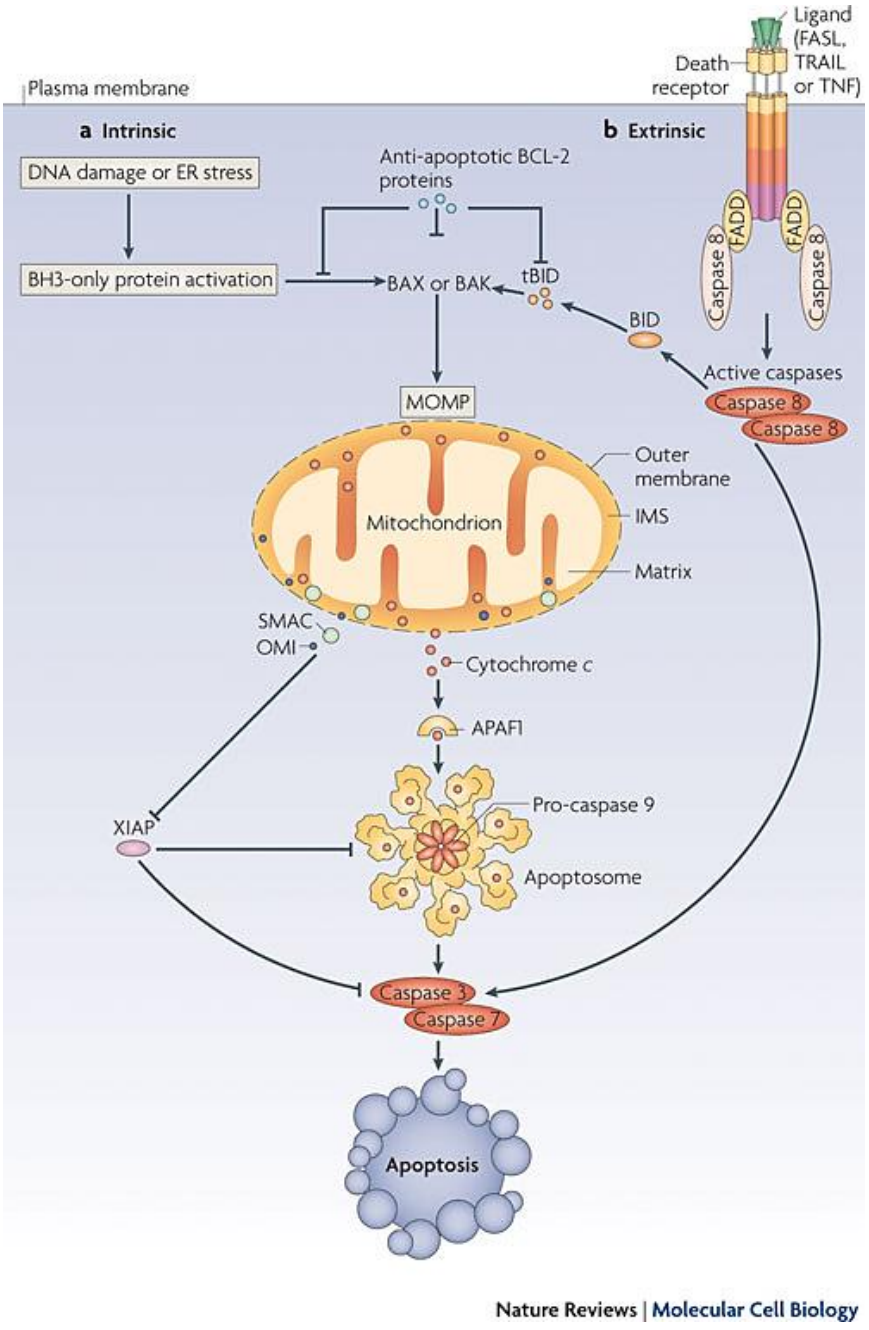


Figure 1.2: Intrinsic and extrinsic mitochondrial apoptosis

a) DNA damage or endoplasmic reticulum (ER) stress are intrinsic apoptotic stimuli that lead to mitochondrial outer membrane permeabilisation (MOMP), and the release of cytochrome *c*. Cytochrome *c* then binds with APAF-1 to form the apoptosome which ultimately leads to the activation of caspases 9, 7 and 3.

b) The extrinsic apoptotic pathway is activated following the ligation of death receptors with their corresponding ligands. This leads to the recruitment of adaptor molecules such as FAS-associated death domain protein (FADD) which then results in the dimerisation and activation of caspase 8, followed by caspase-3 and -7. (Adapted from Tait, W.G. & Green, D.R., Nature Reviews Molecular Cell Biology 11, 621-632, 2010)

pathways by which apoptosis occurs are intrinsic pathway, regulated by intracellular proteins, and the extrinsic pathway, which is triggered by the activation of Fas family of death receptors expressed on the cell surface. The tumour suppressor p53 is involved in triggering both these pathways; however, the intrinsic apoptotic pathway is its principal mechanism of action [283].

1.7.5. Inhibitors of apoptosis

The c-FLIP protein is capable of inhibiting apoptotic signals mediated via the death receptors by directly binding to FADD and caspase-8 [284, 285]. Studies have shown that p53 may also play a role in the extrinsic apoptotic pathway by upregulation or shuttling of DR5 and Fas proteins [283, 286].

The inhibitor of apoptosis proteins (IAPs) are a very important group of proteins capable of inhibiting both the intrinsic and extrinsic apoptotic pathways [287] by direct inhibition of caspases; some of which are nuclear factor kappa B (NFκB) target genes and regulate NFκB anti-apoptotic activity. Eight mammalian IAP family members have been identified to date, namely cellular IAP1 and IAP2 (cIAP1 and cIAP2), X-chromosome-linked IAP (XIAP), neuronal apoptosis inhibitory protein (NAIP), survivin, BRUCE, livin, and testes-specific IAP (Ts-IAP) [288]. A common feature of all IAPs is the baculoviral IAP repeat (BIR) domain located in the N-terminal region, which mediates the interaction with caspases and anti- apoptotic activity. They are approximately 80 amino acids in length and are able to chelate zinc ions [289]. XIAP, cIAP1, and cIAP2 inhibit caspase-9, -7, and -3 directly. Furthermore, it has been shown that XIAP is capable of promoting the degradation of inhibitor κB protein, which leads to

activation of NFκB and its translocation from the cytoplasm to the nucleus [290]. Survivin, together with the hepatitis B X-interacting protein (HBXIP), bind and inactivate procaspase-9, thus preventing activation of the intrinsic pathway [291].

1.8. The DNA damage response and treatment resistance

Treatment of ALL relies on the stratification of patients based on prognostic markers such as clinical and genetic features. Furthermore, poor early blast clearance following remission-induction therapy is indicative of high-risk ALL [162]. Although major improvements in the detection and prediction of treatment outcome in more aggressive forms of ALL have been made over the years, not much was known about the molecular defects that would confer resistance and a poor response to therapy. Various research groups previously investigated differential patterns of gene expression in ALL following treatment with a single cytotoxic agent or a combination of drugs using gene expression arrays [292-295]. Results from such studies provided vast amounts of information to aid the prediction of treatment resistance and patient prognosis in paediatric B-cell ALL; however, the identification of a single gene expression pattern associated with chemoresistance to multiple conventional cytotoxic agents used in the treatment of ALL has not been possible, despite many of these agents having been shown to exert overlapping cellular effects. This, together with the fact the multiplicity of genetic events observed in ALL frequently influence common signalling pathways, have made the discovery of novel therapeutic targets difficult [193, 296].

Many chemotherapeutics drugs used to treat ALL exert their effects by inducing double-strand breaks (DSBs), which is an effect that can be mimicked using ionising radiation (IR). Using IR as a model of DNA DSBs to investigate the integrity of cellular responses in leukaemia cells, studies have shown that DSBs initiate a signalling cascade that results in the activation of both proapoptotic and prosurvival pathways [297, 298]. Whether or not a cell undergoes apoptosis is dependent upon the number of DSBs and the integrity of the DSB response pathway. Usually, above a certain threshold in the number of DSBs, a p53-driven apoptotic response is initiated, and prosurvival signalling overridden. Thus, the integrity of the DSB response plays an important role in the formation and progression of lymphoid tumours, and may modulate chemosensitivity in ALL [299, 300].

1.9. Prosurvival pathways

There are multiple molecules and pathways that modulate apoptosis. In addition to pro-apoptotic signalling there are also prosurvival signalling pathways, which create a dynamic balance of opposing signals that determine the fate of a cell. These pathways do not operate in isolation and a significant degree of overlap exists between them.

1.9.1. The NFκB pathway

The nuclear factor kappa beta (NFκB) protein is a transcription factor with a key role in regulation of the immune system by controlling the expression various cytokines, growth factors, and inhibitors of apoptosis [301]. However, pathological dysregulation of the NFκB pathway has been associated with

inflammatory and autoimmune diseases, as well as many cancers; particularly leukaemias [248]. Five mammalian NFκB family members exist: p50, p52, RelA (p65), c-Rel and RelB, which all have a DNA binding and dimerization domain, known as the Rel homology domain (RHD) [302, 303]. Through the RHD these transcription factors are able to form homodimers and heterodimers that recognise and bind to κB sites located in the promoter and enhancer regions of genes, thereby controlling gene expression. The RHD also allows NFκB to bind inhibitors of NFκB (IκB). IκB sequesters NFκB dimers in the cytoplasm, preventing their translocation to the nucleus [304]. The activation of NFκB dimers involves the phosphorylation of IκB by the IκB kinase (IKK) complex, which causes IκB to be targeted for ubiquitin-mediated degradation by the 26S proteasome [305]. This may occur in response to a wide range of stimuli and ultimately results in nuclear localisation of NFκB dimers, and therefore the expression of NFκB target genes. Unless the stimuli causing activation persist, NFκB provides its own negative feedback loop to terminate gene transcriptional activity by expressing IκBa, which results in the sequestration of NFκB subunits to the cytoplasm. The inhibition of apoptosis by NFκB is mediated by upregulation of several genes that encode anti-apoptotic proteins such as the IAPs, A1, TNF receptor associated factors 1 and 2 (TRAF1 and TRAF2), and caspase-8 FADD-like IL-1β-converting enzyme inhibitory protein (c-FLIP) [306]. Increased expression of the NFκB target genes cIAP1, cIAP2, TRAF1, TRAF2, and c-FLIP results in direct inhibition of capsase-8, whilst upregulation of XIAP prevents the apoptotic activity of caspase-3, -7, and -9 [307, 308]. Multiple anti-apoptotic Bcl-2 family members such as A1, Bcl-2, and Bcl-XL are upregulated

by NFκB and may lead to inhibition of apoptosis by preventing cytochrome *c* release and caspase-9 activation [309, 310].

1.9.2. The JAK-STAT pathway

Studies first identified the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway as a signalling pathway that mediates mammalian cytokine signals [311-314]. To date, seven STAT proteins (STAT1-4, 5A, 5B and 6) and four JAK kinases (JAK1-3 and tyrosine kinase 2 (TYK2)) have been identified. Activation of the canonical JAK-STAT signalling pathway involves the binding of a ligand, such as a cytokine, to the upstream transmembrane receptor. Once activated, the receptor dimerises and provides cross-activation of JAK kinases that are bound to the receptor. The JAK kinase then phosphorylates tyrosine residues located in the cytoplasmic receptor tail. Consequently, STAT transcription factor proteins then dock at these phosphor-tyrosine sites, and are then phosphorylated by the JAK kinases. Phosphorylated STAT proteins dimerise and localise to the nucleus, where they activate transcription and regulate expression of their corresponding target genes [312]. STAT target genes include those encoding antiapoptotic factors such as the B-cell lymphoma 2 (Bcl-2) family of proteins, genes involved in angiogenesis and metastasis factors such as vascular endothelial growth factor (VEGF), and genes involved in cell proliferation such as cyclin D1 and c-Myc [315, 316]. Since many of these genes are involved in proliferation and survival signalling, their dysregulated upregulation by STAT transcription factors is commonly associated with tumour-promotion. JAK-STAT signalling is also modulated by positive and negative regulators, which determine the magnitude and duration

of signals within the pathway [312, 317, 318]. Activated STAT proteins are capable of inducing their own expression in the form of a positive-feedback loop. However, negative regulators of the JAK-STAT pathway including suppressor of cytokine signalling (SOCS), protein tyrosine phosphatases (PTPs), and protein inhibitor of activated STAT (PIAS) also exist, and are responsible for inhibiting JAK-STAT signalling following activation [312, 317, 318]. Studies in *Drosophila* have highlighted the existence of a non-canonical JAK-STAT signalling pathway, where unphosphorylated STAT has a role in directly controlling the stability of heterochromatin and regulating the activity of transcription machinery [319].

1.10. Epigenetic regulation of prosurvival gene transcription

The term ‘epigenetics’ describes the heritable changes to a cellular phenotype that are a result of covalent modifications made to the nucleosome without altering the DNA sequence [320]. The discovery of CpG islands, characterisation of the human DNA methylome, and identification of novel histone modifications, together with the major technological advances in recent years, have greatly improved our understanding of epigenetics and its importance in maintaining normal gene expression [321]. Accumulating data exists to support a significant role of epigenetic modifications in ALL. For example, the gene encoding the negative regulator of the JAK/STAT pathway, SHP1, is frequently silenced by methylation in 24% of B-precursor ALL [322]. Additionally, studies have shown that histone acetylation plays a crucial role in maintaining normal regulation of the complex process of V(D)J recombination

during B-cell development, through a mechanism involving the IL7R/STAT5 signalling axis and the Pax5 transcription factor [323-326].

Chromatin is a macromolecular complex consisting of packaged DNA and histone proteins, and therefore contains all the heritable genetic material of eukaryotic cells [320]. It may be divided into two distinct regions, namely heterochromatin and euchromatin. Heterochromatin is typically very highly condensed and contains mainly inactive genes. In contrast, euchromatin confers a more open structure and contains the majority of active genes. Within chromatin, 147 base pairs of DNA are wrapped around a histone octamer comprising of two of each of histones H2A, H2B, H3, and H4 [320].

The modification of DNA and histones is a dynamic process, with such changes being constantly made and removed by chromatin-modifying enzymes. Epigenetic modifications are critical to the regulation of cellular processes such as gene transcription, DNA repair, and replication, and it is now well known that deregulation of post-translational histone modifications can have serious consequences that contribute to many human cancers [321].

To date, four types of DNA modifications [327, 328], and sixteen different types of histone modifications exist, which serve to alter the structure of chromatin, as well as to provide docking sites for specialised proteins that specifically recognise these changes [320].

1.10.1. Histone acetylation

A major type of histone modification involved in modifying chromatin structure, DNA repair, and gene transcription is the N^{ε}-acetylation of lysine residues. Acetylation functions to remove the positive charge of lysine residues, causing the electrostatic bonds holding histones and DNA together to weaken. Consequently, chromatin adopts a more ‘open’ structure associated with active gene transcription [320, 329, 330]. Additionally, acetylation of lysine residues also functions to provide the docking site required for its recognition by various chromatin ‘reader’ proteins containing bromodomains, and tandem plant homeodomain (PHD) fingers [331].

1.10.2. Histone acetylation readers and their role in regulating gene transcription

The principal readers of N^{ε}-acetylated lysine residues are a family of proteins containing a highly conserved binding motif known as a bromodomain. Over 40 human bromodomain-containing proteins have been identified to date, and function as chromatin remodelers, transcriptional coactivators, histone acetyltransferases, and histone methyltransferases [332]. Until recently, therapeutic targeting of protein-protein interactions with small pharmacological inhibitors had not been possible. However, great advances in this area have meant that it is now possible to develop highly specific inhibitors that target the BET family of bromodomain proteins, consisting of BRD2, BRD3, BRD4, and BRDt [333, 334]. These proteins recognise epigenetic chromatin modifications,

such as poly-acetylated lysine residues of H3 and H4 histone tails, and play an essential role in the regulation of transcription and cell cycle progression [335]. With respect to transcriptional regulation, studies have shown that BRD4 forms part of transcription complexes and remains bound to transcriptional start sites of genes expressed during the M/G1 transition, thus influencing mitotic progression and transcriptional elongation [336]. BRD4 recruits the positive transcription elongation factor complex (P-TEFb), consisting of CDK9 and cyclin T1 subunits, to the acetylated tails of histone H3 and H4. CDK9 subsequently phosphorylates the Ser2 residue of RNA polymerase II, triggering transcriptional elongation [337]. Despite the role in general transcription control, BRD4 activity seems to affect the transcription of a defined subset of growth- and survival-promoting genes [335, 336, 338, 339]. Interestingly, the targeting of BET bromodomains appears to be a promising therapeutic approach following recent success in treating the aggressive and fatal NUT-midline carcinoma; a disease associated with recurrent translocations of BRD3 and BRD4 [334]. Interestingly, such an approach has also proven to be effective in a range of haematological malignancies expressing BET family proteins [340-342], with a common feature of these studies being the downregulation of *MYC* following BET inhibition. The c-Myc protein this gene encodes plays a fundamental role in cell cycle progression and survival, and is one of the most commonly dysregulated genes in cancer, with high expression also frequently observed in a wide range of haematological malignancies, including ALL [343]. Furthermore, studies in mice have provided striking evidence to support that BET inhibition is highly effective

in vivo [344, 345], and may potentially provide an alternative strategy in the treatment of BET protein-expressing malignancies.

1.10.3. The role of PIM kinases in prosurvival gene transcription

The PIM protein family consists of three highly conserved serine/threonine kinase members (PIM1, PIM2 and PIM3) [346]. The *PIM1* gene was first discovered by cloning studies investigating retroviral integration sites in Moloney murine leukaemia virus (MMLV)-induced lymphomas, which showed that over 50% of T-cell lymphomas exhibited integration near the *PIM* gene locus and subsequent upregulation of *PIM1* mRNA [347, 348]. Later studies using transgenic mice confirmed potent cooperating oncogenic activity between *PIM1* and *MYC* [349], as well as with Bcl2, RunX2 and the *E2A-PBX1* fusion gene [350-352]. Interestingly, *MYC* transgenic mice lacking the *PIM1* gene showed that *PIM2* expression was activated to compensate for *PIM1* loss [353]. Similarly, *PIM2* upregulation was found to occur as a late event in MMLV-induced lymphomas [354] and also potently synergised with *MYC* [355].

Significant functional redundancy between PIM kinases has been shown both *in vitro* and *in vivo,* which is due to the high level of amino acid sequence homology, where PIM1 and PIM2 share 61% homology, and PIM1 and PIM3 share 71% homology [346]. All three members of the PIM kinase family are ubiquitously expressed; however, their level of expression can vary between different cell types [356]. For example, PIM1 is most highly expressed in haematopoietic cells, whereas PIM2 presents at higher levels in brain and

lymphoid cells, and PIM3 in breast, brain and kidney cells [356]. Through alternative transcription initiation, two isoforms (34 and 44 kDa) are encoded by the *PIM1* gene that have both been shown to maintain their kinase activity [356]. Similarly, alternative initiation sites within the *PIM2* gene have been reported and give rise to 3 different isoforms (34, 37 and 40 kDa), whilst only one protein is encoded by the *PIM3* gene [357]. The PIM kinases lack a regulatory domain, thus they are mainly regulated at the transcriptional level and so are constitutively active once expressed [358, 359]. PIM1 has also been shown to be regulated via protein stabilisation through binding with heat shock proteins (HSP). For example, the binding of PIM1 protein to HSP90 leads to stabilisation, whereas binding to HSP70 leads to its ubiquitylation and proteasomal degradation [360, 361]. In contrast, protein stabilisation of PIM2 by HSPs has not been reported.

A wide range of cytokines and growth factors, including the interleukins and granulocyte-macrophage colony-stimulating factor (GM-CSF), are known to activate the JAK/STAT signalling pathway, which leads to rapid upregulation of *PIM* genes [346, 362-364]. Similarly, studies have shown that JAK2, BCR/ABL and FLT3 mutants induce the expression of *PIM1* and *PIM2* genes via a mechanism involving the activation of the NFκB prosurvival signalling pathway and downstream STAT transcription factors [365-368]. However, the relationship between PIM kinases and NFkB remains an area of debate within the literature, since it is not clear whether PIM is up- or downstream of NFκB [369, 370].

Although the role of PIM kinases in B-precursor ALL has not yet been explored, studies have shown that PIM1 and PIM2 are frequently upregulated in several other human myeloid and lymphoid leukaemias, and lymphomas, such as acute myeloid leukaemia (AML), chronic lymphocytic leukemia (CLL), mantle cell lymphoma (MCL), diffuse large B-cell lymphoma (DLBLC), marginal zone lymphoma-MALT type (MZL-MALT), follicular lymphoma (FL), and nodal marginal zone lymphoma (NMZL) [357, 365, 371-375]. With respect to the clinical relevance of PIM kinases, high PIM2 expression is associated with aggressive disease in DLBLC patients [376]. Furthermore, recent studies have shown that PIM1 and PIM2 are also upregulated in solid tumours such as prostate cancer, where high levels of PIM1 protein expression (over 50% of prostate cancer patients) positively correlated with an inferior treatment outcome [357, 377].

Studies have shown that on their own, PIM kinases only weakly transform mesenchymal cells to give rise to leukaemia and lymphoma [378, 379]. However, the synergy between PIM family members and other oncogenes such as *MYC,* results in a far greater transforming capacity [353]. Since, on its own, c-Myc protein overexpression leads to the induction of apoptosis, tumours must adapt and gain the capability to overcome this effect [353, 364]. Intriguingly, both PIM1 and PIM2 have been demonstrated to counteract the pro-apoptotic functions of c-Myc by inactivating the pro-apoptotic protein BAD through phosphorylation of its Ser112 residue [380]. In addition, PIM kinases phosphorylate c-Myc, thus increasing its stability and transcriptional activity [346]. Other pro-apoptotic proteins inactivated following PIM-mediated

phosphorylation include ASK1 and PRAS40, which are important for JNK /p38 signalling pathways and the inhibition of mTORC1 kinase activity that regulate cellular growth and proliferation, respectively [381, 382].

More recently, PIM2 has been shown to share similar cellular functions to the Akt kinase downstream of the phosphatidylinositol 3-kinase (PI3K) signalling pathway, since both proteins regulate survival, metabolism and cell size [346, 359, 383, 384]. Interestingly, studies have confirmed that, similar to Akt, PIM2 promotes mRNA translation by phosphorylating and subsequently inactivating a ribosomal translation inhibitor known as the eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) encoded by the *EIF4EBP1* gene [385]. Furthermore, the previously mentioned phosphorylation of proapoptotic BAD by PIM kinases, is also observed upon activation of Akt [359]. These findings, together with the confirmation that PIM kinases act independently of Akt [383], suggest that cytokines and growth factors activate survival pathways with a significant degree of overlap [383].

Intriguingly, recent studies have shown that the PIM kinases also play a role in regulating gene transcription by enhancing BRD4-mediated recruitment of P-TEFb [346, 386]. More specifically, this involves the recruitment of PIM1 to E-box DNA sequences through a direct interaction with c-Myc, which then enables PIM1 to phosphorylate histone H3 at Ser10 (H3S10) [386]. This allows for the binding of 14-3-3 proteins [387], which is then followed by recruitment of the acetyltransferase MYST1 that is responsible for the acetylation of histone 4 at Lysine 16 (H4K16) [388]. Subsequently, H4K16 provides the binding site required for the binding of the BET protein BRD4, which recruits P-TEFb that

functions to phosphorylate RNA Pol II at Ser2, thus releasing stalled RNA Pol II from transcriptional pause sites and promoting transcript elongation [388], as previously described.

Interestingly, this highlights an alternative mechanism of Myc-regulated gene expression, where c-Myc promotes transcript elongation at gene promoters with a pre-formed transcription initiation complex, as opposed to recruiting transcription initiation complexes to the promoter [389]. Although it is not yet clear whether other PIM kinase family members regulate gene transcription in this way, it has been estimated that PIM1 enhances the expression of approximately 20% of all genes induced by c-Myc [346, 386].

Consequently, in recent years, great effort has gone into the generation of pharmacological PIM kinase inhibitors [390]. One study identified the imidazo[1,2-b]pyridazine K00135 as a PIM kinase inhibitor, which demonstrated specific anti-leukaemic activity against primary AML tumour cells *in vitro* [391]. Thus, it remains to be determined whether the targeting of PIM kinases holds any therapeutic advantage in the treatment of B-precursor ALL.

1.11. Aims and objectives

From the overview of the current status of paediatric ALL given in the introduction it is apparent that, despite high cure rates achieved in the last two decades, high levels of toxicity associated with standard chemotherapy and treatment resistance in a subset of patients imposes the need for new therapeutic approaches [1]. The results from our own laboratory suggest that upregulation of prosurvival pathways may represent one of the major factors in B-precursor ALL chemoresistance.

Recent advances in understanding the regulation of transcription suggest two possibilities for the direct targeting of prosurvival cellular machinery:

a) the inhibition of epigenetic readers as factors that recognise chromatin permissive for transcription

b) the inhibition of co-factors that modify and stabilize transcription complexes.

Therefore, the aim of my project was to use these alternative strategies to target prosurvival signaling in a range of B-precursor ALL tumours, and particularly those that exhibit treatment resistance. My approach was based on the hypothesis that B-precursor ALL, including high-risk ALL, can be sensitised to apoptosis by downregulation of survival pathway signaling via the inhibition of gene transcription, and that this can be achieved by targeting the BET family of proteins and PIM kinases.

Thus, the specific aims and objectives of this study were:

- To investigate the role of BET proteins in B-precursor ALL and determine whether pharmacological inhibition of BET proteins leads to the sensitisation of tumour cells, both *in vivo* and *in vitro.*
- To determine the effect of BET protein inhibition on gene transcription in pre-B ALL.
- To characterise the cellular effects of BET protein inhibition in B-precursor ALL.
- To determine whether the PIM kinases pose as suitable candidates for further study, and as potential therapeutic targets in B-precursor ALL.

CHAPTER 2

MATERIALS & METHODS

2. MATERIALS AND METHODS

2.1. Primary ALL patient tumours

The primary tumour samples used in this study were selected according to material availability and derived from patients enrolled in the UKALL 2003 trial, from the Birmingham Children's Hospital, UK.

Following diagnosis, stratification of paediatric B precursor ALL patients into three different risk groups (standard, intermediate and high risk) is defined by diagnostic criteria outlined in the UKALL 2003 protocol http://www.ctsu.ox.ac.uk/research/mega-trials/leukaemia-trials/ukall-2003/protocol-version-7.

The risk groups and the diagnostic features that define them are as follows:

- Standard risk: Patients >1 and <10 years of age, with a diagnostic white cell count (WCC) $<50x10^9$/L, and without hypodiploidy (≤44 chromosomes), BCR-ABL, or rearrangements of the MLL gene.
- Intermediate risk: Patients ≥10 years of age, or that have a diagnostic WCC $≥50x10^9$/L, and that do not harbour lymphoblasts with BCR-ABL, hypodiploidy (≤44 chromosomes), or an MLL gene rearrangement.
- High risk: All patients that exhibit a slow early response (SER) to induction therapy, as well as those patients harbouring lymphoblasts with BCR-ABL, hypodiploidy (≤44 chromosomes), an MLL gene rearrangement, or amplification of RUNX1 (AML1).

Primary ALL samples used in this study were obtained from patients during induction therapy. Standard risk patients were treated on Schedule A (dexamethasone, vincristine, pegylated L-asparaginase, methotrexate, and 6-mercaptopurine), intermediate risk patients on Schedule B (similar to Schedule A, but with the addition of daunorubicin), and high risk patients on Schedule C (similar to Schedule B, but with the administration of higher doses of daunorubicin).

Table 2.1 lists the primary ALL samples used in this study as well as available clinical information associated with the patients from which the samples were derived.

In addition to the ALL tumours, 2 primary paediatric AML tumours (AML-1 and AML-2) were also included as positive controls for JQ1 anti-tumour activity. Clinical information for these patients can be found in Appendix 1.

Clinical data that were available for patients used in the qRT-PCR PIM2 expression screen can be found in Appendix 2.

Patient No.	Age at diagnosis (years.months)	WCC at diagnosis ($x10^9$/L)	Clinical stratification	Cytogenetic profile
ALL-101	5	44.9	HIGH RISK	46 XY
ALL-103	5.3	3.1	LOW RISK	Hyperdiploid
ALL-104	6.4	5.6	LOW RISK	46XYt(12;21)
ALL-105	2	391.3	HIGH RISK	45xy, dic (9;20)+8, -13
ALL-106	2.7	55.9	HIGH RISK	46XY del(1)p332p32, dup(2)q2q3),add(9)p2
ALL-108	6.4	0.6	LOW RISK	46XY, t(12;21)
ALL-109	13	2.7	LOW RISK	46XY
ALL-110	2.5	7.5	HIGH RISK	46XY, t(12;21)
ALL-111	2.7	42.9	LOW RISK	55XY, high hyperdiploid
ALL-112	5.5	6.5	HIGH RISK	Hyperdiploid
ALL-113	1.9	10.7	HIGH RISK	N/K
ALL-114	0.7	394	Infant ALL	MLL - rearrangement
ALL-115	11	26.2	LOW RISK	47-48XX
ALL-116	6.6	5.4	HIGH RISK	46XY, t(12;21)
ALL-117	1.7	56	HIGH RISK	52XY,+X+4+6+7+8+21(2), 46XY(8)
ALL-118	1	27.2	Infant ALL	MLL - rearrangement
ALL-121	2	N/K	HIGH RISK	Hyperdiploid
ALL-122	3.6	3.4	HIGH RISK	46XY, t(12;21)
ALL-123	2.6	12.1	HIGH RISK	54XX +X+4+6+14+17+18+21+21
ALL-124	11	N/K	LOW RISK	46XY, t(12;21)
ALL-126	9	N/K	Relapsed ALL	Hyperdiploid
ALL-129	2.3	87.8	N/K	47,XX,+X,t(3;22)(p25;q13)[9]/46,XX[1]
ALL-130	7.45	40.7	N/K	55,XX,+X,ins(1;?)(q2;?),+4,+6,+10,+14,+17,+18,+21,+21[9]/46,XX[1]
ALL-131	11	72.6	N/K	t(1;19)
ALL-132	9	24.4	N/K	t(1;19)
ALL-133	N/K	N/K	N/K	N/K

Table 2.1: Clinical information for the B-precursor ALL patients used in this study

Tumours were identified by their patient number ('Patient No.'). Data in this table includes the patients' age at diagnosis, diagnostic white cell count (WCC), as well as details of their cytogenetic profiles. This information, together with the measurement of MRD at Day 28, is used to stratify patients diagnosed with B-ALL into different risk groups and ensure they are assigned to the appropriate treatment regimen. (N/K = Not known; data was unavailable).

2.2. Tissue culture techniques

2.2.1. Isolation and cryopreservation of mononuclear cells from bone marrow aspirate

Using aseptic technique, bone marrow aspirate was carefully diluted with 10 ml RPMI culture medium containing L-Glutamine (Invitrogen), supplemented with 10% foetal calf serum (Invitrogen) and 1% penicillin/streptomycin antibiotics (Invitrogen), all pre-heated to 37ºC in a water bath. This was then gently transferred to a universal tube containing 10 ml Lymphoprep (Axis-shield), forming two separate layers with the diluted bone marrow aspirate in the top layer. This was then followed by centrifugation at 1600 rpm for 25 minutes with no brake. The newly-formed, third middle layer containing the lymphoid mononuclear cells was carefully collected using a pasteur pipette, transferred to a new universal tube, and washed twice using supplemented culture medium at 37ºC, centrifuging at 1600 rpm for 5 minutes on a low brake setting. Lymphoid mononuclear cells were counted during the second wash using a haemocytometer. The supernatant was discarded, leaving a pellet of lymphoid cells at the bottom of the universal tube. The contents of the original universal tube still containing the top and bottom layers were discarded, leaving behind residual drops of separated red blood cells.

To prepare cells for cryopreservation, pelleted lymphoid cells were re-suspended in cryopreservation medium at 37ºC, constituting of a mixture of 90% foetal calf serum (Invitrogen) and 10% dimethyl sulphoxide (DMSO) (Sigma), at a cell density of 1.5 x 10^7 cells/ml. Red blood cells were also

prepared by re-suspending all of the red bloods cells in 1 ml cryopreservation medium. Cells were transferred to 1 ml Cryovials (Nunc). Vials containing lymphoid cells and red blood cells were appropriately labelled and placed in plastic containers substantially padded with cotton wool, and then stored at -80ºC. The next day, vials were stored in the liquid nitrogen sample bank, and their location recorded.

When viable cells were needed for experiments, vials containing frozen cells were taken from liquid nitrogen and thawed in a 37ºC water bath, before transferring the contents to a new universal tube, and adding 10 ml culture medium pre-warmed to 37ºC, one drop at a time. Cells were then centrifuged at 1600 rpm for 5 minutes, washed in culture medium, and re-suspended at the required cell density.

2.2.2. Cell line maintenance

The following ALL cell lines were suspended in RPMI culture medium supplemented with 10% foetal calf serum (Invitrogen) and 1% penicillin/streptomycin antibiotics (Invitrogen), maintained in 75 cm^2 vented cell culture flasks (Iwaki), and incubated in humidified incubators at 37ºC, 5% CO_2: NALM-6 and REH were originally provided by Dr. Tony Ford (Institute of Cancer Research, Sutton, UK), and the NALM-17 cell line by Dr. Yoshinobu Matsuo (Fujisaki Cell Center, Japan). Jurkat, SD1, SUPB-15 and TOM-1 cell lines were obtained from DSMZ GmbH, Germany. Cells were split every 7 days at a ratio of 1:10. Details for the cell lines used in this study are summarised in Table 2.2.

Cell line name	Year established	Leukaemia type	Sex & age at diagnosis	Prognostic karyotype	Reference
NALM-6	1976	Pre-B cell ALL	M 19	None	[392]
NALM-17	1978	Pre-B cell ALL	M 9	None	[393]
REH	1975	Common-B cell ALL	F 15	TEL/AML1	[394]
SD1	1991	Pre-B cell ALL	F ?	BCR/ABL1	[395]
SUPB-15	1988	Pre-B cell ALL	M 8	BCR/ABL1	[396]
TOM-1	1987	Pre-B cell ALL	M 54	BCR/ABL1	[397]
Jurkat	1977	T-ALL	M 14	None	[398]

Table 2.2: Cell line information.

Available information relating to the cell lines used in this study. Cell lines were established from leukaemia cells derived from patients diagnosed with ALL. Sex (M = male, F = female), age and the prognostic karyotype of each patient are also provided.

2.2.3. Chemical reagents

The BRD4 inhibitor JQ1, and the PIM kinase inhibitor K00135 (both received as a 10 mM stock dissolved in DMSO), were kindly provided by Prof. Stefan Knapp (Structural Genomics Consortium, University of Oxford, UK). The mitotic inhibitor vincristine (Sigma), the topoisomerase II inhibitor daunorubicin (Sigma), and the synthetic glucocorticoid receptor ligand dexamethasone (Sigma) were dissolved in DMSO at a 10 mM stock concentration. The 26S proteasome inhibitor MG132 (Merck) was dissolved in DMSO at a 100 mM stock concentration. Aliquots of these reagents were stored at -20ºC.

2.3. Protein chemistry techniques

2.3.1. Extraction of total protein from viable cells

Control and drug-treated cells were collected into separate 1.5 ml eppendorf tubes (Eppendorf) and centrifuged at 1600 rpm for 5 minutes at 4ºC. Culture medium was decanted and the pellet washed by re-suspension in ice-cold phosphate buffered saline (PBS). Cells were pelleted once more and PBS poured off, making sure to remove as much residual PBS as possible using a pipette. The pellet was then re-suspended in 30-60 µl UTB lysis buffer (8 M Urea 50 mM Tris-HCl, pH 7.5), depending on size of the pellet. Each sample was then subjected to a single sonication pulse lasting approximately 7 seconds. Lysates were centrifuged at 14000 rpm for 15 minutes at 7ºC. The supernatants were then transferred to clean eppendorf tubes, pre-cooled on ice, leaving behind any cell debris. Protein samples were then snap-frozen in liquid nitrogen and stored at -80ºC until needed.

2.3.2. Total protein quantification using the Bradford assay

Protein concentrations were determined for each sample by constructing a standard curve. This was done by preparing six bovine serum albumin (BSA) (Invitrogen) standards of increasing concentrations (0 (10 µl water), 100, 200, 300, 400, and 500 µg/ml) and adding 10 µl of each standard in triplicate to a 96-well flat-bottomed plate (Iwaki). The protein lysates were then loaded in triplicate onto the 96-well plate at a 1:10 dilution. This was done by first adding 9 µl distilled water to an individual well, followed by 1 µl of the appropriate protein sample. Bradford reagent (Biorad) was then diluted 1:5 with distilled water, followed by addition of 200 µl of the prepared solution to each of the wells containing the BSA standards or protein lysate samples. After 5 minutes, the plate was loaded into a microplate reader (Biorad) and absorbencies measured at a wavelength of 595 nm. The absorbance values obtained were used to plot a standard curve graph with the concentration of BSA (µg/ml) on the *x*-axis and the average absorbance of the triplicate plotted on the *y*-axis. Using the mathematical equation for a straight line $y=mx+c$. the total protein concentration of each lysate was determined using the mean absorbance of the samples from triplicate readings ($x=-c/m$). Between 15 and 30 µg of protein were loaded for each sample.

2.3.3. Western blotting

2.3.3.1. SDS-PAGE

Total protein in lysate samples was separated in an 8% polyacrylamide gel, consisting of 4 ml 0.1 M Tris/0.1 M Bicine (pH 8.3), 10.65 ml 30% acrylamide

(N, N'-methylene-bis-acrylamide) (Bio-Rad), 400 µl 10% (w/v) SDS (Sodium dodecyl sulphate) (Biorad), 80 µl Temed (N, N, N'. N'-tetra-methyl-ethylenediamine) (Sigma), 200 µl 10% (w/v) APS, and 24.65 ml of sterile distilled water. Whilst the gel was left to set, 500 ml running buffer was prepared (0.1 M Tris / 0.1 M Bicine (pH 8.3), 0.1% (w/v) SDS).

Protein samples were prepared on ice, making sure each sample contained the same amount of protein. If large variations of sample volume were observed, samples were made up to the same volume (80 µl max.). Samples were then mixed with the appropriate volume of 4X Sample buffer (0.125 M Tris HCl pH6.8, 20% glycerol, 4% (w/v) SDS, 0.2 M DTT, 0.02% (w/v) bromophenol blue) and denatured for 5 minutes in a heat block at 95ºC. The samples were then briefly centrifuged for 5 seconds, before being loaded into the set polyacrylamide gel, along with a broad range protein molecular weight marker (Fermentas), and electrophoresed in previously prepared running buffer at 28 mA for approximately 5 hours, or until the blue sample buffer visible within the gel had reached the bottom.

2.3.3.2. Electrophoretic protein transfer

Once electrophoresis of protein samples through the polyacrylamide gel was complete, protein was transferred to nitrocellulose membrane (Thermo Scientific) by electrophoresis at 200 mA for 16 hours in a Transphor electrophoresis unit (Hoefer Scientific). The transfer buffer contained 48 mM Tris, 390 mM glycine and 20% (v/v) methanol. Care was taken to ensure, no air bubbles existed between the gel and the nitrocellulose membrane, in order to avoid incomplete transfer of protein, and also ensure that the nitrocellulose

membrane was between the negatively-charged protein in the gel and the anode side of the transfer tank.

2.3.3.3. Immunoblotting for transferred protein

Once transfer of proteins was complete, the nitrocellulose membrane was removed from the transfer tank and left to soak in Ponceau stain (1% Ponceau Red in 3% (w/v) trichloroacetic acid) for approximately 2 minutes on a rocker, before removing the membrane from the Ponceau stain and briefly rinsing it with distilled water. The transferred protein bound to the nitrocellulose membrane was now visible as red bands, allowing for the removal of excess sections of membrane and cutting of the membrane into strips with a scalpel, depending on the size of the proteins being blotted for. Cut strips were then washed in Tris-buffered saline (200 mM Tris HCl, 1.36 M NaCl pH7.6, 0.1% Tween-20 (TBST) for 10 minutes to remove the stain, and blocked in TBST containing 5% milk on a rocker for 1 hour at room temperature. Blocked membranes were then washed in TBST for 10 minutes for a total of three washes, with fresh TBST wash buffer being used with each wash. At the end of the third wash, membranes were incubated with the primary antibody, diluted to the recommended concentration in TBST with 5% milk (Marvel) solution, for 1 hour at room temperature. Antibodies recognising phospho-proteins were diluted to the recommended concentration in TBST with 5% (w/v) BSA (Invitrogen). Following incubation in primary antibody, membrane strips were washed in TBST for 10 minutes, three times. After the third wash, membrane strips were incubated in horseradish peroxidase-labelled secondary antibody conjugate of the appropriate anti-species, and pre-diluted to the recommended

concentration in TBST with 5% milk. Membranes were incubated in secondary antibody for 1 hour a room temperature, after which they were washed 3 times in TBST for 10minutes each wash. Membrane strips were briefly drained of excess liquid and incubated in the enhanced chemiluminescence (ECL) reagent (Milipore), as per manufacturer's directions, for 1 minute. Following incubation in ECL, excess liquid was carefully removed from membrane strips, which were then placed in between two transparent acetate sheets and secured within an X-ray cassette with tape, whilst making sure there were no trapped air bubbles. The reaction between ECL substrate and the horseradish peroxidase conjugate leads to the production of luminol that emits light. Therefore, the ECL-treated membrane strips in the X-ray cassette were taken to a darkroom and exposed to X-ray film (Kodak) and developed in a developing system (Xograph Imaging Systems Compact X4). The length of time the X-ray film was exposed to emitted light was dependent upon the signal strength and often required multiple exposures to attain optimum results. Table 2.3 lists all the antibodies used in this study.

Antigen	Species	Company	Dilution factor	Application in this study	Incubation conditions
β-actin	Mouse monoclonal	Sigma-Aldrich	1:50,000	WB	30 minutes; RT
PARP1	Rabbit monoclonal (45D11)	Cell Signalling	1:1000	WB	24 hours; 4°C
Procaspase-3	Mouse polyclonal	Cell Signalling	1:1000	WB	24 hours; 4°C
Procaspase-7	Mouse monoclonal	Cell Signalling	1:1000	WB	24 hours; 4°C
c-Myc	Mouse monoclonal (9E10)	Calbiochem	1:50	WB	24 hours; 4°C
BRD4	Rabbit polyclonal (H-250)	Santa Cruz	1:200	WB	24 hours; 4°C
p53	Mouse monoclonal (DO1)	Donated by R Grand	1:30,000	WB	24 hours; 4°C
phospho-p53 (Ser15)	Rabbit polyclonal	Cell Signalling	1:1000	WB	24 hours; 4°C
Mouse IgG	Rabbit polyclonal	Dako	1:10,000	WB	1 hour; RT
Rabbit IgG	Goat polyclonal	Dako	1:30,000	WB	1 hour; RT
CldU/BrdU	Rat monoclonal (BU1/75)	Serotec	1:400	IF	1 hour; RT
IdU/BrdU	Mouse monoclonal	BD Pharmingen	1:25	IF	1 hour; RT
Rat IgG	AlexaFluor 555-conjugated Goat IgG	Molecular Probes	1:400	IF	30 minutes; RT
Mouse IgG	AlexaFluor 488-conjugated Goat IgG	Molecular Probes	1:400	IF	30 minutes; RT
BrdU	Mouse monoclonal (Bu20a)	Dako	1:50	F	45 minutes; RT
Mouse IgG	Fluorescein-conjugated Horse IgG	Vector Laboratories	1:50	F	1 hour; RT
PIM2	Rabbit polyclonal	Cell signalling	1:1000	WB	24 hours; 4°C

Table 2.3 Antibodies information.

Details for the antibodies used in this study and experimental conditions under which they were used.

(WB = Western blot, IF = Immunofluorescence, F = Flow cytometry, RT = Room temperature).

2.3.4. FITC Annexin V / propidium iodide flow cytometric analysis for the detection of apoptosis

Cells were seeded into a 6-well plate (Iwaki) at a cell density of $1x10^6$ cells/ml and exposed to 1 µM JQ1 inhibitor for 0, 24, and 48 hours (where '0 hours' cells represented the negative control and were treated with 0.01% (v/v) DMSO instead of JQ1. Two additional plates were prepared in this way so as to provide a triplicate reading. JQ1 inhibitor was added to cells in a way that would allow for harvest of cells at the same time.

At time of harvest, 2 ml of cell suspension for each treatment were transferred into individual flow cytometry sample tubes using a pasteur pipette. Cells were then washed by adding 3 ml ice-cold phosphate-buffered saline (PBS), and centrifuging at 2000 rpm for 5 minutes. The supernatant was then discarded, and cells washed with PBS once more. The required volume of 1X binding buffer was prepared using the 10X binding buffer contained in the apoptosis detection kit (BD Pharmingen) and diluting it with distilled water, after which 100 µl 1X binding buffer was added to each tube. I then added 5 µl of FITC Annexin V and/or PI to the appropriate tube. Tubes were then vortexed, wrapped in aluminium foil and incubated for 10 minutes in the dark at room temperature. 500 µl 1X binding buffer was then added to each tube and flow cytometry performed using the Beckman Coulter XL sorter. Gating of the fluorescence signal emitted in FITC Annexin V labelled and PI labelled cells was performed prior to analysis of test samples in order to be able to distinguish between the two different fluorophores and allow for the separation of cell populations into viable (FITC Annexin V and PI negative), early apoptotic (FITC Annexin V

positive, PI negative), and late apoptotic or dead cells (both FITC Annexin V and PI positive). A total of 15,000 events were collected per sample.

2.4. Microarray study

The microarray experiment in this study was conducted in collaboration with Dr. Simone Sharma at Institute of Child Health Microarray Facility, University College London (UCL).

Procedures in sections 2.4.3 – 2.4.5 were performed according to instructions detailed in product-specific NuGen user guides (http://www.nugeninc.com/nugen/index.cfm/support/user-guides/).

2.4.1. RNA extraction

A total of 8 primary ALL tumour cells were treated with either DMSO or 1 μM JQ1, and then total RNA isolated using the RNeasy Mini kit (Qiagen).

First, cells were centrifuged at 300 x *g* for 5 minutes and culture medium completely removed by careful pipetting, after which 600 μl of the supplied Buffer RLT was added to 0.5-1 x 10^7 cells. Cells were homogenised by passing the lysate through an RNase-free syringe fitted with a 0.9 mm syringe needle at least five times. One volume of 70% ethanol was then added to each lysate, and mixed well by pipetting. Up to 700 μl of the lysate was transferred to an RNeasy spin column placed in a 2 ml collection tube. The samples were then centrifuged at 10,000 rpm for 15 seconds, and the flow-through discarded. This step was repeated for the remainder of the lysate, if the sample volume exceeded 700 μl.

At this stage, the optional on-column DNase digestion using the RNase-free DNase kit (Qiagen) was incorporated into this protocol to eliminate genomic DNA contamination. This was carried out as per manufacturer's instructions.

Following DNase digestion, 500 µl Buffer RPE were added to each spin column containing the RNA samples, which were then centrifuged at 10,000 rpm for 15 seconds. The flow-through was discarded and an additional 500 µl Buffer RPE were added to the spin columns, and then centrifuged at 10,000 rpm for 2 minutes. The columns were placed in clean 1.5 ml collection tubes and centrifuged at full speed for 1 minute to remove any residual flow-through. The columns were then placed in clean 1.5 ml sample tubes (Eppendorf), and the RNA eluted by adding 30 µl RNase-free water directly to each column and centrifuging at 10,000 rpm for 1 minute, and repeating the elution step once more by passing the eluate through the same column. RNA samples were immediately placed on ice, and the RNA quality and concentration was determined using the ND-1000 Spectrophotometer (NanoDrop). RNA with an $OD_{260nm/280nm}$ ratio between 1.8 and 2.0 was considered of sufficient quality for downstream microarray applications.

2.4.2. RNA quality control

The 2100 Bioanalyzer (Agilent) was used to determine the integrity of RNA samples. The algorithm used by this machine allows for the calculation of a RNA integrity number (RIN), which is considered to be a reliable and standardised method for the assessment of RNA quality for microarray applications [399]. Only RNA samples with a RIN value greater than 8 were

deemed to be good quality RNA and chosen for use in this microarray study [400].

2.4.3. First and second strand cDNA synthesis

A total of 40 ng RNA per sample were treated using the Ovation PicoSL WTA kit, which serves as a fast and simple method for preparing cDNA for whole genome expression analysis, optimised for analysis on Affymetrix Array Plates.

The Ovation PicoSL WTA kit utilises Ribo-SPIA® technology to amplify cDNA from very small amounts of sample RNA within the picogram range, and consisted of three steps:

1- Generation of first strand cDNA: The unique first strand DNA/RNA chimeric primers hybridise to both the 5' end of the poly-A tail sequence and randomly across RNA transcripts in the sample. The reverse transcriptase (RT) enzyme then generates the first strand cDNA by extending the 3' DNA end of each primer, producing a cDNA/mRNA hybrid molecule that contains a specific RNA sequence at the 5' end of the cDNA strand that is required for the generation of the second strand cDNA.

2- Generation of a DNA/RNA heteroduplex double strand cDNA (second strand cDNA): Priming sites for DNA polymerase to produce a second strand cDNA are made by fragmenting the mRNA within the cDNA-mRNA complexes produced in the last step. This allows for the generation of second strand cDNA that contains a specific complementary 5' DNA sequence for the chimeric primer mix to bind to. What is produced, is a double-stranded cDNA molecule with a unique DNA-RNA heteroduplex at one end.

3- Amplification of DNA using SPIA® technology: This is a rapid DNA amplification process that involves the use of SPIA DNA/RNA chimeric primers RNase H and DNA polymerase to produce large quantities of cDNA with a sequence that is complementary to the mRNA in the original ALL sample.

2.4.4. Generation of ST-cDNA

Sense transcript-cDNA (ST-cDNA) was generated using the WT-Ovation Exon Module kit (NuGen), in order to render it suitable for use in the downstream Encore Biotin Module (NuGen).

2.4.5. Fragmentation, labelling, and hybridisation of ST-cDNA to oligonucleotide probe array

ST-cDNA was then fragmented and labelled using the Encore Biotin Module (NuGen). This process involved two steps, whereby cDNA was chemically and enzymatically fragmented into cDNA products of 50 to 100 base pairs in length, followed by enzymatic conjugation of a biotin-labelled nucleotide to the 3-hydroxyl end of the fragmented cDNA.

Subsequently, 2.5 µg of fragmented and labelled ST-cDNA were then hybridised to GeneChip® Human Gene 1.0 ST Arrays (Affymetrix) for 16 hours at 45ºC, as per NuGen's instructions. The GeneChip® Human Gene 1.0 ST Arrays cover a total of 36,079 total RefSeq transcripts each. The arrays were then washed and stained with a fluorescent biotin-binding molecule (streptavidin-phycoerythrin) using the GeneChip® Fluidics Station 450 (Affymetrix).

2.4.6. Oligonucleotide probe array scan

The Human Gene 1.0 ST Arrays were then scanned using the GeneChip Scanner 3000 (Affymetrix), under the control of the Microarray Suite program (MAS 5.0). The scanner indirectly quantifies the amount of biotinylated ST-cDNA for each probe set by using a laser to induce fluorescence of phycoerythrin molecules that are conjugated to streptavidin, which in turn binds biotin molecules. The dedicated software package measures the fluorescence and calculates an intensity value for each of the probe sets and using a statisctical expression algorithm, determines the expression level of each transcript within a sample.

2.4.7. Analysis of microarray data

Data was analysed with the help of Dr. Wenbin Wei at the School of Cancer Sciences, University of Birmingham.

2.4.7.1. Normalisation of data

Experiments involving the use of several high density oligonucleotide arrays often introduce variation of non-biological origin between arrays, and it is therefore important to remove this variation by normalisation, so that gene expression data may be compared between samples [401].

Normalisation of microarray data in this study was achieved using the default settings of the Robust Multichip Analysis (RMA) probe set summarisation algorithm available as part of the Expression Console v1.2.1 software package (Affymetrix).

2.4.7.2. Data filtration and statistical tests

Following normalisation, data was filtered to remove any statistically insignificant expression values, thus making data interpretation easier.

For univariate analysis, data was filtered in two ways: the first filtration method involved ordering only the DMSO-treated primary ALL tumours according to *in vitro* sensitivity to the cytotoxic effects of JQ1 (as measured in cytotoxicity assay experiments), and then ranking genes according to the fold difference in gene expression between ALL tumours most sensitive and least sensitive to JQ1. This was in aid of identifying genes predictive of JQ1 sensitivity.

The second filtration method, also used as part of univariate data analysis, involved the ranking of genes according to fold gene expression change between primary ALL samples treated with either DMSO or 1 µM JQ1.

These filtration processes were achieved using an empirical Bayes approach, using the Linear Models for MicroArray (LIMMA) statistical package within the BioConductor computer program [402]. For both filtration methods parameters were set to exclude genes with a p-value greater than 0.001 and a fold change less than 2.

For multivariate analysis, consisting of the gene set enrichment analysis performed on microarray data from this study (Section 2.4.7.4), normalised expression data were exported to the Excel program (Microsoft) and filtered by removing genes that were not expressed at all or at very low levels that could be considered background noise. Genes with a p-value greater than 0.05 and a fold change less than 1.5 were excluded.

2.4.7.3. Gene clustering

For the generation of heatmaps microarray data was imported from GeneSpring and loaded into the Cluster 3.0 computer program. Complete linkage clustering was then performed on the data, and then visualised as heatmaps that were gereated using the TreeView software program (http://rana.lbl.gov/EisenSoftware.htm). The heatmaps allowed for the visualisation of patterns of differential gene expression patterns between DMSO- and JQ1-treated primary ALL tumours, as well as genes that correlated with JQ1 $EC_{50.}$

In experiments involving PIM2, microarray data previously obtained in our lab [247] were re-analysed. Heatmaps allowed for the visualisation of genes that were expressed with a significant association to PIM2 expression.

2.4.7.4. Gene Set Enrichment Analysis

With the help of Dr. Wenbin Wei, Gene Set Enrichment Analysis (GSEA) [403] was performed on filtered microarray data and allowed for the identification of differentially expressed genes that belonged to, and enriched, functionally related gene sets representing major cellular processes and biological pathways.

Using the javaGSEA Windows application (http://www.broadinstitute.org/gsea/), GSEA was conducted on the filtered dataset, which determined the enrichment of each gene in gene sets constituting the Molecular Signatures Database (MsigDB) 1.0 gene set database. The application parameters were set to identify only those gene sets with a minimum of 10 genes and a maximum of 500 genes. The

javaGSEA application also calculated the level of significance (p-value) for each enriched gene set, and a false discovery rate (FDR) [403], which indicated the probability that an observed result was a false positive. Once GSEA was complete, enriched gene sets were ranked according to the p-value and FDR, and gene sets with a p-value less than 0.05 and an FDR rate less than 0.25 (25% chance of a false positive result) excluded. Using such stringent cut-off values resulted in the selection of only those gene sets with highly significant gene enrichment.

2.5. Molecular genetics techniques

2.5.1. Quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR)

2.5.1.1. cDNA synthesis

The reverse transcription system (Invitrogen) was used to convert 1 µg total RNA into single-stranded cDNA.

The first step involved placing PCR reaction tubes on ice and adding 1 µl dNTP mix (each of the four different dNTPs at a concentration of 10 mM) (Invitrogen), 1 µl random primers (Promega), 1 µg of RNA, and RNase-free water (Qiagen) up to a total volume of 12 µl. The sample tubes were briefly vortexed and centrifuged, and then incubated at 65ºC for 5 minutes.

The second step involved the addition of 1 µl Superscript II reverse transcriptase (Invitrogen), 1 µl RNaseOUT recombinant ribonuclease inhibitor (Invitrogen), 2 µl dithiothreitol (DTT) (Invitrogen), and 4 µl 5X First-Strand Buffer (250 mM Tris-HCl, 375 mM KCl, 15 mM $MgCl_2$) (Invitrogen) to each sample tube containing 12 µl of reaction mix and RNA from the first step. Samples were

then incubated at 25ºC for 5 minutes, 42ºC for 50 minutes, and finally 80ºC for 15 minutes. Synthesised cDNA was then stored at -80ºC until further use. When cDNA samples were needed for experiments and thawed for the first time, samples were diluted 1:5 with distilled water.

2.5.1.2. Primer design

Primers were designed for specific genes, based on the mRNA sequence for a given gene, using the OligoPerfect Designer online tool (Invitrogen). Parameters were set to generate primers with an annealing temperature of 60ºC and maximum length of 100 bases. Lyophilised primers were reconstituted with sterile distilled water to a stock concentration of 200 µM. To avoid unnecessary freeze-thawing, primer stocks were diluted 1:10 with sterile distilled water. Sequences for the primers used in this study can be found in Table 2.4.

Primer name	Primer sequence
IL7R Forward	5’-AGG CAC TTT ACC TCC ACG AG-3’
IL7R Reverse	5’-GTT GGA AGT GAA TGG ATC GC-3’
BIRC3 Forward	5’-TCC GGC AGT TAG TAG ACT ATC CA-3’
BIRC3 Reverse	5’-GGG AAG AGG AGA GAG AAA GAG C-3’
EIF4EBP2 Forward	5’-GAG AAT TGC GAC GAT CCA AC-3’
EIF4EBP2 Reverse	5’-TCA TGA CTA TTG CAC CAC GC-3’
TNFSR4 Forward	5’-TTG AAT TCG AGG ATA CCG ATG-3’
TNFSR4 Reverse	5’-GTA ATT CAG GGA CTG GGG CT-3’
PPP1R13B Forward	5’-GAC AGG TTG TTT CCG GTG TT-3’
PPP1R13B Reverse	5’-GAC TCT CCC CGC GAT GAT-3’
MYC Forward	5’-AGG GAT CGC GCT GAG TAT AA-3’
MYC Reverse	5’-TGC CTC TCG CTG GAA TTA CT-3’
B-ACTIN Forward	5’-CAC CAT TGG CAA TGA GCG GTT C-3'
B-ACTIN Reverse	5’-AGG TCT TTG CGG ATG TCC ACG T-3’
PIM2 Forward	5’-TGG GCA TCC TCC TCT ATG AC-3’
PIM2 Reverse	5’-ATT AGG GCA CAG CAG TCT GG-3’

Table 2.4: qRT-PCR primer sets used in this study.

Primers were designed using the OligoPerfect primer design online tool (Invitrogen). Primers were all designed to exhibit optimum annealing at approximately 60ºC, in order to be most efficiently used in downstream qRT-PCR experiments using the same protocol.

2.5.1.3. SYBR Green RT-PCR reaction preparation

Control cDNA synthesised from NALM-6 cell line template mRNA was used in test PCR reactions to test primer sets using the FastStart Taq DNA Polymerase kit (Roche) with a range of annealing temperatures from 58ºC to 68ºC, as well as different concentrations of primers. Reactions were run on a 4% agarose gel and visualised in order to confirm PCR products were present, that only one single PCR product was made per primer set, and that a high PCR product yield was obtained.

After testing the primers, PCR reactions were prepared in MicroAmp Fast Optical 96-Well Reaction Plates (Applied Biosystems) to which 4 µl of cDNA were added to each well, along with 16 µl of a prepared Master mix consisting of 0.25 µl forward primer (250 nM), 0.25 µl reverse primer (250 nM), 10 µl Fast SYBR Green mix (Applied Biosystems), and 5.5 µl distilled water, giving a total volume of 20 µl per reaction. The reaction plate was then placed in a 7500 Fast Real-Time PCR System (Applied Biosystems) and thermal cycling conditions set as 1 cycle at 95ºC for 20 seconds for activation of the DNA polymerase enzyme, 40 cycles at 95ºC for 3 seconds for denaturation, followed by 60ºC for 30 seconds for annealing and extension. A heat dissociation phase was also added on to the end of the thermal cycling protocol within the RT-PCR system software, to provide a dissociation curve that measured the level of amplicons relative to a range of melting temperatures from 60ºC to 95ºC, and therefore confirm primer specificity, as characterised by a single peak above 75ºC. Ct values generated for each gene of interest were then normalised to the β-actin loading control gene, so that relative expression levels could be calculated. Fold change in

transcript levels of a given gene were then calculated by dividing the relative expression levels after drug treatment by the relative expression before drug treatment.

2.6. Cell biology techniques

2.6.1. Measurement of cytotoxicity

Primary leukaemic cells and cell lines were seeded into opaque 96-well plates at a density of $5x10^4$ cells per well, and treated with either dimethylsufoxide (DMSO) or increasing concentrations of JQ1 (0.001 to 10 µM), with or without the additional agent (dexamethasone or vincristine). Following incubation at 37ºC for 48 hours (primary ALL tumours) or 72 hours (cell lines), the CellTiter-Glo Luminescent Cell Viability Assay (Promega) was used according to the manufacturer's instructions to indirectly evaluate the loss of cellular viability in response to drug treatment based on quantitation of the amount of ATP released by cells following lysis using the supplied reagents.

In cytotoxicity experiments involving PIM inhibitor K00135, the same conditions were used as above, only K00135 concentrations ranging from 0.01 to 100 µM were used instead, unless stated.

The Prism 4 (GraphPad) software package was used to calculate half maximal effective concentrations (EC_{50}) and to analyse results by Student's unpaired t-test where appropriate. A p-value of <0.05 was taken as significant.

Calcusyn software was used to measure the degree of synergy, as indicated by calculated combination indices (CI), between two compounds used to treat tumour cells (CI<1 synergistic, CI=1 additive, CI>1 antagonistic).

2.6.2. Fluorescent DNA fibre-labelling technique

NALM-6 cells were pre-treated with DMSO or 1 μM JQ1 inhibitor for 1 hour and 24 hours prior to labelling. Dual labelling of replication tracts was performed by pulse-labelling cell cultures in exponential growth with 25 μM chloro-deoxyuridine (CIdU) for 20 minutes, followed by 250 μM iodo-deoxyuridine (IdU) for a further 20 minutes.

To prepare DNA fibre-spreads on microscope slides, 2 ml of each cell suspension at a cell density of 200 cell/ml were spread on Silane-prep slides (Sigma-Aldrich), close to and in parallel to the label. The sample was allowed to evaporate until almost dry, after which 10 μl of spreading buffer (0.5% SDS in 200 mM Tris–HCl (pH 7.4), 50 mM EDTA) was pipetted on top of the sample, causing cells to swell and rupture. After 10 minutes had elapsed, the slide was tilted at an angle of 15º to allow the cell lysate to slowly travel down the slide. The DNA spreads were then left to air-dry, before being fixed in 3:1 methanol/acetic acid for 2 minutes and left to air-dry once more overnight. The slides were then stored at -20ºC for 24 hours.

For immunodetection of CIdU- and IdU-labelled tracts, acid-treated fibre spreads were incubated with a mix of rat anti-BrdU monoclonal antibody, clone

BU1/75 (ICR1) (Serotec), and mouse anti-BrdU monoclonal antibody. Slides were fixed in 4% paraformaldehyde and incubated with a mix of AlexaFluor 555-conjugated goat anti-rat and AlexaFluor 488-conjugated goat anti-mouse immunoglobulin G (IgG) (Molecular Probes). Fibres were examined using a Nikon Eclipse E600 microscope with a 60X lens, images recorded using the Volocity software package (Perkin Elmer) and the lengths of green (AlexaFluor 488) and/or red (AlexaFluor 555) labelled patches measured using the ImageJ software application.

2.6.3. Cell cycle analysis

Staining of ALL cell line cells with PI and analysis by flow cytometry was used to measure the cell cycle distribution of cells treated with either DMSO or 1 µM JQ1 for 24 hours. This technique uses light scattering from the passing of sample cells through a laser, and fluorescence emitted following the excitation of the PI dye, which binds and intercalates into DNA, to quantify the DNA content in cycling cells.

ALL tumour cells were treated in a 6-well plate (Iwaki) at a density of $2x10^6$/ml. Using a Pasteur pipette, 3 ml of cell suspension were transferred to flow cytometry sample tubes and centrifuged at 2000 rpm for 3 minutes, and the supernatant discarded. Cells were then washed by re-suspending cells in 3 ml ice-cold PBS, before centrifuging again and re-suspending cells in 3 ml ice-cold 70% ethanol/PBS, drop-by-drop whilst gently vortexing. At this stage cells were either stored at -20ºC for up to a week or used immediately.

Ethanol-fixed samples were stained on the day of cytometric analysis, by first centrifuging cells at 2000 rpm for 5 minutes at 4ºC, re-suspending the formed pellet in 3 ml ice-cold PBS, and then centrifuging cells once more before re-suspending pellets in a 1 ml PI/RNase solution (25 µg/ml PI, 0.1 mg/ml RNase). PI-satined cells were then wrapped in aluminium foil and incubated in the dark at room temperature. Samples were then analysed using an Accuri C6 Flow Cytometer (Becton Dickinson) with an excitation wavelength of 488 nm. Using the latest version of WinMDI, the proportion of cells in the G1, S, and G2 phases of the cell cycle were determined.

2.6.4. Bromodeoxyuridine (BrdU) incorporation cell proliferation assay

NALM-6 cells were treated with either DMSO or 1 µM JQ1 for 24 hours. $3x10^6$ NALM-6 cells were pulse-labelled with 10 µM BrdU (Sigma-Aldrich) for 20 minutes prior to harvesting cells at the 24 hour timepoint, washed in PBS, and then fixed in 80% ice-cold ethanol overnight at -20ºC. Cells were then incubated in 15 mM pepsin for 20 minutes at 37ºC, before being pelleted and incubated with 2 N HCl for 20 minutes at room temperature. The pellet was then washed in buffer Bu (0.1% FCS, 0.1% Tween 20, 0.1 M Hepes in PBS) and incubated with mouse anti-BrdU antibody, clone Bu20a (Dako) diluted to 1:50 in buffer Bu for 45 minutes. Cells were then centrifuged and re-suspended in anti-mouse-FITC antibody (Vector laboratories) diluted at 1:50 with buffer Bu. To stain total DNA, cells were pelleted and re-suspended in PBS containing 25 µg/ml propidium iodide (Sigma-Aldrich) and 50 µg/µl RNase (Sigma-Aldrich), before

performing analysis of samples by flow cytometry using an Accuri C6 Flow Cytometer (Becton Dickinson).

2.7. ALL xenograft mouse model studies

Two *in vivo* experiments were performed in this study. Animals were treated in accordance with UK Home Office guidelines, Schedule 1 (http://www.legislation.gov.uk/ukpga/1986/14/pdfs/ukpga_19860014_en.pdf).

In the first experiment $1x10^6$ NALM-6 cells were injected subcutaneously into 16 NOD/Shi-*scid*/IL-2Rγ^{null} (NOG) mice. Upon evidence of visible tumours, animals received either 50 $mgkg^{-1}$ JQ1 (n=8) or vehicle alone (10% (w/v) 2-hydroxy-propyl-β-cyclodextrin) (Sigma-Aldrich) (n=8) via intra-peritoneal (IP) injection for 5 days per week for a period of 2 weeks. Tumour volume was measured manually thrice weekly using a calliper.

In the second experiment, a xenograft model of a primary B precursor ALL tumour (ALL 105) was created by the intravenous injection of $1x10^6$ cells via the tail vein. Once levels of human CD45 reached 1% in the peripheral blood, mice received either 50 $mgkg^{-1}$ JQ1 (n=7) for 5 days per week over a period of 4 weeks, or vehicle alone (n=7). The proportions of cells positive for hCD45, hCD10, hCD19, and hCD34 were assessed on weekly blood samples. At the end of drug treatment spleens were harvested and tumour burden determined by spleen size. In addition, percentages of hCD45+CD34+CD19+CD10+, hCD45+CD34+CD19+CD10-, and hCD45+CD34-CD19+CD10 subpopulations were calculated from the proportion of gated live cells, and compared between the vehicle-treated and JQ1-treated animals.

CHAPTER 3

Results I: Targeting BET proteins in pre-B ALL

3. RESULTS I: TARGETING BET PROTEINS IN PRE-B ALL

3.1. Sensitisation of ALL tumour cells to the BET bromodomains inhibitor JQ1

Although remarkable improvements in the treatment of B precursor ALL have been made over the past few decades, a significant number of patients still suffer from disease relapse and toxicity associated with the use of conventional chemotherapeutic agents. This highlights the highly heterogenous nature of ALL, where variable treatment responses are observed and resistance to treatment sometimes ensues. It is therefore clear to see that the requirement exists for a novel, more targeted approach in treating these patients.

The bromodomain-containing BRD4 protein is a member of the BET family of proteins, and has recently emerged as novel therapeutic target in malignancies such as AML and NUT-midline carcinoma [344]. Studies have shown that BRD4 forms part of transcription complexes and remains bound to transcriptional start sites of genes expressed during the M/G1 transition of the cell cycle, thus promoting mitotic progression and transcriptional elongation [336].

Taking these findings into consideration, I aimed to explore the possibility of targeting BET proteins in ALL, including BRD4 that has previously been implicated in the pathophysiology of haematopoietic malignancies, using the highly specific pharmacological inhibitor JQ1, as an alternative strategy to downregulating multiple prosurvival signaling pathways and cellular processes, and thereby sensitising tumours to apoptosis.

3.1.1 Expression of BRD4 in ALL cell lines and primary tumours

To establish whether or not pharmacological inhibition of BRD4 was a feasible approach, I began by determining the protein expression levels of BRD4 in lysates prepared from a panel of 6 ALL cell lines, representing diverse disease subtypes. These included BCR-ABL1^{+} ALL (SD1, SUPB-15, and TOM-1), apoptotic-resistant BCR-ABL1^{-} ALL (NALM-6, REH), and apoptotic-sensitive ALL (NALM-17). I also tested a panel of 10 primary ALL tumours (ALL-103, ALL-104, ALL-105, ALL-106, ALL-108, ALL-109, ALL-110, ALL-111, ALL-112, and ALL-113) of which 5 were low-risk MRD (ALL-103, ALL-104, ALL-108, ALL-109 and ALL-111) and 5 were high-risk MRD (ALL-105, ALL-106, ALL-110, ALL-112 and ALL-113). I used SDS-PAGE followed by the Western-blotting technique to visualise BRD4 protein.

I observed high levels of 152 kDa BRD4 protein expression across all ALL cell lines and primary tumours analysed, irrespective of their phenotype (Figure 3.1a & b). Although it was not possible to improve the western blot due to restricted primary tumour material, BRD4 expression did not appear to correlate with clinical features (age, WCC, cytogenetic abnormalities, or risk stratification) that were associated with the patients from which these tumours were derived (Table 2.1).

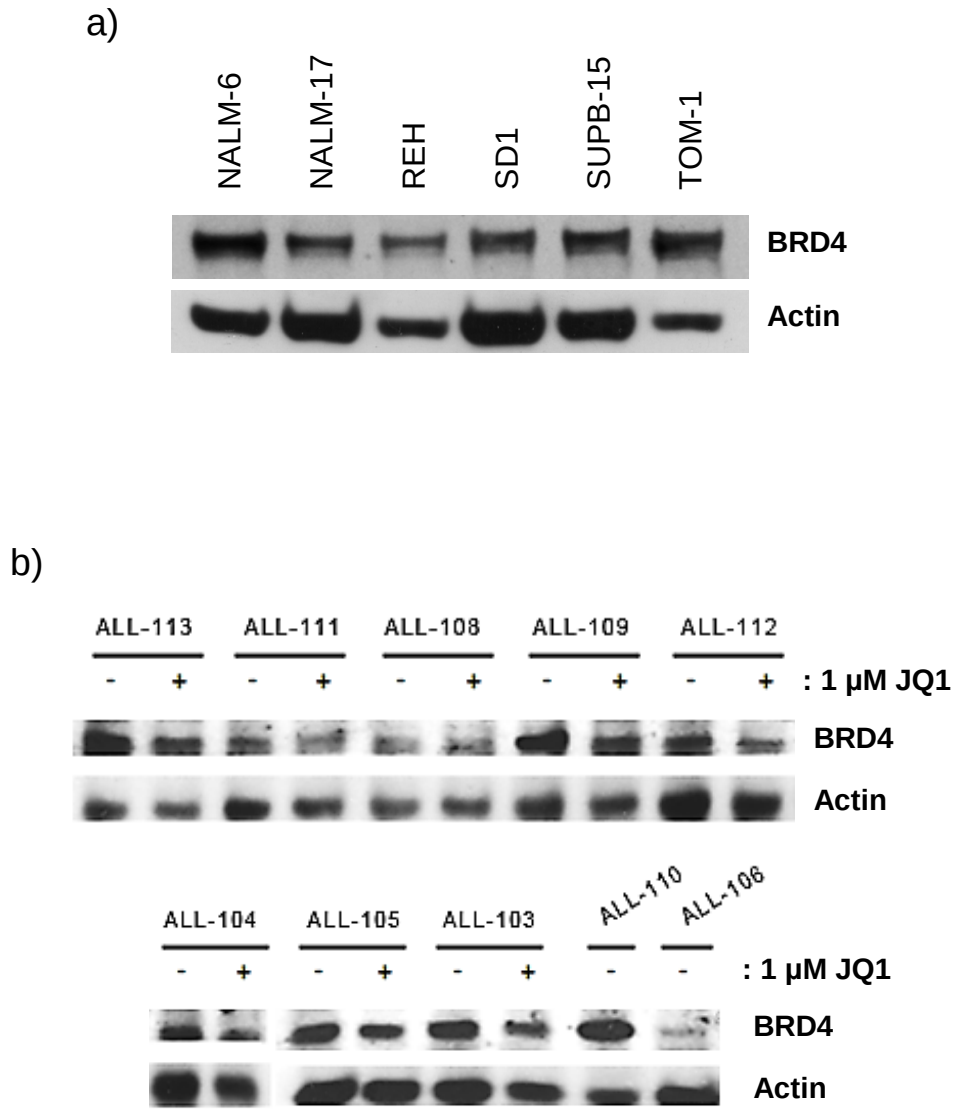


Figure 3.1: Expression of BRD4 protein in ALL cell lines and primary tumours.

Whole protein lysates were prepared from 6 ALL cell lines and 10 primary ALL tumours and probed for 152 kDa BRD4 protein expression by Western blotting. B-actin was used as a loading control.

a) High levels of BRD4 expression are seen across all ALL cell lines in this panel, namely NALM-6, NALM-17, REH, SD1, SUPB-15 and TOM-1.

b) Although some degree of inter-patient variability in the level of BRD4 expression exists, all primary ALL tumours express BRD4 protein. Tumour cells treated with 1 μM JQ1 for 6 hours do not show any significant decrease in BRD4 expression relative to β-actin.

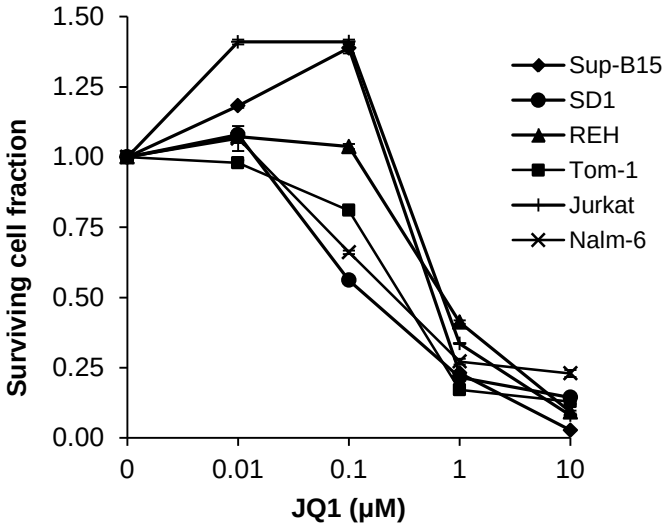
3.1.2 *In vitro* cytotoxic effects of JQ1-mediated inhibition of BET proteins in ALL cell lines and primary tumours

Since all the ALL cell lines and primary tumours analysed expressed high levels of BRD4 protein (Figure 3.1), I decided to use a luminescent ATP-based cytotoxicity assay to measure the effect of JQ1 treatment on cell viability. The panel of cell lines tested were SUPB-15, SD1, REH, TOM-1, Jurkat and NALM-6. Cell viability was measured 72 hours following exposure to increasing concentrations of JQ1 inhibitor only. I also addressed JQ1-mediated cytotoxicity in a panel of 25 non-cycling primary B precursor ALL tumours that included representatives of low-risk and high-risk MRD phenotypes, infant and relapsed ALL, as well as non-tumour PBMC obtained from 3 healthy donors. Since recent studies demonstrated potent sensitisation of primary AML tumours following BRD4 inhibition [344], I also tested for JQ1 cytotoxicity in 2 primary AML tumours.

In all the ALL cell lines tested, I found that JQ1 led to an impressive decrease in cell viability at 72 hours (Figure 3.2a), with EC_{50} values ranging from 0.17–1.00 µM (Table 3.1). Interestingly, treatment of primary ALL tumours with a cytotoxic dose of 1 µM JQ1 inhibitor did not have an impact on BRD4 expression levels, which was consistent with the JQ1 mechanism of action involving the inhibition of BRD4 activity as opposed to decreasing its expression.

In contrast to ALL cell lines that were uniformly sensitive, incubation of primary ALL tumours with 1 µM JQ1 demonstrated a moderate to substantial loss of cell viability following 48 hours (Figure 3.2b). I calculated that 36% (9/25) of primary

a)



b)

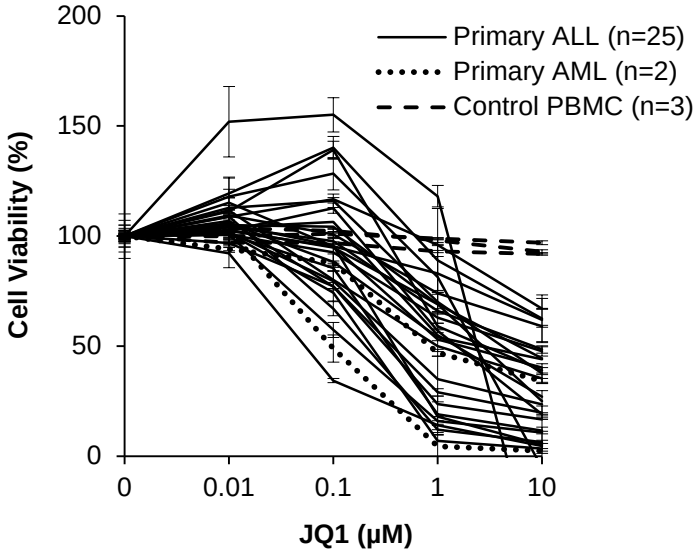


Figure 3.2: JQ1-induced cytotoxicity in a panel of ALL cell lines and a range of primary tumours.

Effects of JQ1 treatment on cell viability were measured using a luminescent ATP-based cytotoxicity assay.

a) Cell lines NALM-6, NALM-17, REH, SD1, SUPB-15 and TOM-1 incubated with 1 μM JQ1 for 72hr show a dramatic loss of viability. Assays were performed in triplicate. Data are presented as mean ± SEM; n=3.

b) Compared to PBMC obtained from two healthy individuals, representative primary ALL and AML tumours show a differential loss of viability upon 48hr incubation with 1 μM JQ1. Data are presented as mean ± SD.

ALL tumours showed EC_{50} values < 1 µM (2 high-risk MRD, 4 low-risk MRD, 1 infant ALL, and 2 tumours with unknown risk stratification), which was a cytotoxic dose comparable to that observed in ALL cell lines (Table 3.2). Furthermore, I also noticed that primary tumours with an EC_{50} value between 1 and 10 µM, for which I had complete clinical information, were all MRD high-risk ALL tumours. Interestingly, lower sensitivity to JQ1 was observed in 16% (4/25) of primary ALL tumours, where EC_{50} values were >10 µM (Table 3.2). Of these JQ1-resistant tumours, one was high-risk ALL (ALL-123), and three were low-risk ALL (ALL-103, ALL-109, ALL-124).

I calculated the mean EC_{50} for all primary tumours and control PBMC treated with JQ1, and found that high-risk ALL tumours had a lower mean EC_{50} of 5.63 ± 5.32 µM, compared to low-risk ALL tumours with a mean EC_{50} of 7.58 ± 6.31 µM (Table 3.2). This suggested JQ1 cytotoxic activity favourably targeted high-risk ALL tumours, which are those derived from patients that frequently exhibit chemoresistance and are deemed as having an unfavourable clinical prognosis.

Infant ALL tumours (ALL-114 and ALL-118) were also sensitive to JQ1-treatment (0.17 and 2.0 µM, respectively). Infant ALL is characterised by potential chemoresistance, frequently occurring rearrangements of the Mixed-Lineage Leukaemia (*MLL)* gene, and a poor clinical prognosis [404, 405]. Since MLL fusion proteins activate the transcription of several oncogenes through binding with BRD4 [406], and the JQ1 inhibitor has been shown to exert potent anti-leukaemic activity against MLL-rearranged AML [344], it is possible that JQ1 is also effective in eliminating infant ALL tumour cells.

Cell line	median JQ1 EC_{50} (±SD) (μM)
SUPB-15	0.237 (±0.019)
SD1	0.173 (±0.023)
REH	1.004 (±0.012)
TOM-1	0.398 (±0.019)
Jurkat	0.326 (±0.006)
NALM-6	0.552 (±0.021)

Table 3.1: Half-maximum-effect concentrations (EC_{50}) calculated for a panel of ALL cell lines treated with JQ1 inhibitor.

JQ1 EC_{50} values (±S.D.) were calculated for SUPB-15, SD1, REH, TOM-1, Jurkat, and NALM-6 using the Prism 4 software package (GraphPad Inc.), which were derived from data values obtained for each cell line in cytotoxicity experiments following 72h incubation of cells with a log-scale range of JQ1 concentrations.

EC_{50} values for JQ1-treated ALL cell lines were generally in the low nanomolar range (0.173-1.004 μM).

	Patient	JQ1 EC50 (μM)	Sub-group mean JQ1 EC50 ± SEM (μM)
High Risk ALL (n=12)	ALL-101	1.80	5.63 ± 5.32
	ALL-105	0.11	
	ALL-106	5.83	
	ALL-110	3.38	
	ALL-112	4.30	
	ALL-113	0.72	
	ALL-116	5.82	
	ALL-117	5.75	
	ALL-121	2.81	
	ALL-122	1.45	
	ALL-123	34.17	
	ALL-126	1.47	
Low Risk ALL (n=6)	ALL-103	23.08	7.58 ± 6.31
	ALL-104	0.18	
	ALL-108	0.73	
	ALL-109	20.22	
	ALL-111	0.79	
	ALL-115	0.49	
Infant ALL (n=2)	ALL-114	0.17	1.08 ± 0.75
	ALL-118	2.00	
Clinical stratification not known	ALL-129	0.12	1.17 ± 0.57
	ALL-130	1.45	
	ALL-131	2.64	
	ALL-132	1.17	
	ALL-133	0.42	
AML (n=2)	AML-1	0.06	1.89 ± 1.49
	AML-2	3.72	
Healthy control	PBMC-1	886.08	594.78 ± 283.48
	PBMC-2	303.49	

Table 3.2: Half-maximum-effect concentrations (EC_{50}) calculated for a panel of primary leukaemia tumours treated with JQ1 inhibitor.

Calculated EC_{50} values were derived from cytotoxicity experiment data, where primary tumours were treated with a range of log-scale JQ1 concentrations for 48h.

ALL tumours, particularly those derived from high-risk patients, were sensitised to clinically achievable JQ1 concentrations (JQ1 EC_{50} <10 μM). Primary AML tumours were used as a positive control and were also sensitised to JQ1.

Healthy PBMCs were not sensitised to JQ1 and showed high JQ1 EC_{50} values.

The healthy PBMCs did not exhibit a significant loss of cell viability even with 10 µM JQ1 (Figure 3.2b), whereas both primary AML samples (AML-1 and AML-2) showed a significant loss of viability with a mean EC_{50} of 1.89 ± 1.49 µM (Table 3.2).

These results indicate that JQ1 activity has a profound impact on the viability of multiple ALL cell lines and primary ALL tumours *in vitro*, regardless of their phenotype. This data also suggests that JQ1 is capable of sensitising high-risk ALL more potently than low-risk ALL. The observation that primary AML tumours are highly sensitive to the effects of JQ1 treatment in the results I reported, is supported by published data demonstrating the *in vivo* anti-tumour activity of JQ1 in an AML xenograft model [344]. Finally, results showing that JQ1 treatment does not exert cytotoxic effects in healthy control PBMCs, are indicative of JQ1 activity being tumour-specific and potentially safe to test in animal studies.

3.1.3 Combined *in vitro* cytotoxic effects of JQ1 together with conventional chemotherapeutic agents in ALL cell lines and primary tumours.

Since treatment with JQ1 alone induced substantial *in vitro* cytotoxic effects in ALL cell lines and a large panel of ALL tumours, I decided to investigate the combined cytotoxic effects of JQ1 together with conventional cytotoxic agents used to treat ALL, namely dexamethasone, vincristine, and daunorubicin.

I treated REH, TOM-1, and NALM-6 cells with a subcytotoxic dose of JQ1 (0.1 µM) in combination with log scale increments in drug concentration of each of the conventional cytotoxic agents named above, ranging from 0.0001 to 10 µM, and measured cell viability at 72 hours using the ATP-based cytotoxicity assay.

Notably, treatment of TOM-1 cells with the JQ1/dexamethasone combination showed enhanced cytotoxicity compared to dexamethasone treatment alone (Figure 3.3), and was highly synergistic with dexamethasone concentrations ranging from 0.0001 to 0.1 µM (Table 3.3).

In contrast, I observed no increased cytotoxicity in REH cells when 0.1 µM JQ1 was combined with increasing concentrations of dexamethasone (Figure 3.4). There was no dose-dependent response following dexamethasone treatment, therefore use of the Calcusyn software package to calculate synergistic effects was not possible, since a dose-effect curve is a requirement. This may have been due to the fact REH cells are known to lack expression of the gonadotrophin receptor via which dexamethasone exerts its cytotoxic effects

[407]. I did not observe any synergistic effects in REH cells treated with a combination of JQ1 with either vincristine or daunorubicin (data not shown).

Similarly, treatment of NALM-6 cells with the JQ1/dexamethasone combination also led to greatly enhanced cytotoxic effects compared to dexamethasone treatment alone (Figure 3.5), and exhibited the greatest cytotoxic response to dexamethasone compared to REH and TOM-1. Furthermore, I observed highly synergistic effects across all the different concentrations of dexamethasone I used to treat NALM-6 cells (ranging from 0.0001 to 10 μM) (Table 3.3).

Given that combined JQ1/dexamethasone treatment appeared to induce the greatest sensitisation of ALL cell lines (TOM-1 and NALM-6) to dexamethasone (Figures 3.3 & 3.5) in comparison to combinations with either vincristine or daunorubicin, and the availability of primary tumour material was limited to be able to test multiple drug combinations, I selected dexamethasone as the drug to test for synergistic effects in combination with 0.1 μM JQ1 in three representative primary ALL tumours (ALL-129, ALL-130, and ALL-132). I did this by measuring cell viability at 48 hours after drug treatment using the ATP-based cytotoxicity assay.

I found that the JQ1/dexamethasone treatment combination led to increased sensitisation to dexamethasone in all three primary ALL tumours (ALL-129, ALL-130 and ALL-132), when compared to tumour cells treated with dexamethasone alone (Figure 3.6-3.8). I observed the greatest sensitisation in ALL-129 and ALL-130, with the greatest differential in surviving cell fractions being at lower concentrations of dexamethasone, ranging between 0.0001 and

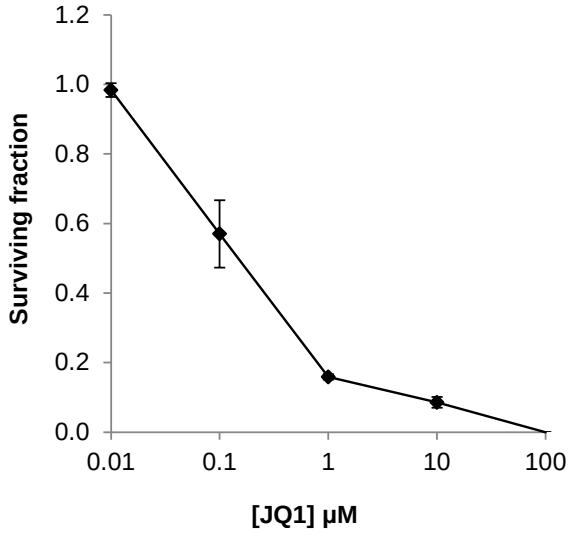
0.01 μM. Furthermore, I confirmed that treatment with the JQ1/dexamethasone combination demonstrated highly synergistic effects in all three primary ALL tumours using concentrations of dexamethasone ranging between 0.0001 and 1 μM (Table 3.4).

These results indicate that JQ1 activity leads to increased *in vitro* sensitisation of ALL cell lines and primary tumours to conventional chemotherapeutic agents used in the clinic.

3.1.4 Summary

In conclusion, I showed that BRD4 protein is expressed at high levels in multiple ALL cell lines and primary ALL tumours. I also showed that treatment of both cell lines and primary tumours with JQ1 induced cytotoxicity, irrespective of the phenotype or associated risk stratification. Furthermore, the data I have reported shows there may be potential benefits from treating ALL tumours with a combination of JQ1 with chemotherapeutics such as dexamethasone. This data supports the rationale for further work to elucidate the mechanism of JQ1-mediated cytotoxicity in ALL, and determine whether or not these anti-tumour effects are translatable in an *in vivo* model of ALL.

a)



b)

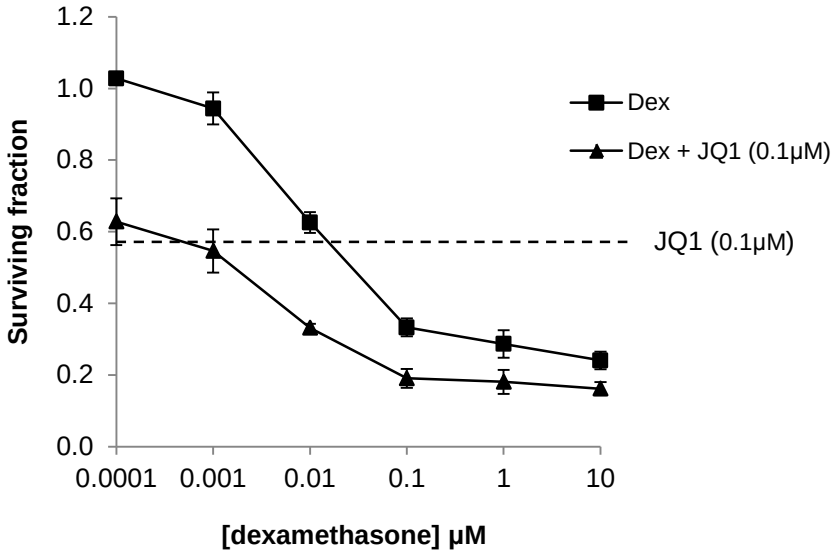


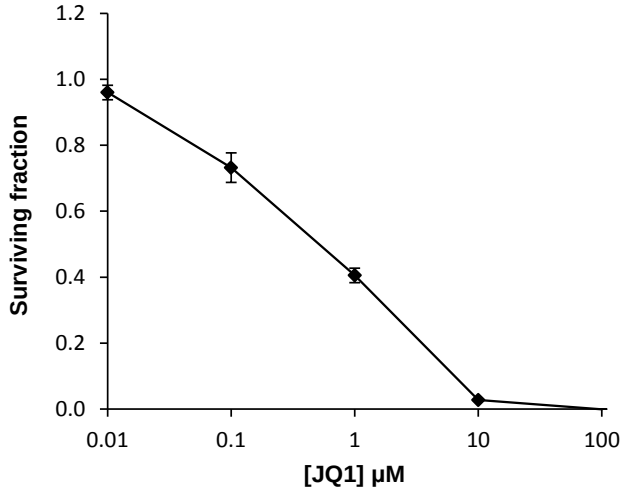
Figure 3.3: *In vitro* cytotoxic effects of combined treatment with JQ1 and dexamethasone in the TOM-1 cell line

Cell viability was measured in triplicate using a luminescent ATP-based cytotoxicity assay. Data are presented as mean ± SEM; n=3.

a) JQ1 sensitises TOM-1 cells (72h incubation), with a surviving fraction of 0.59 at 0.1 µM JQ1.

b) Treatment of TOM-1 cells with the JQ1/dexamethasone drug combination led to significant synergistic cytotoxicity.

a)



b)

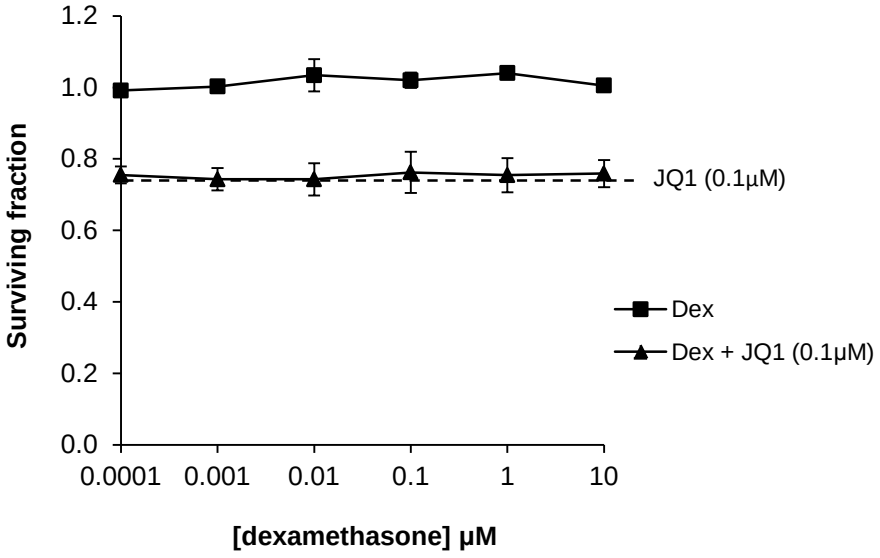


Figure 3.4: *In vitro* cytotoxic effects of combined treatment with JQ1 and dexamethasone in the REH cell line

Cell viability was measured in triplicate using a luminescent ATP-based cytotoxicity assay. Data are presented as mean ± SEM; n=3.

a) JQ1 sensitised REH cells (72h incubation), with a surviving fraction of 0.76 at 0.1 µM JQ1.

b) REH cells are resistant to the cytotoxic effects of dexamethasone. Subsequently no synergistic effects were observed following treatment with a combination of JQ1/dexamethasone.

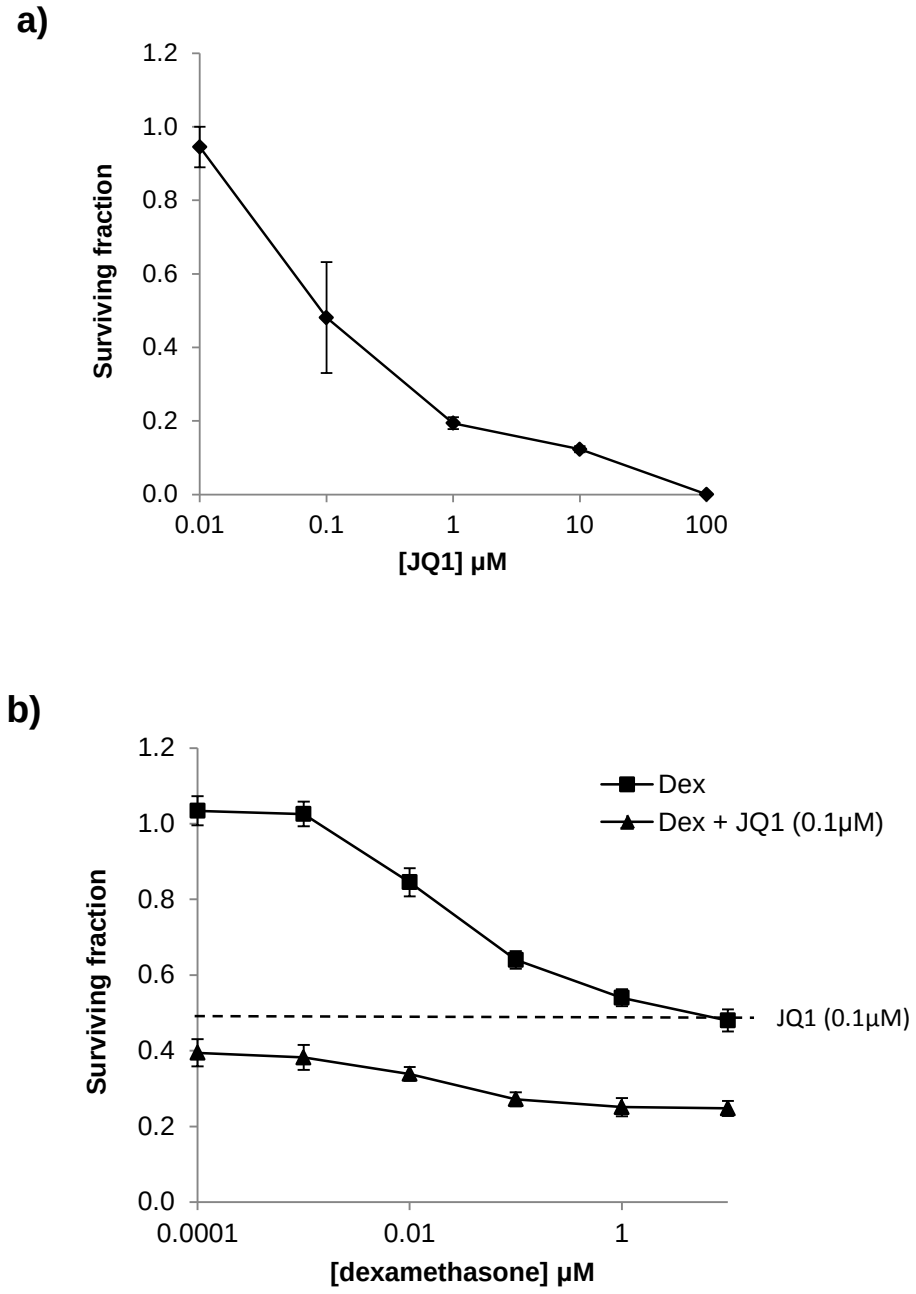


Figure 3.5: *In vitro* cytotoxic effects of combined treatment with JQ1 and dexamethasone in the NALM-6 cell line

Cell viability was measured in triplicate using a luminescent ATP-based cytotoxicity assay. Data are presented as mean ± SEM; n=3.

a) JQ1 sensitises NALM-6 cells (72h incubation), with a surviving fraction of 0.48 at 0.1 µM JQ1.

b) Treatment of NALM-6 cells with the JQ1/dexamethasone drug combination led to significant synergistic cytotoxicity.

	Dexamethasone (μM)	Combination Index JQ1 (0.1 μM)	Synergism JQ1 (0.1 μM)
TOM-1	0.0001	0.44	***
	0.001	0.34	***
	0.01	0.25	***
	0.1	0.53	***
	1	4.43	*
	10	36.77	*
NALM-6	0.0001	0.33	***
	0.001	0.31	***
	0.01	0.26	***
	0.1	0.18	***
	1	0.17	***
	10	0.19	***

Table 3.3: Combination indices for JQ1 and dexamethasone in TOM-1 and NALM-6 cell lines.

Combination indices (CI) were calculated using the Calcusyn software (Biosoft™), in line with the Chou and Talalay drug interations mathematical model [408].

The CI is a measure of drug interaction, with CI>1, CI=1 and CI<1 representing antagonistic (*), additive (**) and synergistic (***) effects, respectively.

Synergistic cytotoxic effects were observed in TOM-1 cells treated with 0.1 μM JQ1 together with 0.0001-0.1 μM dexamethasone.

Synergistic cytotoxic effects were observed in NALM-6 cells treated with 0.1 μM JQ1 together with 0.0001-10 μM dexamethasone.

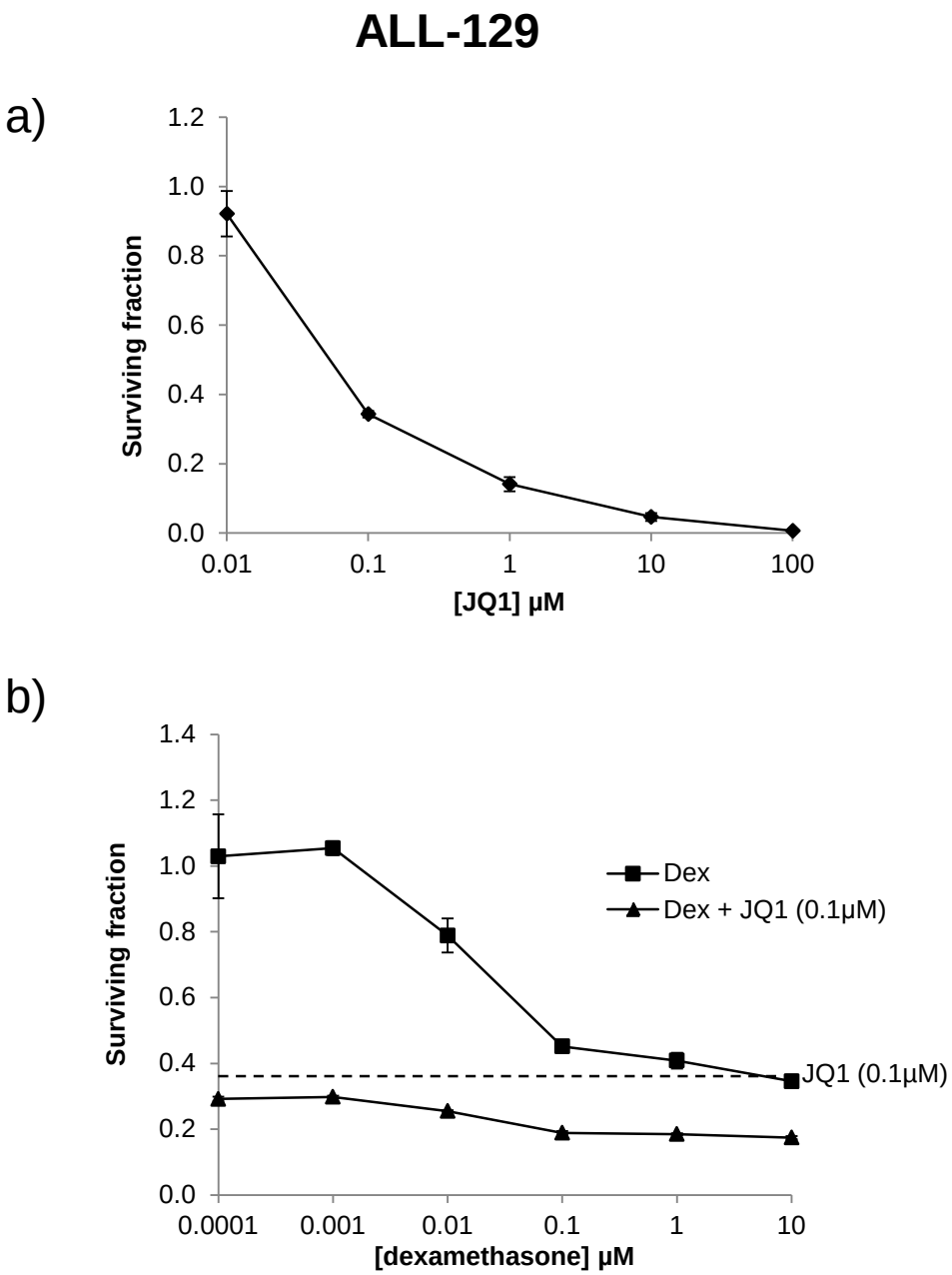


Figure 3.6: *In vitro* cytotoxic effects of combined treatment with JQ1 and dexamethasone in ALL-129 primary tumour cells.

Cell viability was measured in triplicate using a luminescent ATP-based cytotoxicity assay. Data are presented as mean ± SEM; n=3.

a) JQ1 sensitises ALL-129 tumour cells (48h incubation), with a surviving fraction of 0.37 at 0.1 µM JQ1.

b) Treatment of ALL-129 cells with the JQ1/dexamethasone drug combination led to significant synergistic cytotoxicity using 0.0001-10 µM dexamethasone.

ALL-130

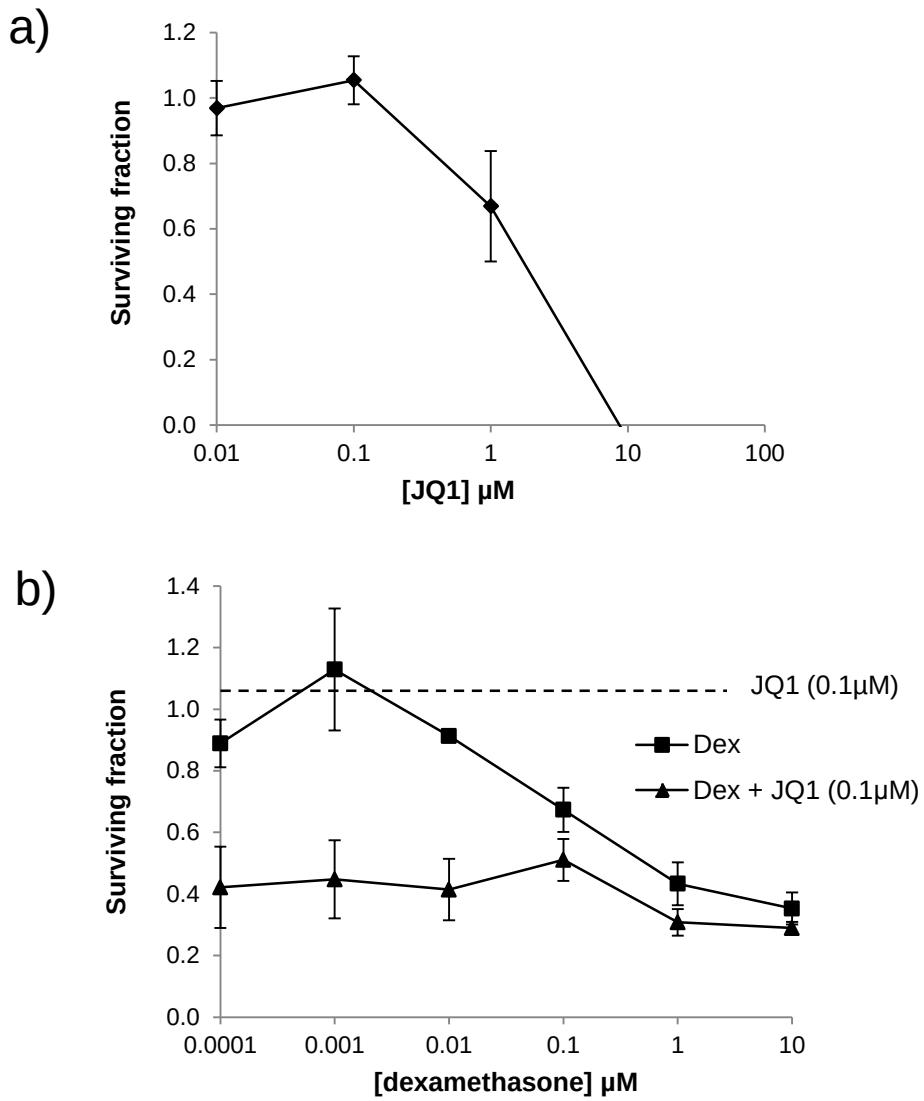


Figure 3.7: *In vitro* cytotoxic effects of combined treatment with JQ1 and dexamethasone in ALL-130 primary tumour cells.

Cell viability was measured in triplicate using a luminescent ATP-based cytotoxicity assay. Data are presented as mean ± SEM; n=3.

a) JQ1 sensitises ALL-130 tumour cells (48h incubation), with a surviving fraction of 1.06 at 0.1 µM JQ1.

b) Treatment of ALL-130 cells with the JQ1/dexamethasone drug combination led to significant synergistic cytotoxicity using 0.0001-1 µM dexamethasone.

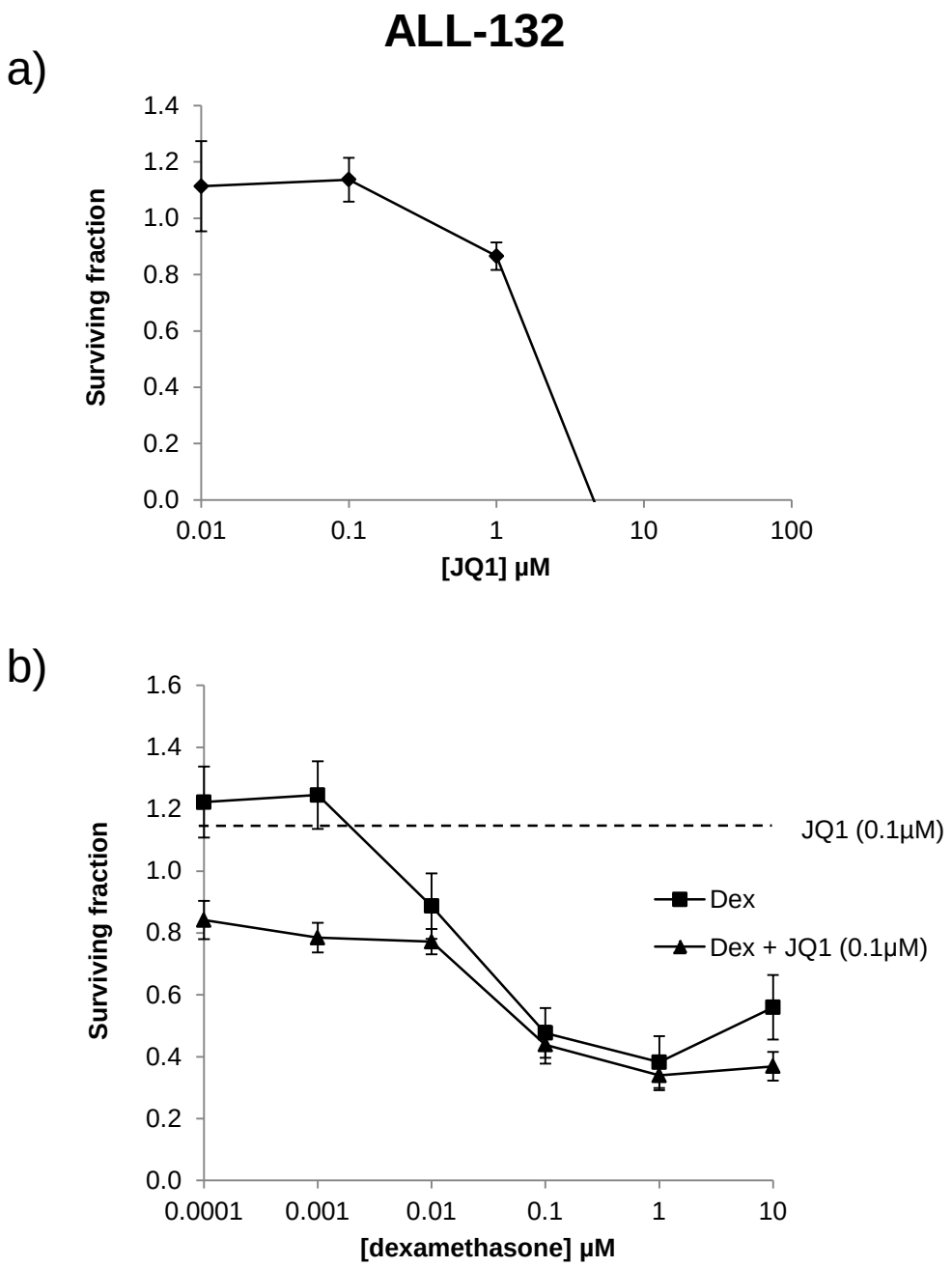


Figure 3.8: *In vitro* cytotoxic effects of combined treatment with JQ1 and dexamethasone in ALL-132 primary tumour cells.

Cell viability was measured in triplicate using a luminescent ATP-based cytotoxicity assay. Data are presented as mean ± SEM; n=3.

a) JQ1 sensitises ALL-132 tumour cells (48h incubation), with a surviving fraction of 1.26 at 0.1 µM JQ1.

b) Treatment of ALL-132 cells with the JQ1/dexamethasone drug combination led to significant synergistic cytotoxicity using 0.0001-0.1 µM dexamethasone.

	Dexamethasone (μM)	Combination Index JQ1 (0.1 μM)	Synergism JQ1 (0.1 μM)
ALL-129	0.0001	0.266	***
	0.001	0.001	***
	0.01	0.211	***
	0.1	0.135	***
	1	0.210	***
	10	0.838	***
ALL-130	0.0001	0.066	***
	0.001	0.001	***
	0.01	0.069	***
	0.1	0.168	***
	1	0.192	***
	10	1.143	*
ALL-132	0.0001	0.112	***
	0.001	0.141	***
	0.01	0.469	***
	0.1	0.342	***
	1	1.286	*
	10	15.398	*

Table 3.4: Combination indices for JQ1 and dexamethasone in primary ALL tumours.

Combination indices (CI) were calculated using the Calcusyn software (Biosoft™), in line with the Chou and Talalay drug interactions mathematical model [408].

The CI is a measure of drug interaction, with CI>1, CI=1 and CI<1 representing antagonistic (*), additive (**) and synergistic (***) effects, respectively.

Synergistic cytotoxic effects were observed in ALL-129 tumour cells treated with 0.1 μM JQ1 together with 0.0001-10 μM dexamethasone.

Synergistic cytotoxic effects were observed in ALL-130 tumour cells treated with 0.1 μM JQ1 together with 0.0001-1 μM dexamethasone.

Synergistic cytotoxic effects were observed in ALL-132 tumour cells treated with 0.1 μM JQ1 together with 0.0001-0.1 μM dexamethasone.

3.2 Gene expression profiling and transcriptional biomarkers of JQ1 sensitisation

Although the bromodomain-containing protein BRD4 has been shown to play a key role in regulating cell growth and proliferation, the underlying mechanisms by which it regulates transcription of genes involved in cell growth had, until recently, remained largely unknown. We now understand that whereas most transcription factors such as RNA polymerase II, Oct1, Pax 3, and E2F1 dissociate from chromosomes during mitosis, causing global transcription to cease, BRD4 protein remains bound to mitotic chromosomes to provide cellular 'memory' to daughter cells [336]. This allows for the reloading of RNA polymerase II and many other transcription factors onto chromosomes, and re-initiation of inherited patterns of gene expression following mitosis. Studies in NIH3T3 and MEF cells have shown that knockdown of the BRD4 gene using specific shRNA leads to a block at G1 and therefore cell cycle arrest, and that BRD4 knockdown significantly reduces the expression of cell cycle genes, such as cyclin D1 and cyclin D2 [335].

Data to support alternative roles for BRD4 and the regulation of transcription of genes independent of cell cycle regulation are relatively limited. However, a recent study has provided evidence that Lys310-acetylated NFκB recruits BRD4 in a complex with p-TEFb, which consists of CDK9 and cyclin T1, and RNA polymerase II to form the transcription machinery complex required for the expression of various pro-inflammatory NFκB genes, frequently associated with the aetiology of many cancers [409]. Our laboratory has previously published data confirming the deregulation of apoptotic pathways in primary ALL tumours,

partly attributed to the upregulation of multiple prosurvival signaling pathways such as the NFκB pathway [247, 248]. Taking all these reported findings into consideration, together with the fact that I showed JQ1-mediated BRD4 inhibition to not only be cytotoxic to proliferating cell lines, but also to largely non-cycling primary ALL tumour cells *in vitro*, I hypothesised that BRD4 also regulates the expression of genes involved in prosurvival signaling and inhibition of apoptosis in ALL, and not only genes associated with cell-cycle progression.

To test my hypothesis and further investigate the mechanism of JQ1-mediated cytotoxicity in ALL, I decided to make use of global gene expression microarray technology to identify the effect that BRD4 inhibition has on gene transcription in 8 primary ALL tumours before and after 6 hour exposure to 1μM JQ1, using Affymetrix GeneChip Human Gene 1.0 ST Arrays. I decided to use a 6 hour JQ1-exposure time point since I was only interested in the changes in expression of primary response genes, and wanted to reduce the likelihood of secondary response gene activation.

3.2.1 RNA quality control

Before starting the microarray experiment I needed to assess the integrity and quality of the RNA extracted from primary ALL tumour cells, to ensure that results obtained were reliable and truly representative of gene expression patterns within sample tumour cells. I made use of the Agilent 2100 Bioanalyser system to assess RNA quality, which is based on the RNA Integrity number (RIN), determined by the 2100 Expert system software. High RIN values represent good quality RNA, which is seen as two distinct bands (representing 28S and 18S eukaryotic ribosomal subunits) and as two distinct peaks on the electropherogram (Figure 3.9b). It has been shown that RNA samples with RIN values below 7 are likely to reflect RNA degradation (Figure 3.9). Thus, I used carefully extracted RNA with RIN values greater or equal to the recommended value of 8 [400]. A total of 8 primary ALL tumour samples were selected for this microarray study (ALL-101, ALL-103, ALL-104, ALL-105, ALL-106, ALL-108, ALL-109 and ALL-113), which represented a spectrum of different sensitivity to JQ1 based on EC_{50} values obtained in *in vitro* cytotoxicity experiments.

The primary RNA I extracted proved to be high quality, which increased the likelihood of successful microarray hybridisations and the collection of high integrity data from which to investigate the effects of JQ1-mediated BRD4 inhibition on gene transcription in primary ALL (Figure 3.10 and 3.11).

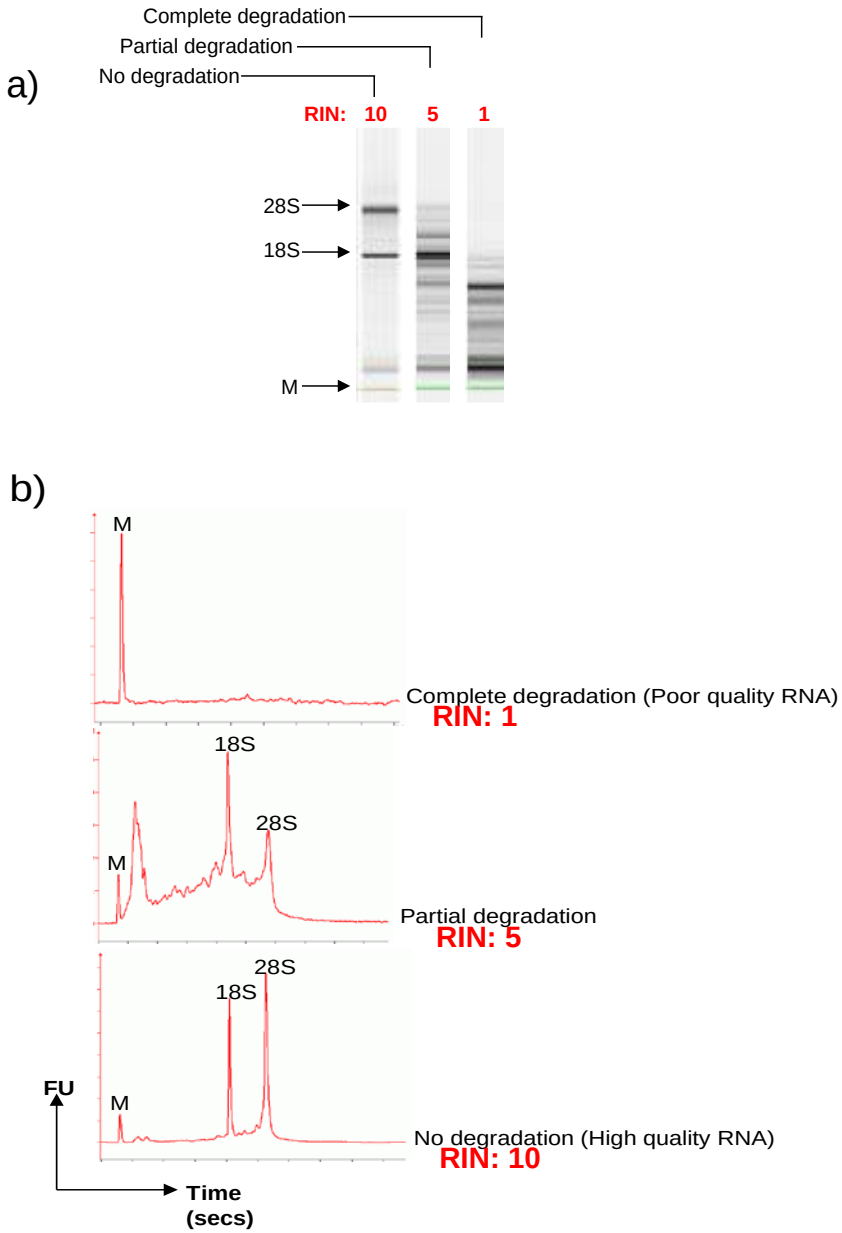


Figure 3.9: Interpretation of data generated by the Agilent 2100 bioanalyser in the assessment of RNA quality.

This system uses voltage-induced size separation in gel-filled channels and microcapillary electrophoresis to separate RNA and allow for the assessment of RNA quality. The system generates an electropherogram, and then uses an algorithm to calculate an RNA integrity number (RIN) [399, 410]. RNA samples with RIN values below 7 are of insufficient quality for downstream microarray applications, and a RIN value of 8 or greater is recommended [400].

a) Good quality gel-separated RNA is visualised as two distinct bands, comprising of 28S and 18S ribosomal RNA r(RNA) species, with high RIN values.

b) A typical electropherogram of good quality RNA is shown as two single peaks for 18S and 28S rRNA, and high RIN values. (M = marker, FU = fluorescence units)

Figure 3.10: Quality assessment of primary ALL RNA samples using the Agilent 2100 bioanalyser: Gel electrophoresis.

RNA extracted from 16 primary ALL tumour samples (8 control DMSO-treated and 8 1µM JQ1-treated samples) was gel-electrophoresed by the bioanalyser.

Two distinct 18S and 28S rRNA bands were observed for all tumour RNA samples, with no observable degradation.

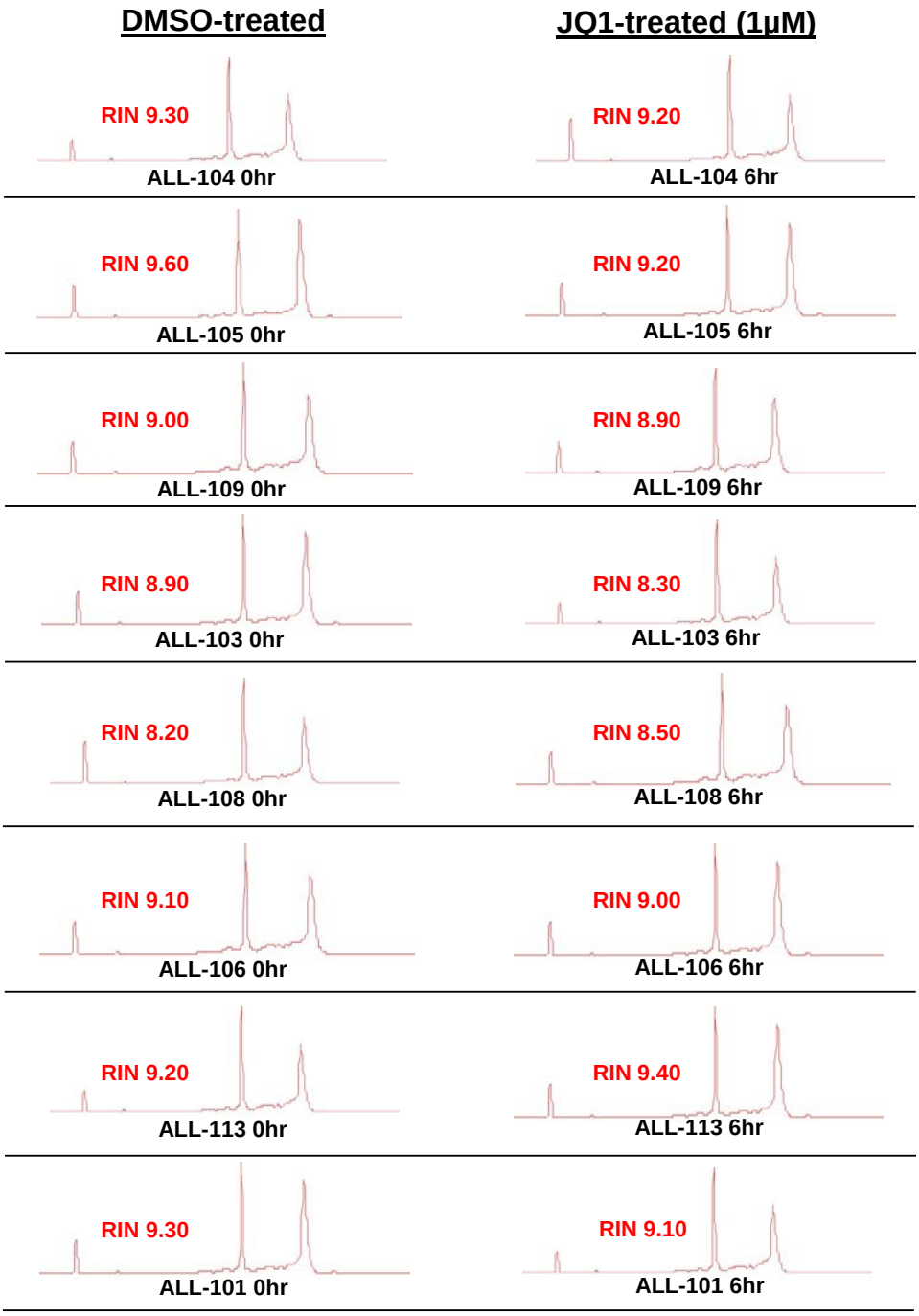


Figure 3.11: Quality assessment of primary ALL RNA samples using the Agilent 2100 bioanalyser: Electropherogram and RIN calculation.

Laser-induced fluorescence of RNA is detected by the bioanalyser, and generates an electropherogram (plotted as fluorescence over time), which is used to calculate the RIN value for each RNA sample.

Aside from the marker peak on the far left of each electropherogram, two distinct 18S and 28S rRNA peaks were observed for each RNA sample.

All RNA samples (DMSO- and JQ1-treated) showed RIN values greater than 8 and were of very high quality for downstream microarray experiments.

3.2.2 Analysis of single gene expression changes in primary ALL tumours following BRD4 inhibition

Univariate analysis of microarray results is used to explore the most significant changes in the expression of individual genes between two different treatment conditions. I used the univariate analysis approach to investigate which genes were most differentially expressed in 8 primary ALL tumours upon inhibition of BRD4 following 6 hour exposure to the cytotoxic concentration of 1 μM JQ1, compared to the same tumours treated with DMSO. With the assistance of the bioinformatics expert, Dr. Wenbin Wei, array data were analysed using the Affymetrix Expression Console with the default settings of "Default: RMA-Sketch". I then identified genes most differentially expressed, with p-values less than 0.001 and a fold change greater than 2 using the limma analysis package [402].

With expression data filtered using the above mentioned statistical parameters, I identified a total of 96 differentially downregulated genes in JQ1-treated tumour cells (Figure 3.12). Downregulated genes included those involved in the inhibition of apoptosis such as *BIRC3*, which is an NFκB target gene encoding cIAP2 protein, commonly overexpressed in haematological malignancies and implicated in caspase inhibition [343, 411, 412]. The *FAIM3* gene, encoding the anti-apoptotic factor Toso, was also significantly downregulated by JQ1 and is known to inhibit and regulate death receptor signaling [413]; an apoptotic pathway shown to be upregulated in lymphoblasts treated with anthracyclines commonly used to treat ALL [414]. Intriguingly, and consistent with recently published data [345], I found that JQ1 also caused downregulation of the *IL7R*

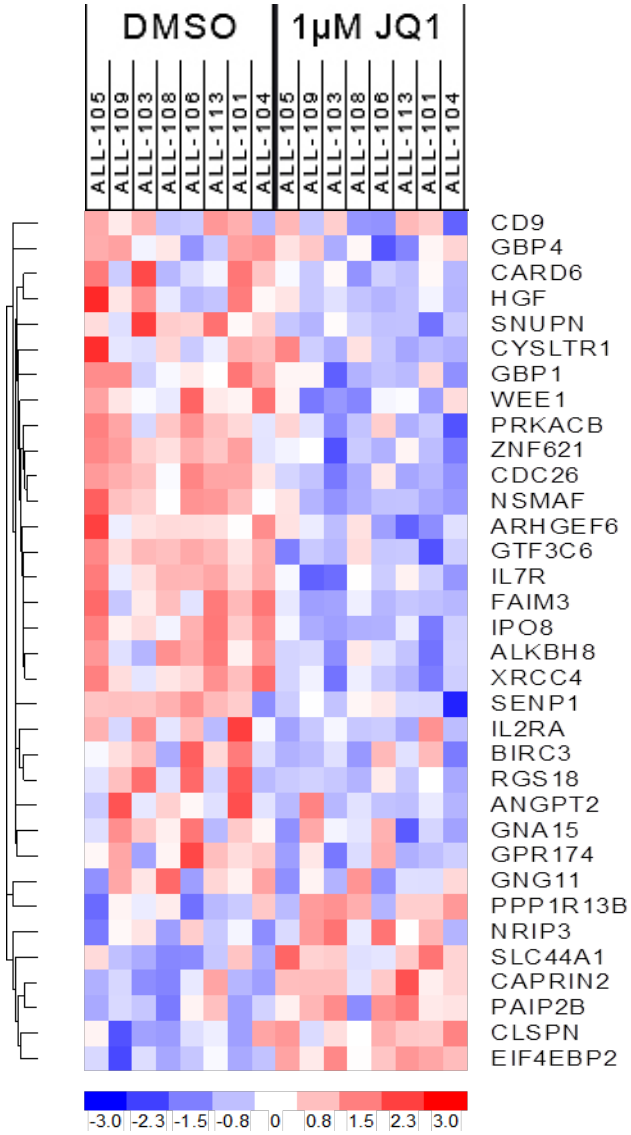


Figure 3.12: Global gene expression profiling to investigate the effects of JQ1 on gene transcription in primary ALL tumours

Transcript expression heat-map was generated using dChip (http://www.dchip.org/) with the default settings, and shows a selected subset of genes downregulated or upregulated in 8 primary ALL tumours following 6 hour exposure to 1 μM JQ1.

Downregulated genes include those belonging to multiple pro-survival signaling pathways such as NFκB and JAK/STAT, as well as c-Myc target genes and members of the interleukin gene family.

Each column represents a different patient sample and each row represents a single gene. Colour changes within a row indicate expression levels relative to the average of the same population. Red indicates up-regulation, blue down-regulation.

gene, which was the third most differentially expressed gene among JQ1-treated primary ALL tumours (3.91-fold decrease, $p<0.001$). The *IL7R* gene encodes the interleukin-7 receptor (IL-7R), which mediates prosurvival signalling via the JAK/STAT pathway and has been shown to be constitutively expressed in B-cell precursor ALL [197]. For a fully functional cytokine receptor to form, IL-7R must heterodimerise with either the IL-2R γ (IL2RG) subunit to form the IL-7 receptor, or with the cytokine receptor-like factor 2 (CRLF2) to form a receptor for thymic stromal lymphopoietin (TSLP), both of which are involved in abnormal activation of the STAT5 transcription factor [415, 416] . Furthermore, CRLF2 deregulation is observed in up to 10% of paediatric ALL and is associated with an unfavourable prognosis [192].

Another cytokine receptor gene, *IL2RA,* encoding a protein subunit of the cell surface receptor complex to which the cytokine IL-2 binds has been shown to activate the JAK-STAT signalling pathway [417]. The *IL2RA* gene was also significantly downregulated following JQ1 treatment in all primary ALL tumours tested, suggesting that JQ1 targets multiple points in JAK/STAT pathway.

Additionally, I found that the *SENP1*, *ALKBH8*, and *CARD6* genes were also downregulated in JQ1-treated primary ALL, and encode proteins frequently associated with the promotion of cancer cell survival [418-422], as well as the *MYC* gene commonly overexpressed in haematological malignancies and key to cell proliferation [343, 412].

I found that 127 genes were significantly upregulated following treatment of primary ALL cells with JQ1 inhibitor (Figure 3.12). These included the

EIF4EBP2 gene, encoding a protein involved in the regulation of mTor signaling through repression of growth-promoting mRNA translation [423], as well as the *PAIP2B* gene, with a role in inhibiting translation of mRNA. The *PPP1R13B* gene was also upregulated following JQ1 treatment, and encodes the ASPP1 protein. Interestingly, ASPP1 cooperates with p53 to induce apoptosis and is associated with a poor prognosis in ALL patients that lack its expression [424].

In summary, univariate analysis of microarray data in this experiment revealed that JQ1-mediated BET protein inhibition in primary ALL led to downregulation of genes involved in apoptosis inhibition and survival, as well as the upregulation of genes with a role in apoptosis induction and translational repression.

3.2.3 Microarray validation of differential responses in primary ALL tumours at 6 hours following JQ1-mediated BRD4 inhibition using qRT-PCR

Due to the nature of microarray studies, involving the measurement of expression levels of thousands of genes all at the same time, there is potential for false positive results, which imply a given gene is up- or downregulated following a particular treatment, when in fact it is not. For the same reasons as to why it is necessary to repeat experiments, and apply statistical significance tests to data, the validation of microarray data using a different experimental technique provides confidence that an observed result is not a false positive.

To validate the differential responses observed in the microarray in this study, I selected 6 candidate genes (*TNFSF4, IL7R, BIRC3, MYC, EIF4EBP2,* and *PPP1R13B*) and decided to use qRT-PCR as the method of validation, since it is a highly sensitive and efficient technique. I selected *TNFSF4* because this gene exhibited the greatest average differential response between untreated and JQ1-treated cells, and the highest statistical rank in the microarray analysis, with a 4.82-fold decrease in transcript expression ($p<0.01$). The *IL7R* gene also ranked highly with respect to statistical analysis, playing a major role in precursor B cell proliferative signaling and being directly involved in the activation of the JAK-STAT pathway [425]. *BIRC3* is the NFκB target gene encoding the antiapoptotic cIAP2 protein [426]. *MYC* was selected since it is commonly overexpressed in leukaemia and potently drives cell proliferation. Recent studies have also confirmed that downregulation of BRD4 activity leads

to reduced expression of c-Myc protein in AML [344]. *EIF4EBP2* is the gene encoding the 4EBP2 protein that is responsible for inhibiting growth-promoting mTOR signaling [427], whilst *PPP1R13B* encodes the ASPP1 protein implicated in the regulation of the apoptotic function of the *TP53* tumour-suppressor gene in ALL and is associated with a poor prognosis when expressed at abnormally low levels [424]. Changes in expression of the above mentioned genes after 6 hour exposure to 1 µM JQ1, measured by qRT-PCR, were generally consistent across tumours in the microarray, and have functions relevant to cell survival and apoptosis. Thus, JQ1-mediated modulation of the expression of all 6 genes might explain JQ1-induced cytotoxicity observed in ALL cells.

I used 10 primary tumours (ALL-119, ALL-120, ALL-121, ALL-122, ALL-123, ALL-124, ALL-125, ALL-126, ALL-127, and ALL-128) for RT-PCR validation that were not used in the microarray, in order to confirm whether or not the changes in gene expression following JQ1 treatment observed in the microarray results were reproducible by RT-PCR.

After RT-PCR quantification of selected gene mRNA transcripts in primary tumours, I found that there were uniform patterns of differential expression similar to those seen in the microarray data. *IL7R, BIRC3, TNFSF4* and *MYC* were significantly downregulated following 6 hour JQ1 exposure, whilst *EIF4EBP2* and *PPP1R13B* were significantly upregulated (Figure 3.17).

In summary, the RT-PCR results I obtained suggest that changes in gene expression following JQ1 treatment are reproducible irrespective of the method

used or the primary tumour analysed, and therefore that results derived from the analysis of my microarray data are reliable.

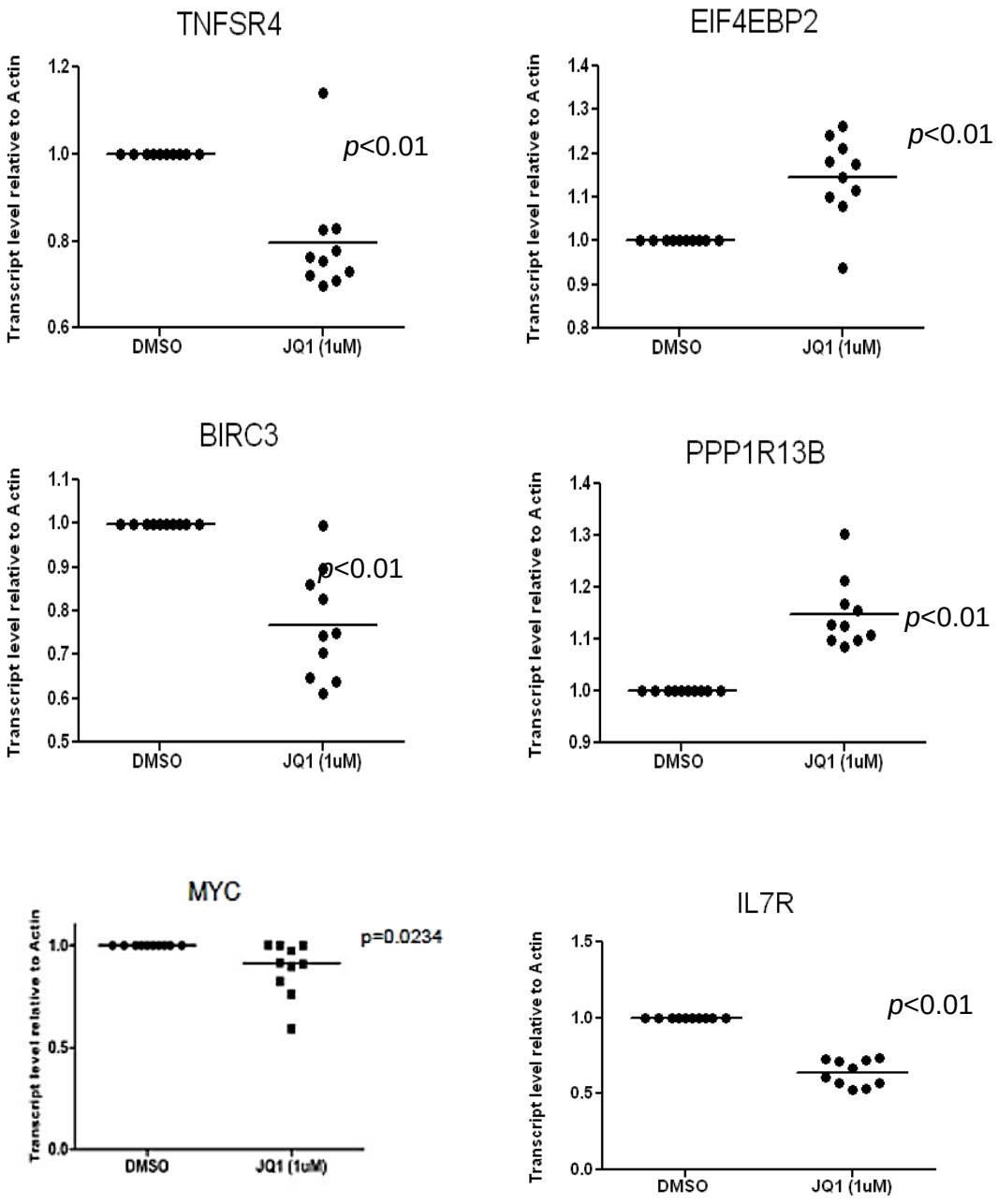


Figure 3.17: Validation of microarray data using the qRT-PCR technique

RNA was extracted from 10 primary tumours (ALL-119, ALL-120, ALL-121, ALL-122, ALL-123, ALL-124, ALL-125, ALL-126, ALL-127, and ALL-128) that were not included in the microarray experiment.

Microarray results were validated by qRT-PCR using primer sets recognising *TNFSR4*, *EIF4EBP2*, *BIRC3*, *PPP1R13B*, *MYC* and *IL7R* mRNA. Ct values were normalised to β-actin mRNA. Graphs represent mRNA levels in DMSO- and JQ1-treated tumours.

Pre-treatment of primary ALL tumours with 1 µM JQ1 leads to the differential expression of all selected validation genes in a similar fashion to that observed in the microarray experiment.

3.2.4. GSEA analysis of differentially regulated signaling pathways in primary ALL tumours following JQ1-mediated BET protein inhibition

Multivariate analysis of microarray data is an approach used to assess whether or not there are synchronised changes in the expression of multiple genes belonging to common signaling pathways. As a wealth of expression data is generated from a microarray study, multivariate analysis can help identify which signaling pathways are affected by a particular treatment. Thus, I decided to use this method to analyse my microarray data to provide further insight as to the cellular signaling pathways being modulated in primary ALL tumours following treatment with JQ1 inhibitor.

More specifically, I used gene set enrichment analysis (GSEA) [403] to look for concordant expression changes among genes grouped according to the biological pathway or function they are associated with; otherwise known as ‘gene sets’. An advantage of this method of analysis over analysis on an individual gene level is that GSEA does not depend upon important individual genes standing out, but instead can detect modest yet consistent changes in gene expression within gene sets. This approach prevents the overlooking of differentially expressed genes even if only a selection of genes within a gene set is actually significant [428].

With this in mind, the GSEA software package I used allowed me to rank all the genes according to their significance level (with respect to the differential expression observed when comparing genome-wide expression profiles of

DMSO- and JQ1-treated ALL tumours), and then calculating an enrichment score (ES) for each biological pathway, which is dependent upon the significance ranking of the member genes in that pathway. In other words, this establishes the proportion of genes that are significantly under- or overexpressed within their respective gene sets. This proportion is considered to be significant if the calculated false discovery rate (FDR) is less than 25% [429], or if the p-value is less than 0.05 [430, 431]. Use of the FDR value to determine significance is usually more stringent than use of the p-value; however, I decided to use both the FDR and p-value to determine the significance of differentially regulated gene sets.

I aimed to identify biological processes or signaling pathways that were differentially regulated in primary ALL tumours following JQ1 treatment. Using GSEA I identified 771 out of 1886 gene sets that were downregulated following exposure to 1 μM JQ1, of which 172 gene sets were significantly downregulated exhibiting an FDR < 25%. %. Consistent with previous univariate analysis, I identified gene sets representing the prosurvival signaling pathways NFκB and JAK-STAT, as well as the c-MYC pathway and multiple cytokine signaling pathways (IL2, IL7, IL10, and IL17) (Table 3.5; Figure 3.13 and 3.14). The ‘BRCA/BRCA1 pathway’ and ‘cell proliferation’ gene sets were also downregulated, but marginally failed to meet the significance criteria, with them having a significant p-value but not FDR. GSEA also identified 1115 out of 1886 gene sets to be upregulated following JQ1 treatment, of which 175 gene sets were significantly enriched at FDR < 25%. Noticeably, a significant proportion of

gene sets upregulated included those involved in gluconeogenesis, glycolysis, and Hif1a signaling.

Gene set / pathway name	Number of genes in pathway	NOM *P*	FDR *q*
IL17 PATHWAY	13	0	0.010
CELL SURFACE RECEPTOR LINKED SIGNAL TRANSDUCTION	121	0	0.010
NFkB PATHWAY	106	0	0.080
INFLAMMATORY RESPONSE PATHWAY	29	0.002	0.020
CYTOKINE CONNECTION	15	0.02	0.100
IL7 PATHWAY	16	0.014	0.100
MYC PATHWAY	43	0.004	0.079
IL10 PATHWAY	13	0.039	0.140
IL2RB PATHWAY	34	0.012	0.140
STRESS SPECIFIC	44	0.012	0.150
CELL ADHESION	132	0.006	0.150
IL1 PATHWAY	62	0.014	0.170
JAK/STAT SIGNALING PATHWAY	150	0.01	0.200
VEGF	23	0.05	0.220
BRCA/BRCA1 PATHWAY	93	0.02	0.264
CELL PROLIFERATION	196	0.01	0.270

Table 3.5: GSEA pathways downregulated in primary ALL tumours following JQ1 treatment.

GSEA of microarray expression data obtained from 8 primary ALL tumours incubated with either DMSO or 1 μM JQ1 for 6 hours show that, in addition to the MYC pathway, JQ1-treatment leads to the downregulation of multiple cytokine and prosurvival signaling pathways, including the NFκB and JAK-STAT pathways, which are significant at FDR <0.25 (25%).

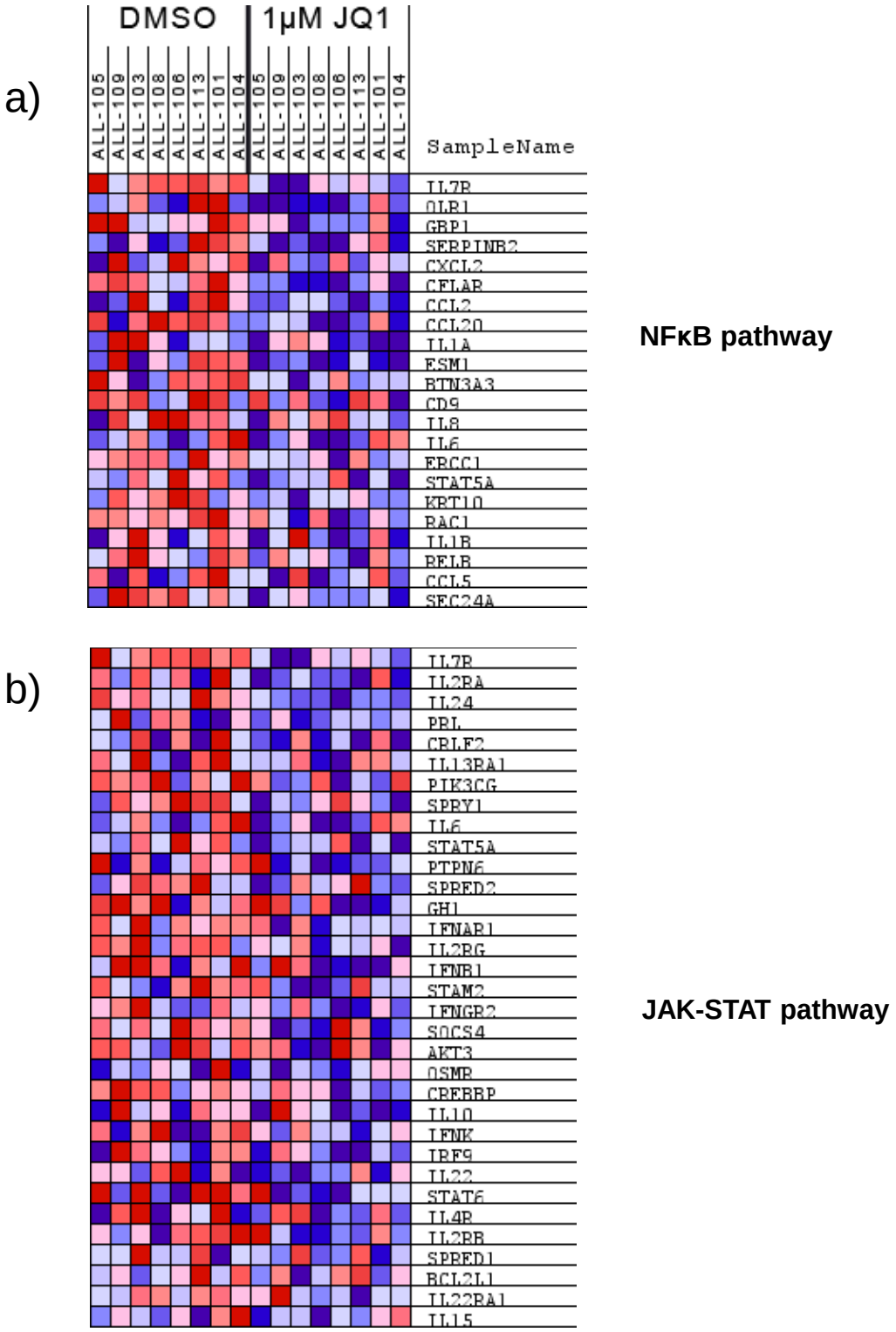


Figure 3.13: GSEA of the NFκB and JAK-STAT pathways in primary ALL tumours in response to JQ1 treatment.

Both the prosurvival signaling a) NFκB and b) JAK-STAT pathways are significantly downregulated (FDR <25%) in primary ALL tumours cells exposed to 1μM JQ1 for 6 hours.

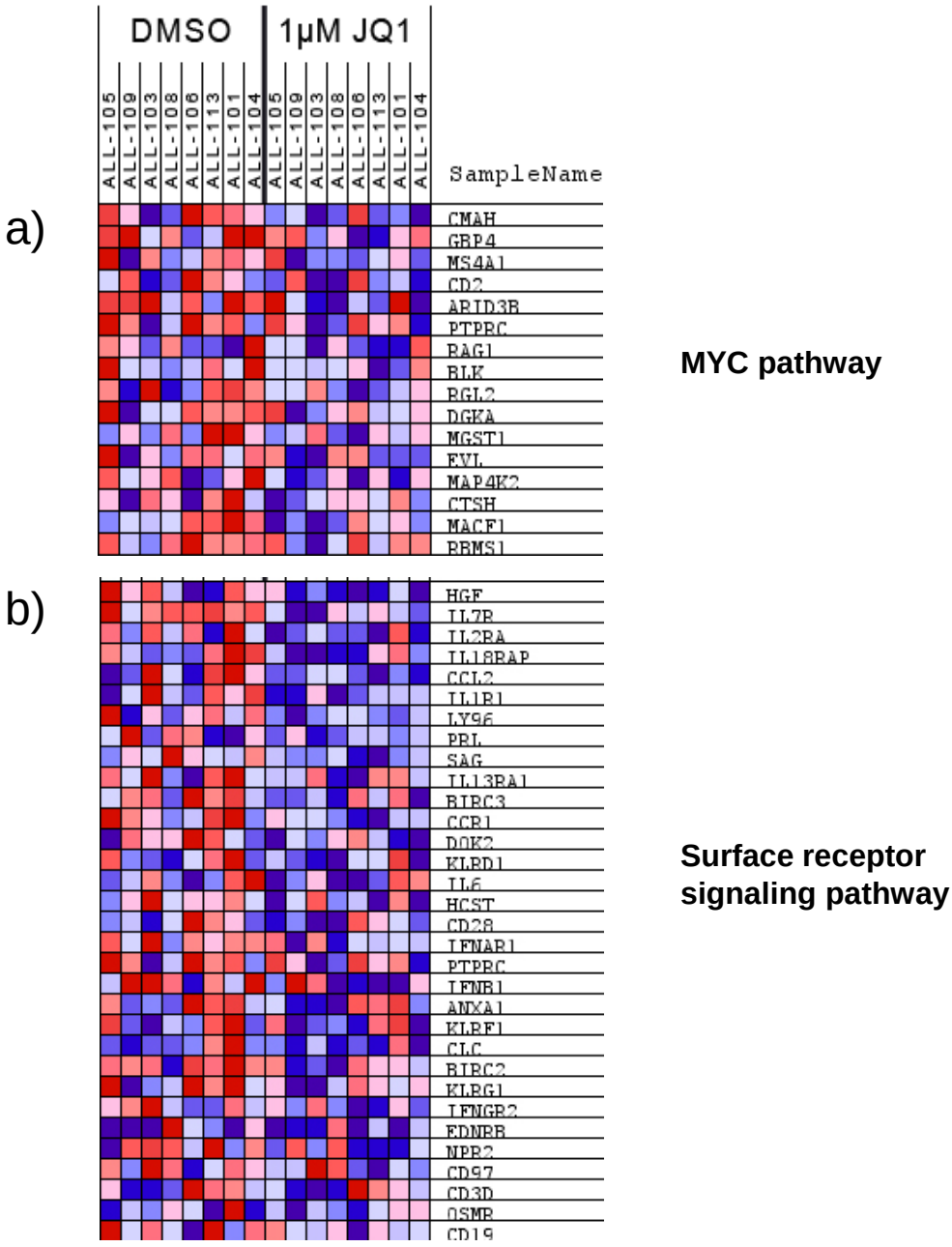


Figure 3.14: GSEA of the MYC and Surface receptor signaling pathways in primary ALL tumours in response to JQ1 treatment.

Both the a) MYC and b) Surface receptor signaling pathways are significantly downregulated (FDR <25%) in primary ALL tumour cells exposed to 1μM JQ1 for 6 hours.

3.2.5 Identification of biomarkers predictive of JQ1 sensitivity and resistance

Cytotoxicity data presented in section 3.1.1 show that, although primary ALL tumours ubiquitously express the BET protein BRD4 and a large proportion of ALL tumours are sensitised to the cytotoxic effects of JQ1 treatment, a considerable number of tumours still exhibit moderate sensitisation or resistance to BET inhibition.

Upon consideration of the above observations, I proceeded to identify a way by which to predict tumour response to JQ1, so that unnecessary administration of this inhibitor to ALL patients most unlikely to respond would be avoided as this would only increase the risk of toxic side-effects, without providing any therapeutic advantage.

Subsequently, I decided to look for biomarker genes predictive of JQ1 sensitivity using the microarray data I had previously obtained (section 3.2.2). With the help of Dr. Wenbin Wei, I ranked primary ALL tumours according to JQ1 EC_{50} values from cytotoxicity experiments in section 3.1.2 and, using normalised and filtered gene expression values derived from untreated tumours, selected those genes most significantly associated with JQ1 EC_{50} using Spearman's rank correlation coefficient statistical analysis (coefficient ≥ 0.6; $p<0.05$) (Figure 3.15).

Of these genes, I found that expression of the *PLAUR* and *REL* genes showed a strong positive correlation with JQ1 EC_{50} values in primary ALL tumours

(Figure 3.16), where the highest expression levels were observed in those tumours most resistant to the cytotoxic effects of JQ1 treatment.

The *PLAUR* gene encodes the receptor for urokinase plasminogen activator (uPAR), which is anchored to the cell surface and is frequently overexpressed in human cancers [432-434]. The uPAR plays a role in the urokinase-catalysed activation of plasminogen, which results in extracellular matrix degradation and facilitates tumour invasion [435]. Furthermore, studies have shown that uPAR expression is associated with blast cell dissemination to the peripheral blood and inferior treatment outcome in AML [436].

The *REL* gene encodes c-Rel, which belongs to the rel subfamily of NFκB transcription factors, and is frequently overexpressed in Hodgkin's lymphoma (HL) and non-Hodgkin's B-cell lymphomas (NHLs) [437], and promotes B-cell proliferation and survival [438].

Given their close association with JQ1 resistance in primary ALL tumours, quantification of either *PLAUR* or *REL* gene transcripts could be used to predict tumour sensitivity to JQ1. Importantly, this could be quickly determined using the qRT-PCR technique, which would require a very low number of cells from a biopsy.

I next decided to perform GSEA on the EC50-ranked microarray data derived from untreated primary tumours, in a bid to identify biological processes or signaling pathways that may be upregulated and contributing to the resistance to JQ1 observed in a subset of tumours.

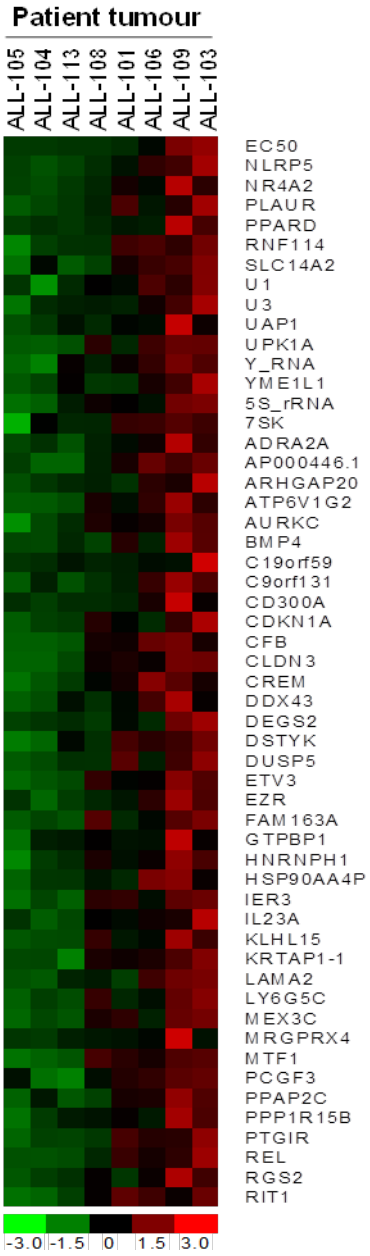


Figure 3.15: Transcriptional biomarkers predictive of JQ1 sensitivity and resistance in primary ALL

The Affymetrix Expression Console™ software was used to normalise microarray gene expression values from untreated primary ALL tumours that were ranked according to JQ1 EC_{50} values derived from *in vitro* cytotoxicity experiments.

ALL tumours are ranked from low to high EC_{50} values (green to red), seen on the top row of the above heatmap.

The heatmap shows a subset of genes that positively correlate with JQ1 EC_{50} using Spearman's rank correlation coefficient (coefficient ≥0.6; $p<0.05$), and may allow ALL tumour sensitivity to treatment with JQ1 to be predicted.

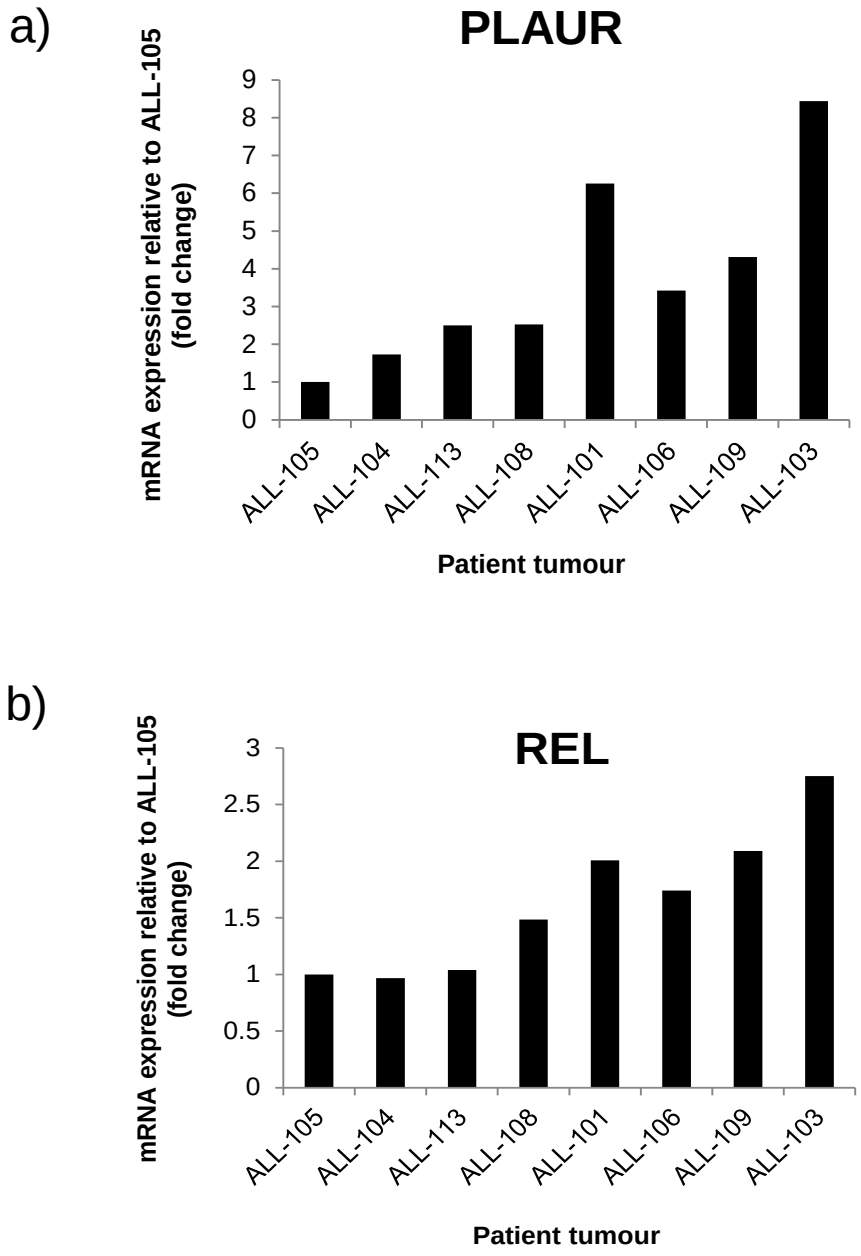


Figure 3.16: The *PLAUR* and *REL* genes as biomarkers predictive of JQ1 sensitivity

Primary ALL tumours analysed in the JQ1 microarray were ranked according to JQ1 EC_{50} values derived from in vitro cytotoxicity experiments.

Expression levels of the a) *PLAUR* and b) *REL* genes in DMSO-treated tumours were plotted as mRNA expression relative to transcript levels in ALL-105 tumour cells, which expresses the lowest level of each biomarker gene and is most sensitive to the cytotoxic effects of JQ1.

Expression of *PLAUR* and *REL* genes negatively correlates with JQ1 sensitivity.

I found that 721 of 1335 gene sets were differentially upregulated in JQ1-resistant primary tumours in comparison with JQ1-sensitive tumours. Of these, 121 gene sets were significant (FDR <25%; p<0.05) and included gene sets representing NFκB, RAS and NOTCH prosurvival signaling pathways, as well as pathways that regulate cytokine signaling and gene transcription (Table 3.6).

Since RAS and NOTCH pathways are upregulated in untreated JQ1-resistant primary ALL tumours, and I did not observe their downregulation in tumours following JQ1 treatment, it is possible that cross-talk from RAS and NOTCH signaling pathways may be promoting ALL tumour cell survival and contribute towards JQ1-resistance [439-441].

In addition, two gene sets representing pathways that regulate the ribosome and ribosomal protein expression were also upregulated in JQ1-resistant tumours (Figure 3.15).

Overall, the *PLAUR* and *REL* genes appear to be suitable candidate biomarker genes predictive of JQ1 sensitivity in B-precursor ALL. Furthermore, JQ1 resistance may be mediated via the upregulation of multiple converging prosurvival signaling pathways and expression of ribosomal proteins involved in protein translation.

3.2.6. Summary

In conclusion, microarray data presented in this section were generated using high quality primary RNA samples, and qRT-PCR validation results were concordant with the changes in gene expression observed in the microarray.

The inhibition of BET proteins induced gene expression changes in primary ALL tumours that would be expected to favour the induction of apoptosis. This involved the downregulation of multiple prosurvival pathways, such as the NFκB and JAK-STAT signaling pathways.

Importantly, the *PLAUR* and *REL* genes were identified as potential biomarker genes predictive of B-precursor ALL tumour sensitivity to JQ1.

Overall, these results support my hypothesis in that targeting the BET family of proteins poses as an alternative strategy by which to inhibit prosurvival gene transcription to promote the induction of apoptosis in pre-B ALL.

Gene set / pathway name	Number of genes in pathway	NOM *P*	FDR *q*
RIBOSOME ASSEMBLY	67	0.000	0.002
TNFα-MEDIATED NFKB SIGNALING	18	0.000	0.021
RIBOSOMAL PROTEINS	81	0.000	0.029
TACI GENE EXPRESSION SIGNATURE (Moreaux *et al*. 2004)	288	0.000	0.032
RAS ONCOGENIC SIGNATURE	199	0.000	0.035
NFKB SIGNALING	88	0.005	0.085
TRANSCRIPTION FACTORS	56	0.000	0.110
GENE TRANSCRIPTION	63	0.011	0.143
CYTOKINE-CYTOKINE RECEPTOR INTERACTION	197	0.006	0.156
CELL COMMUNICATION	99	0.006	0.188
NOTCH SIGNALING PATHWAY	37	0.025	0.191

Table 3.6: GSEA pathways upregulated at baseline level in primary ALL tumours most resistant to JQ1 activity

Gene expression data was obtained from 8 DMSO-treated primary ALL tumours ranked according to JQ1 $EC_{50.}$

GSEA was performed on microarray gene expression data to identify pathways most strongly associated with JQ1 EC_{50} using Spearman's rank correlation coefficient (coefficient ≥ 0.6; $p<0.05$).

Multiple prosurvival pathways (including RAS and NFκB pathways), gene transcription pathways, and ribosome metabolism and assembly pathways are upregulated in primary ALL tumours with the highest JQ1 EC_{50} values (most resistant to JQ1 activity). These pathways are significant at FDR <0.25 (25%) and a nominal p-value (NOM P) <0.05.

3.3. Cellular effects of JQ1 on ALL tumour cells

I proceed to elucidating some of the cellular effects by which cytotoxicity is exerted in ALL tumour cells following JQ1-mediated BET inhibition (Section 3.1.2).

Since I showed in section 3.1.1 that the BET protein family member BRD4 is frequently expressed in B-cell precursor ALL, and other studies have confirmed BRD4 plays a crucial role in positively regulating the expression of genes involved in growth and cell cycle progression [442], I began by investigating the effects of JQ1 treatment on the cell cycle of cycling ALL tumour cells (section 3.3.1).

Based on data from cell cycle analysis experiments that hinted towards a JQ1-mediated impact on S-phase tumour cells, as well as the reported findings that BRD4 interacts with the RFC-140 essential for DNA replication [443], and plays a distinct role in Merkel cell polyomavirus (MCV) DNA replication [444], I also investigated the impact of JQ1 on the DNA replication process in ALL (section 3.3.2).

Finally, I investigated the effects of JQ1 treatment on apoptosis in ALL tumour cells, in order to gain a better understanding of how ALL tumour cells are eliminated.

3.3.1. Investigating JQ1-mediated effects on the cell cycle and c-Myc downregulation.

I decided to use propidium iodide staining and flow cytometry to obtain cell cycle profiles for TOM-1, SUPB-15, SD1, and NALM-6 ALL cell lines following 24 hour treatment with either DMSO or 1 μM JQ1. I found that JQ1 treatment led to a consistent accumulation of cells in the G1 phase of the cell cycle in all cell lines compared to control (Figure 3.18). This was accompanied by a marked reduction in S-phase cells in the SUPB-15, SD1, and NALM-6 cell lines. However, there was no reduction in the proportion of cells in the G2 phase of the cell cycle. Taking these observations into consideration, it is possible that JQ1 activity may be affecting cell cycle proteins regulating transition to the G2 phase, as well as clearly inducing G1 arrest. The significant reduction in the proportion of S-phase cells is of particular interest and may be indicative of an impact on the DNA replication process. Furthermore, since the S-phase population of cells are not completely reduced, it was important to investigate whether DNA replication is still active within these cells (section 3.3.2).

The c-Myc protein is tightly regulated throughout the cell cycle and plays a vital role in the facilitation of G_1/S progression through the induction of G1 cyclin/CDK kinase activity [445, 446]. Thus, downregulation of c-Myc is commonly associated with cell cycle arrest and the accumulation of cells in the G_1 phase of the cell cycle [445]. A wide range of haematological malignancies exhibit deregulated expression of c-Myc, including B- and T-ALL, AML, chronic lymphocytic leukemia (CLL), follicular lymphoma, diffuse large B-cell lymphoma, multiple myeloma, and Burkitt lymphoma, with previous studies showing that

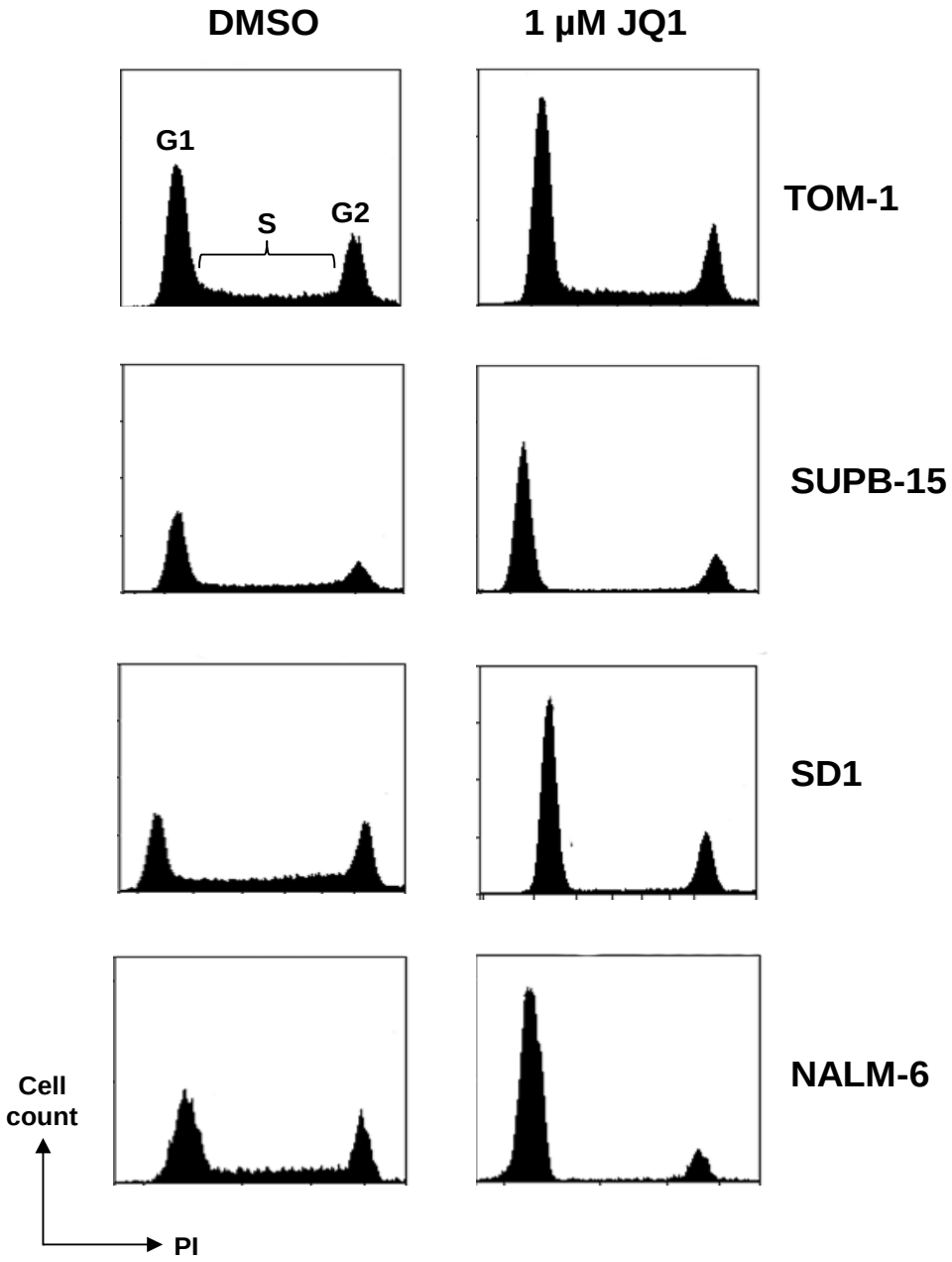


Figure 3.18: Cell cycle analysis in DMSO- and JQ1-treated ALL cell lines

ALL cell lines TOM-1, SUPB-15, SD1 and NALM-6 were treated with either DMSO or 1µM JQ1 for 24 hours. Cells were then analysed by FACS using the PI dye to stain total DNA content. Graphs are plotted as cell count over PI stain intensity.

G1-arrest is shown in all ALL cell lines tested following JQ1 treatment.

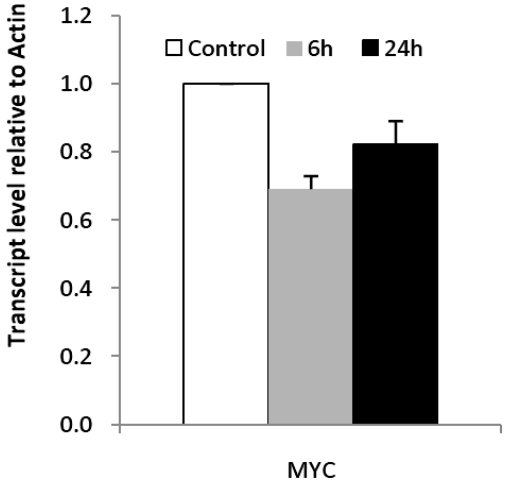
A reduction of S-phase cells is also shown in SUPB-15, SD1 and NALM-6 cell lines.

overexpression of c-Myc in many of these diseases is associated with a poor clinical prognosis [447]. Consequently, a pharmacological strategy to downregulate c-Myc expression and activity is long sought after but not yet available.

In recent years, c-Myc has been implicated as a downstream target of BRD4 [339, 442]. Additionally, treatment of leukaemic cells with JQ1 in the context of AML led to rapid, marked reductions of Myc at both the transcriptional and protein levels. Taking these findings into consideration, together with the observation that JQ1 treatment induces potent G_1-arrest, and that data from my microarray experiment shows *MYC* transcripts were downregulated in primary ALL tumours following exposure to JQ1, I decided to further investigate the regulation of Myc expression in ALL.

Using the preB-ALL NALM-6 cell line, I quantified *MYC* mRNA levels by RT-PCR at 6 and 24 hours after initial exposure of cells to 1 µM JQ1. I found that at 6 hours *MYC* transcript levels were reduced by approximately 30% compared to control DMSO-treated NALM-6 cells (Figure 3.19a). However, at 24 hours *MYC* transcript levels had increased again, this time exhibiting a 20% reduction compared to control. This was unexpected since studies have shown *MYC* transcript levels to be substantially downregulated by lower concentrations of JQ1 up to at least 48 hours in multiple AML cell lines [344]. I then decided to use Western blotting to see how JQ1 affected c-Myc protein expression in a panel of ALL cell lines. Interestingly, exposure to 1 µM JQ1 led to complete downregulation of c-Myc protein at 24 hours across all cell lines tested and was maintained up to at least 96 hours (Figure 3.19b).

a)



b)

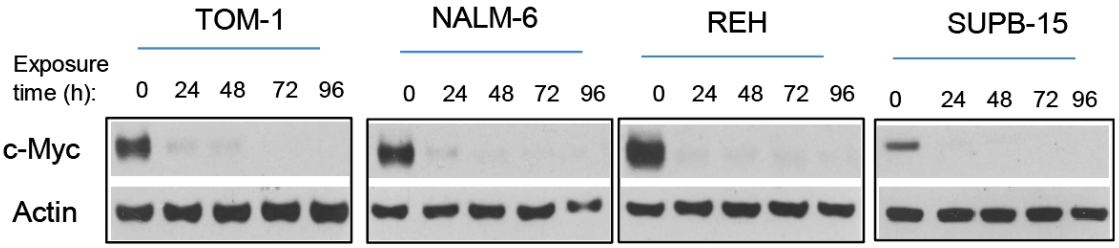


Figure 3.19: Myc downregulation in ALL cell lines following JQ1 treatment

a) NALM-6 cell were treated with either DMSO or 1µM JQ1 for 6 and 24 hours. Total RNA was extracted from samples and Myc mRNA was measured by qRT-PCR.

JQ1 induces transient downregulation of Myc mRNA (6hr) at the transcriptional level, before increasing again after 24hr exposure to JQ1.

b) TOM-1, NALM-6, REH and SUPB-15 cells were treated with 1µM JQ1 for 0 (DMSO control), 24, 48, 72 and 96 hrs. Protein lysates were analysed by Western blotting, where samples were probed for c-Myc and β-actin (loading control).

1µM JQ1-treatment leads to complete and sustained downregulation of c-Myc protein by 24hr in all ALL cell lines analysed.

Since Myc expression at the transcriptional level did not mirror expression at the protein level in ALL cell lines, and c-Myc protein downregulation following 24 hour exposure to 1 μM JQ1 was a common feature, I decided to investigate whether or not JQ1-mediated c-Myc protein downregulation was due to modulation of protein stability and the targetting of c-Myc for proteasomal degradation. This idea is consistent with the fact BRD4 has previously been reported to regulate the stability of the papillomavirus-encoded E2 protein [448]. I treated NALM-6 and TOM-1 cells with either DMSO (control), the 26S proteasome inhibitor MG132 (10 μM) alone, JQ1 (1 μM) alone, or a MG132/JQ1 combination for 24 hours, and then assessed the levels of c-Myc protein expression by Western blotting. I found that in both ALL cell lines, treatment with JQ1 alone caused complete reduction of c-Myc, as was expected; however, treatment with the MG132/JQ1 combination completely abrogated JQ1 activity with respect to c-Myc downregulation (Figure 3.20). This suggests that the MG132 inhibitor prevents proteasomal degradation of c-Myc protein in ALL tumour cells exposed to JQ1, and that JQ1 is capable of modulating the stability of c-Myc protein via a post-translational mechanism involving the 26S proteasome, thus highlighting a role for BET proteins in the reguation of cellular proteins at the post-translational level, in addition to the transcriptional level.

In summary, JQ1 treatment leads to cell cycle G_1 arrest in all ALL cell lines tested and it is possible that JQ1 activity has an impact on DNA replication machinery. Treatment with JQ1 also induces modest and temporary downregulation of *MYC* at the transcriptional level, but instead appears to

mainly exert the observed loss in c-Myc protein expression by regulating protein stability at the post-translational level.

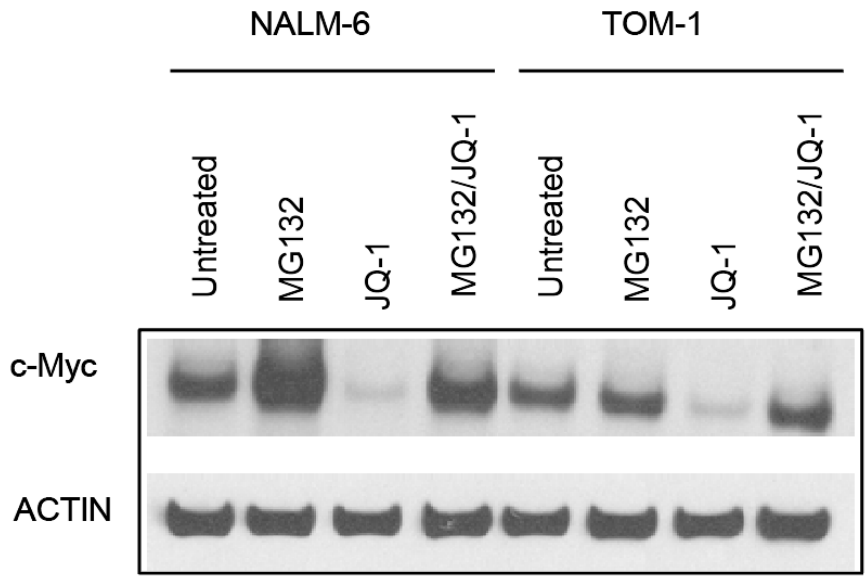


Figure 3.20: MG132-mediated inhibition of the proteasome abrogates downregulation of c-Myc protein by JQ1

NALM-6 and TOM-1 cells were either untreated, treated with 10 μM MG132 only, 1 μM JQ1 only, or treated with an MG132/JQ1 inhibitor combination for 24hr. Protein lysates were analysed by Western blotting, where samples were probed for c-Myc and β-actin (loading control).

Treatment of ALL tumour cells with 1 μM JQ1 leads to complete downregulation of c-Myc in both cell lines, whereas treatment with either MG132 or MG132/JQ1 combination does not. This suggests JQ1-mediated c-Myc protein downregulation is dependent upon activity of the proteasome.

3.3.2 Investigating the effects of JQ1 exposure on the DNA replication process

Each time a eukaryotic cell divides, accurate replication of the complete genome must be precisely coordinated in order to maintain genome integrity. The origins of replication are the sites at which multiple proteins assemble in an ordered manner during late mitosis and early G_1 to form the pre-replicative machinery, giving rise to replication forks [449]. Abnormal modifications to this highly organised process may lead to unscheduled DNA synthesis and genomic instability; thus promoting tumorigenesis [450]. Studies have shown that c-Myc plays an important role in DNA replication origin initiation via a non-transcriptional mechanism involving the direct interation with minichromosome maintenance (MCM) proteins that constitute the pre-replicative complex crucial to the regulation of replication origins [451, 452]. Furthermore, it has also been reported that the BET protein BRD4 directly interacts with the largest subunit, RFC-140, that constitutes part of the highly conserved five-unit replication factor C (RFC) complex essential for DNA replication [443]. Interestingly, the role of BET proteins in replication is not only restricted to mammalian cells. A recent study has shown that BRD4 interacts with the MCV Large T antigen (LT) and is critical for successful viral DNA replication of the MCV genome [444].

Upon consideration of the above mentioned observations, it is possible that JQ1 activity has an impact on the DNA replication process in ALL cells. This was also supported by the observation that transcription of the *CLSPN* gene encoding the protein Claspin was upregulated in the microarray in primary ALL tumours treated with JQ1 (Figure 3.12), since Claspin is a critical mediator of

the DNA-damage response pathway [453]. The Claspin protein is expressed following increased levels of stress and blocks in DNA replication, and is responsible for the phosphorylation and consequent activation of Chk1, which in turn activates the S-phase checkpoint [454].

The significant reduction in the proportion of S-phase cells following JQ1 treatment of ALL cell lines I previously showed (Figure 3.18), also suggests a specific effect on replication. In light of this observation, I was particularly intrigued to investigate the status of replicative activity in the very small proportion of remaining S-phase cells, despite being treated with JQ1, as active replication in these cells might indicate JQ1-resistant ALL clones capable of re-expansion.

I therefore decided to test my hypothesis that JQ1-induced cytotoxic effects observed in proliferating ALL cells are partly due to a direct impact on DNA replication, rather than just consequences of cell cycle arrest. I did this by using a fluorescent DNA fibre-labelling technique and the BrdU incorporation assay to investigate the effects of JQ1 on replicating NALM-6 cells.

The DNA fibre-labelling technique involves the use of halogenated nucleotide analogues that are incorporated into newly synthesised DNA of proliferating cells. Once extracted DNA fibres are stained with specific antibodies conjugated to either red or green fluorescent dyes, they can be visualised by fluorescent microscopy. With the use of dedicated computer software, this approach allows for the identification and quantification of ongoing replication forks, fork terminations, new origins of replication, as well as the determination of the

direction and the rate at which replication forks progress [455] (See Appendix 8.6 for schematic showing possible replication structures).

I began by investigating fork progression rates, derived from the measurement of the fluorescent red and green portions of each individual DNA fibre within the captured photographic images. I decided to treat NALM-6 cells with 1 µM JQ1 for a short exposure time of 1 hour, insufficient to induce G1/S cell cycle arrest, so as to exclude the possibility that any replication effects I observed using a longer exposure time would not be attributable to cell cycle effects. Upon measuring the individual fibres extracted from NALM-6 cells treated with either DMSO or 1 µM JQ1 for 1 hour, I found that treatment of cells with JQ1 led to greatly decreased fork progression rates compared to control cells (Figure 3.21a). This indicated that JQ1 may have a direct effect on the DNA replication process and that it was unlikely that the effects observed after just 1 hour were due to JQ1-mediated downregulation of genes involved in cell cycle progression.

I then investigated the effect of JQ1 on fork progression rates at a 24 hour timepoint, since this was the same timepoint at which I observed a significant reduction in the proportion of S-phase NALM-6 cells by PI/FACS cell cycle analysis (Figure 3.18). I found that 24 hour exposure to 1 µM JQ1 also caused a decrease in fork progression rates compared to control cells (Figure 3.21b).

To further confirm that the observed reduced rates of fork progression were due to the inhibition of replication in S-phase cells and not derived from a JQ1-mediated effect on the G_1/S checkpoint and reduced entry to the S-phase, I

used the BrdU incorporation assay to quantify the level of BrdU uptake by S-phase NALM-6 cells following exposure to increasing concentrations of JQ1. I found that JQ1 treatment did not affect the entry of cells into the S-phase of the cell cycle, but did reduce the proportion of S-phase cells in a dose-dependent manner (Figure 3.22).

I next wanted to gain further insight into the type of replication structures that were present in NALM-6 cells treated with 1 μM JQ1 by quantifying the total number of 1^{st} label origins, 2^{nd} label origins, 1^{st} label terminations/stalled forks, and 2^{nd} label terminations. I found that 24 hour exposure of NALM-6 cells to 1 μM JQ1 led to greater than 13% increase in the proportion of DNA fibres characteristic of stalled replication forks compared to DMSO-treated cells (Figure 3.23). No significant changes were observed for the other types of replication structures.

The data presented in this section lend support toward my hypothesis that JQ1 activity has a direct effect on DNA replication. I showed that treatment of a proliferating ALL cell line with JQ1 led to reduced fork progression rates and an increase in the frequency of stalled replication fork structures, as a result of increased replication stress. Finally, BrdU incorporation assay data also indicates that the observed JQ1-mediated effects on replication are independent of G_1/S checkpoint control.

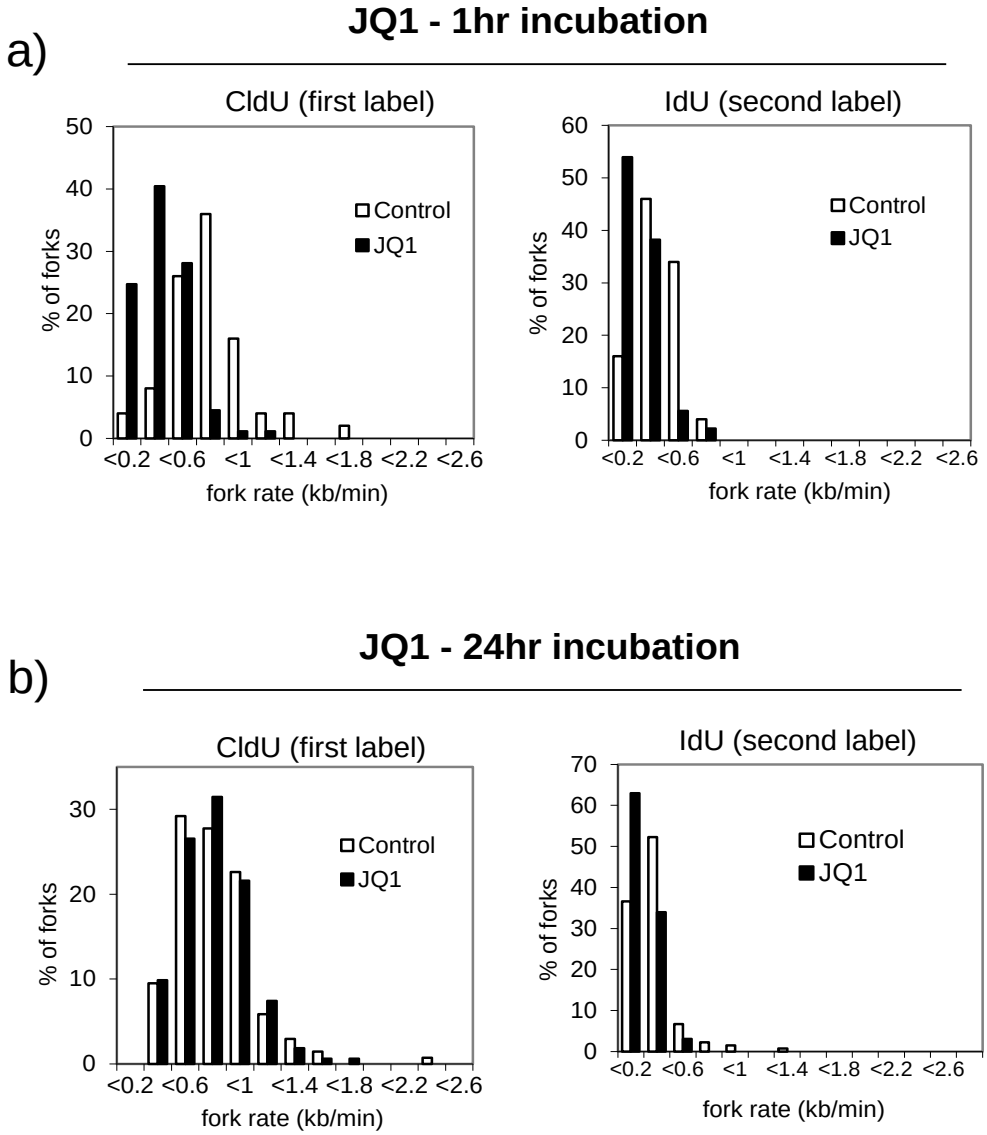


Figure 3.21: Effect of JQ1 treatment on DNA replication fork progression in ALL tumour cells

NALM-6 cells were pre-treated with either DMSO (control) or 1 µM JQ1 for 1hr and 24hr. Cells were then labelled with CldU (first label/red fluorescent dye) and IdU (second label/green fluorescent dye) for 20 minutes each. Photographs were taken of visualised replication fibres in each treatment. Individual fibres were measured using ImageJ software, and distribution of fork speeds calculated.

a) Treatment of NALM-6 cells with 1 µM JQ1 for 1hr causes a reduction in replication fork progression, as demonstrated in analysis of both CldU and IdU labels.

b) Treatment of NALM-6 cells with 1 µM JQ1 for 24hr causes a reduction in replication fork progression, as demonstrated in analysis of the IdU label. This is not demonstrated by analysis of the CIdU label.

These results, involving a short 1hr incubation of cells with JQ1 inhibitor, suggest JQ1 activity has a direct impact on DNA replication fork progression in NALM-6 cells.

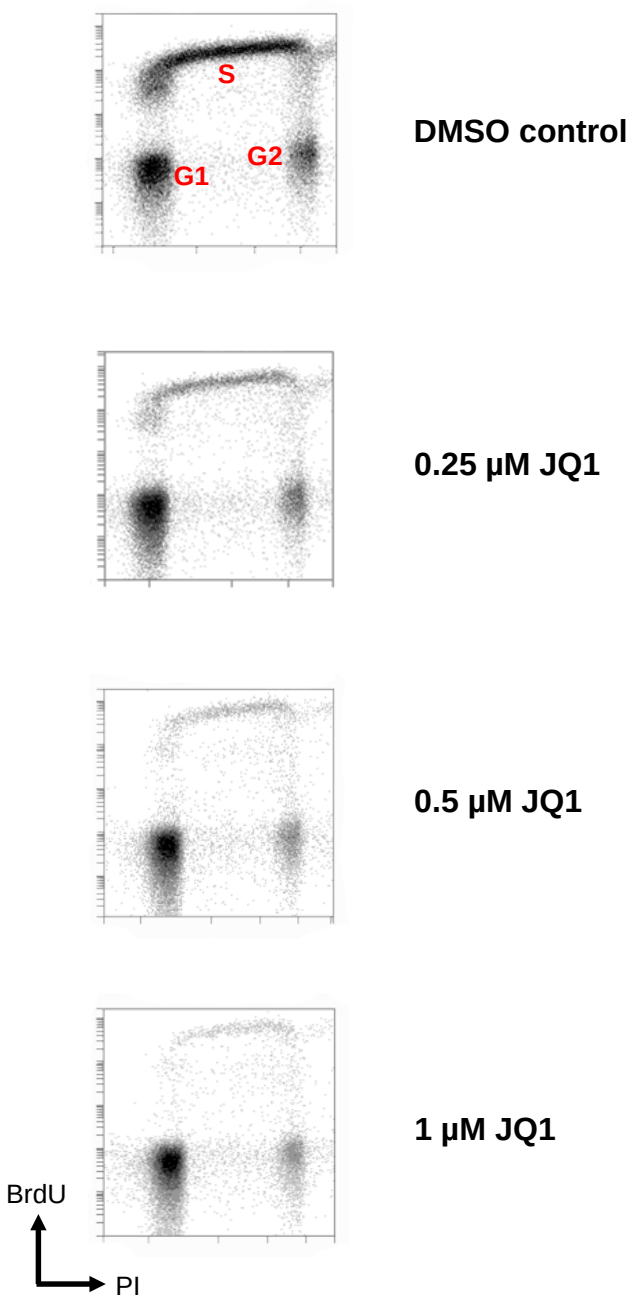


Figure 3.22: JQ1 treatment induces specific reduction of replicating S-phase cells

BrdU incorporation by FACS analysis shows a normal S-phase cell distribution in NALM-6 cells (DMSO-treated control), which reduces in intensity when treated with increasing concentrations of JQ1. This suggests JQ1 specifically targets DNA replication.

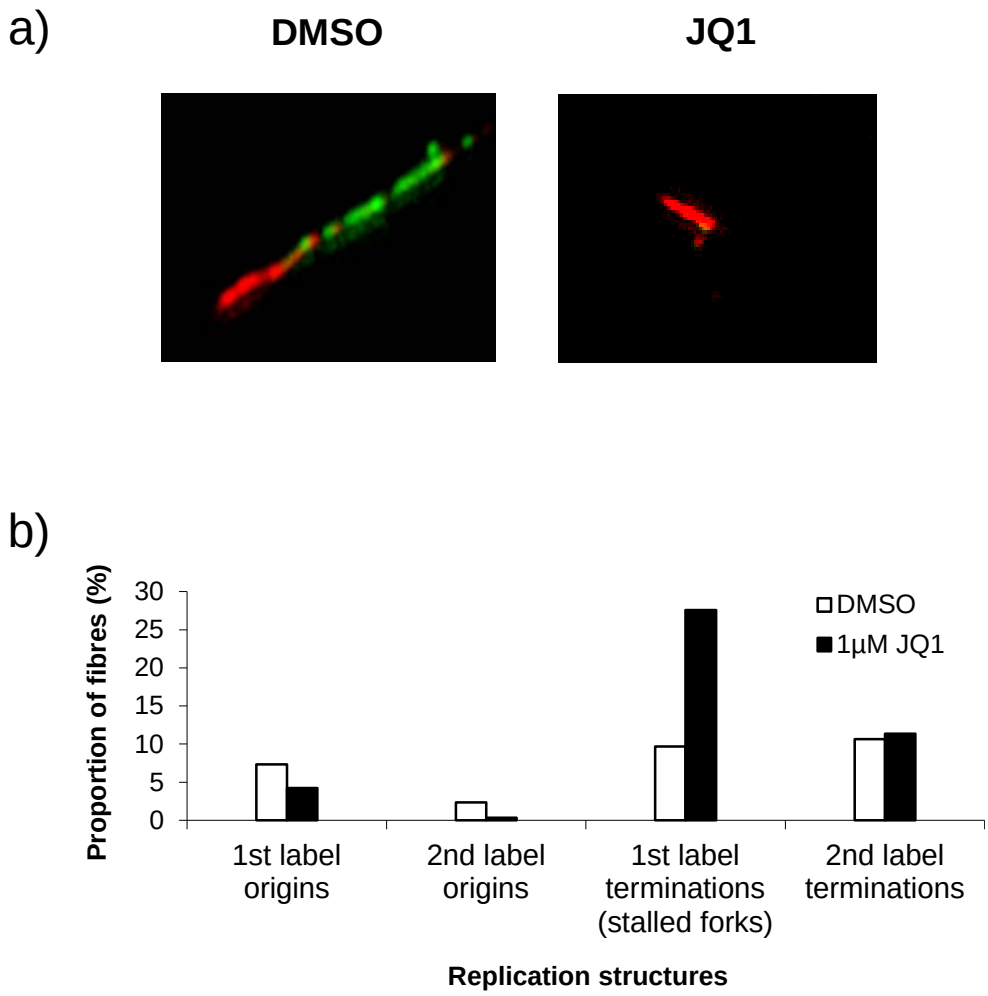


Figure 3.23: JQ1 causes DNA replication stress in S-phase ALL tumour cells

NALM-6 cells were pre-treated with either DMSO (control) or 1 μM JQ1 for 24hr and pulse-labelled with CldU (red) and IdU (green) for 20 minutes each.

a) JQ1-treated DNA replication fibre (right) shows reduced incorporation of CIdU, and complete lack of IdU incorporation, compared to replication fibres extracted from DMSO-treated NALM-6 cells (left) that incorporated both dyes. This indicates JQ1 perturbs replication fork progression.

b) Four different replication structures could be derived from the measurement of both CIdU and IdU stained replication fibres.

Treatment of cells with JQ1 causes an increase (~13%) in the proportion of 1st label terminations, which represent stalled replication forks.

3.3.3 Effect of JQ1 on apoptosis in ALL tumour cells

Most therapeutic agents kill tumour cells via the induction of apoptosis, and the knowledge we have acquired regarding programmed cell death has allowed for the design of novel agents that specifically target malignant cells for killing more efficiently. The induction of apoptosis is considered to be an ideal mechanism of killing for novel agents, since apoptotic cells are removed in a very controlled manner via phagocytosis, as opposed to necrotic cell death, where cells lyse and release their contents into the extracellular space, triggering an unfavourable inflammatory response [245].

Our group previously reported that apoptotic resistance observed in primary ALL tumours *in vitro* following DNA damage correlates with high-risk patient stratification and a poor clinical response *in* vivo, indicating that overcoming this apoptotic resistance with novel agents could potentially improve the treatment outcome of high-risk patients [247]. Our group also reported that despite a normal damage-induced upregulation of p53 in the primary tumours tested, those resistant to apoptosis exhibited upregulation of multiple prosurvival pathways [248]. Deregulation of prosurvival pathways is often associated with the abnormal expression of antiapoptotic proteins such as Mcl-1, Bcl-2, as well as many of the IAP family members. Mcl-1, is a known therapeutic target in acute and chronic lymphoid malignancies [456], and is upregulated in many cases of relapsed ALL [457]. Deregulated expression of Bcl-2 is also reported to correlate with a poor response to chemotherapy in AML and CLL [458, 459]. The *BIRC3* gene encodes the antiapoptotic protein cIAP2, which is frequently

overexpressed in Hodgkin lymphoma and many haematological malignancies including ALL [460, 461]. Overexpression of survivin, an antiapoptotic protein encoded by the *BIRC5* gene, has been reported in primary ALL tumours and is associated with high risk of clinical relapse [462].

Taking the above reported findings and the importance of understanding a therapeutic agent's mechanism of cell killing, particularly when combined with other agents, I first determined whether or not JQ1 induced killing by apoptosis. I used Annexin V / PI staining and flow cytometric analysis, and found that incubation of NALM-6 cells with 1 µM JQ1 for 72 hours revealed a differential increase (~11%; $p < 0.01$) in the percentage of apoptotic cells compared to DMSO-treated control cells (Figure 3.24a). I then used the Western blotting technique to confirm this observation by treating preB-ALL cell lines with 1 µM JQ1 and probing for PARP1, procaspase-3 and procaspase-7 over a 96 hour timecourse. I found that JQ1 treatment led to cleavage of full-length PARP1, procaspase-3 and procaspase-7 proteins, as seen in the BCR-ABL1$^+$ SUPB-15 cell line at 72 hours, and in the REH cell line at the later timepoint of 96 hours (Figure 3.24b). Since procaspase-3 and -7 require cleavage in order to act as the effector caspases of apoptosis and induce cleavage of substrates such as PARP1, these results indicate that the cytotoxic effects of JQ1 activate the apoptosis pathway. The delayed onset of apoptosis seen in REH when compared to SUPB-15 may be a reflection of the less prominent JQ1 cytotoxic effects observed in experiments using ATP-based cytotoxicity assays.

Next, I wanted to further investigate the mechanism of apoptosis induction following JQ1 treatment in ALL cell lines by measuring p53 expression levels in the NALM-6 cell line with wild-type p53. I used Western blotting to measure the level of total p53 in NALM-6 cells treated with 1 µM JQ1 over a 72 hour timecourse. In this experiment I also included DMSO-treated NALM-6 cells as a negative control, and NALM-6 cells treated with 100 nM daunorubicin as a positive control for p53 upregulation and activation. I found that treatment with JQ1 did not cause upregulation of p53 protein throughout the timecourse, as seen in the DMSO control, and in contrast to the cells treated with the DNA-damaging agent daunorubicin, where p53 upregulation occurred at 6 hours post-treatment and persisted up to 72 hours (Figure 3.25a).

Subsequently, I tested for phosphorylation of p53 at Ser15, which is a phosphorylation site targeted by the mediators of the DNA damage response, ATR and ATM [463]. Phosphorylation of p53 at Ser15 leads to p53 transactivation and p53-mediated transcription of genes involved in cell cycle arrest such as *CDKN1A* that encodes the p21 protein, as well as other genes involved in DNA repair and apoptosis [464-466]. Whereas treatment with daunorubicin led to phospho-p53 (Ser15) upregulation at 6 hours through to 72 hours exposure (Figure 3.25a), I found that JQ1 and DMSO did not lead to the phosphorylation of p53 (Ser15). Similarly, JQ1 and DMSO failed to induce p21 upregulation, whereas exposure to daunorubicin led to moderate p21 upregulation after 6 hours, the levels of which continued to increase, peaking at 48 hours, before decreasing again at 72 hours post- treatment (Figure 3.25a).

These results suggest that JQ1 induces via p53-independent apoptosis in ALL cells.

Thus, to further elucidate the p53-independent mechanism of apoptosis induced in JQ1-treated NALM-6 cells, I decided to probe for four key anti-apoptotic proteins, Bcl-2, Mcl-1, BIRC3 (cIAP2), and BIRC5 (survivin) and look for any changes in expression of these proteins following JQ1 treatment. I found that following 24 hour exposure of NALM-6 cells to 1 µM JQ1, Mcl-1 expression levels decreased steadily until 96 hours and that no significant change was observed in the expression levels of Bcl-2 and cIAP2 (Figure 3.25b). However, the expression of survivin was completely lost within 48 hours of incubation with 1 µM JQ1.

Since I did not observe any significant impact of JQ1 on the transcription of *BIRC5* in the microarray data, I addressed the possibility that the loss of survivin protein expression was due to protein degradation. Using Western blotting I measured the levels of survivin in NALM-6 cells treated with either DMSO, 1 µM JQ1, 10 µM MG132, or a combination of JQ1/MG132. I found that JQ1 treatment alone caused complete downregulation of survivin, compared to no change in expression in cells treated with DMSO or MG132 alone (Figure 3.26). In contrast, treatment of NALM-6 cells with JQ1 combined with the proteasome inhibitor MG132 completely abolished the ability of JQ1 to downregulate survivin, as was seen with c-Myc protein expression.

3.3.4 Summary

Taken together, in terms of cellular killing, I found that JQ1 treatment of ALL cell lines induced p53-independent apoptosis that involved the downregulation of the antiapoptotic protein survivin by modulating the stability of this protein at the post-translational level.

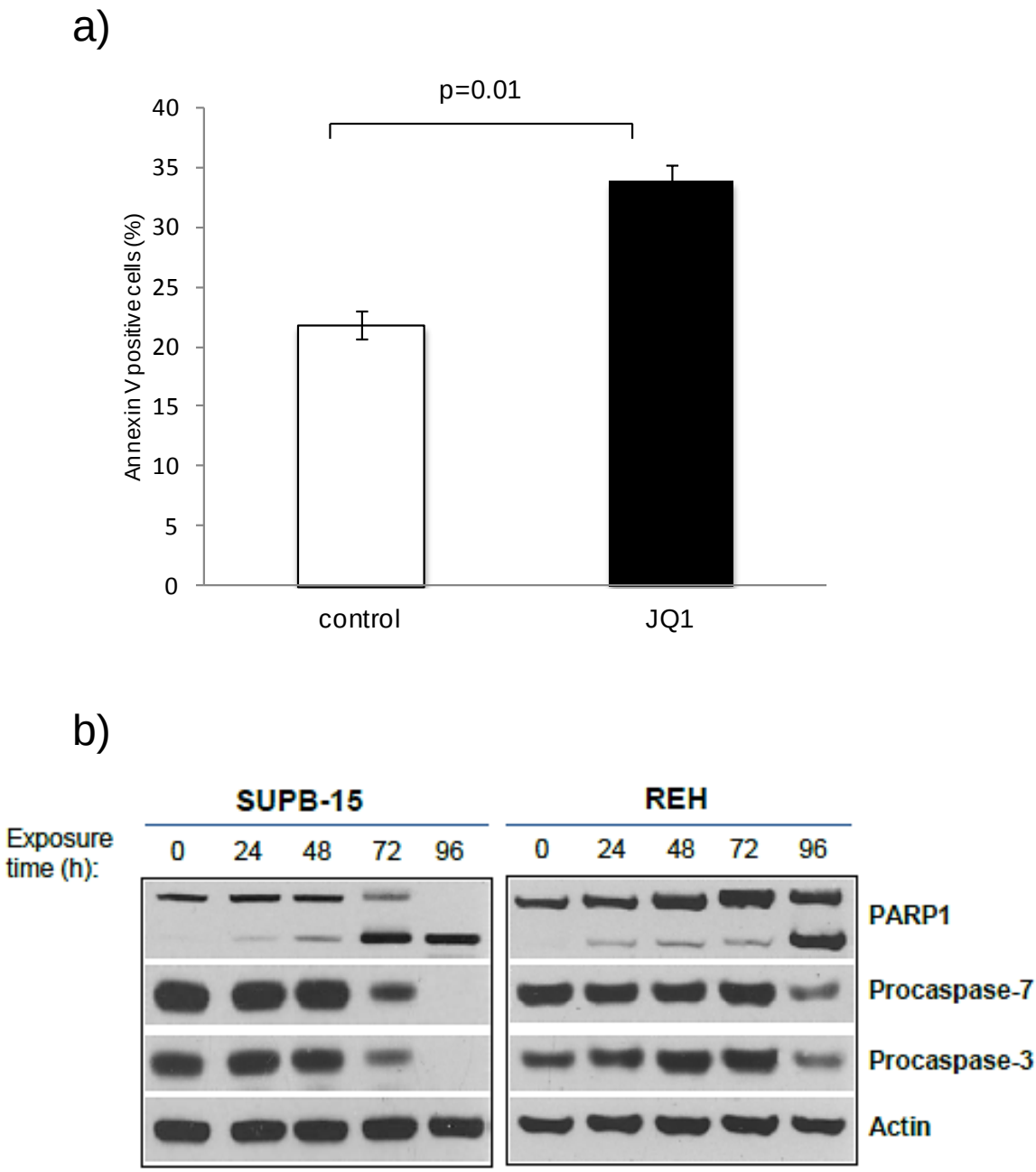
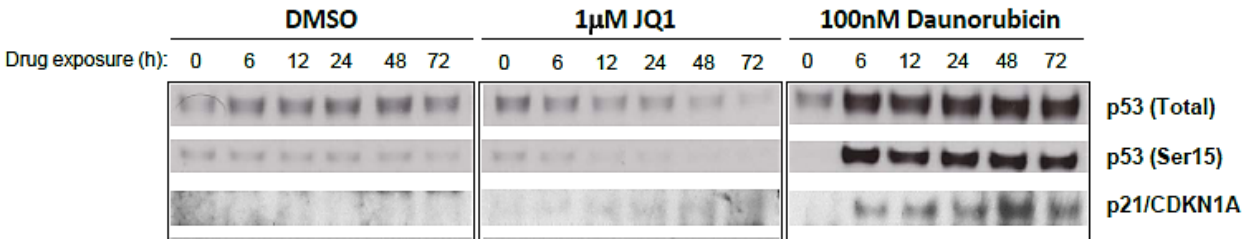


Figure 3.24: JQ1 induces caspase-dependent apoptosis in ALL tumour cells

a) Annexin V/PI staining and FACS analysis shows differential induction of apoptosis in NALM-6 cells treated with 1 µM JQ1 over a 72h incubation period compared to DMSO-treated control cells.

b) Western blotting shows the induction of apoptosis in ALL cell lines SUPB-15 and REH as measured by cleavage of proteins PARP1, procaspase-3 and -7 over a 96hr incubation period with 1 µM JQ1. Actin was probed for as a loading control.

a)



b)

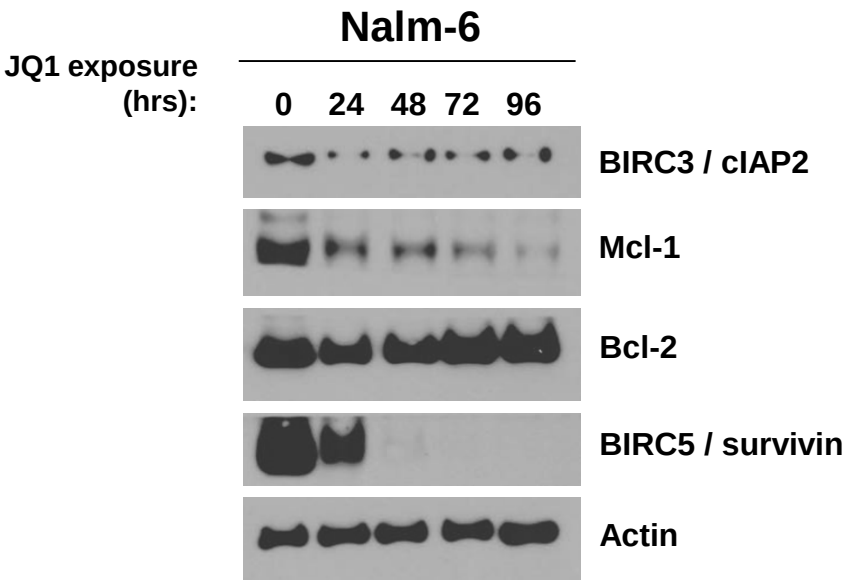


Figure 3.25: Induction of apoptosis by JQ1 involves a p53-independent mechanism

a) Western blotting shows no evidence of either p53 upregulation, p53 phosphorylation or significant p21 upregulation in NALM-6 cells incubated with 1 µM JQ1 over 72hr. Treatment with the DNA-damaging agent daunorubicin (100 nM) induces expression of both total and phosphorylated p53, as well as the expression of the p53 target p21.

b) Western blot shows that 24hr exposure of NALM-6 cells to 1 µM JQ1 leads to the downregulation of Mcl-1 protein expression levels, which decrease steadily until 96 hours. The expression of survivin was completely lost within a 48hr incubation with 1 µM JQ1. No significant change was observed in the expression levels of Bcl-2 and cIAP2 protein. Actin was used as a loading control.

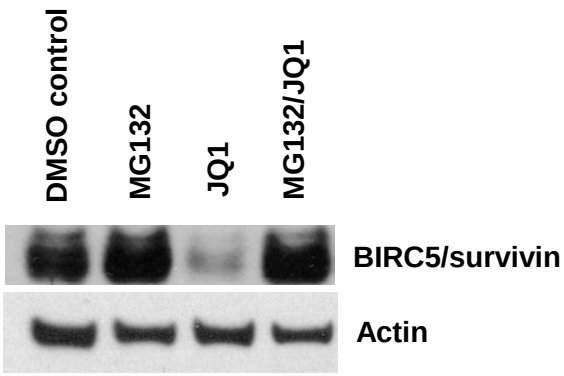


Figure 3.26: Co-treatment of ALL tumour cells with JQ1 and the proteasome inhibitor MG132 abrogates JQ1-mediated downregulation of survivin

JQ1 abolishes protein expression of survivin in NALM-6 cells, which is rescued following combined JQ1/MG132 treatment. This suggests the downregulation of survivin expression following JQ1 treatment is at the post-translational level.

3.4. Investigation of JQ1 activity in an ALL xenograft model

Many murine models have been developed to aid our understanding of human cancer development and responses to novel therapies before they reach the clinic. One of the most commonly used models is the human tumour xenograft, whereby human tumour cells are transplanted under the animal's skin or into the organ type from which the human tumour cells originated from [467]. The recipient mice must be immunocompromised so that the human tumour cells are not rejected by the host immune system. Efforts to develop mouse strains with increased capacities for enhanced human tumour engraftment have led to the discovery and use of severe combined immunodeficiency (SCID) mouse strains. The NOG (NOD/Shi-scid/IL-2Rγnull) mouse is a more recently developed generation of SCID mice, accepting heterologous cells far more easily than other immunodeficient murine models such as the nude mouse and NOD/SCID mouse, since NOG mice have dyfuctional macrophage and dendritic cells, and lack T-, B- and NK-cell activity [468]. Depending on the number of cells injected, leukaemia cells typically engraft over a period of 8 weeks, in which time *in vivo* responses to specific therapies may be tested [469]. The greatest advantage of using a human tumour xenograft model is that tumours will consist of human and not mouse tumour cells, therefore maintaining the original human tumour's molecular features including chromosomal abnormalities, whilst providing a more realistic representation of how effective the therapy would be if given to the patient [469, 470]. Indeed, studies looking at whether or not results from tumour xenograft models translated to responses

observed in the respective patients revealed a very high degree of predictive power both in the response to chemotherapeutic agents and resistance in patients [471]. Therefore, it is not surprising that cancer therapeutic agents are rarely clinically approved unless shown to exhibit anti-tumour activity in a preclinical *in vivo* mouse model [470].

Additional aspects to consider, when using murine models to investigate leukaemia, are that exposure to some drugs may impose selective pressure on leukaemic subclones or may target specific immunophenotypic subpopulations of cells more than others, causing a shift in the expression of immunophenotypic markers [472]. This phenomenon is sometimes observed in the clinic and creates difficulties in the monitoring of MRD to promptly predict the risk of relapse in patients receiving treatment [473, 474]. The CD34 marker is an antigen expressed in human haematopoietic stem cells and progenitor cells, with expression decreasing as the cell differentiates. Important studies in ALL have provided evidence to support the existence of disease-propagating pre-leukaemic stem cells that evolve through the acquisition of additional genetic abnormalities [475-478]. This is also supported by studies in paired relapse and diagnostic primary ALL samples, which provide evidence for the selection of leukaemic stem cells with acquired additional abnormalities [80, 479].

In hindsight, the fact that the proportion of leukaemic subpopulations are subject to change and the evolving pre-leukaemic stem cell is likened to a constantly moving therapeutic target [480], highlights the importance in monitoring

subpopulations by measuring expression of surface markers when testing therapeutic agents in animal studies.

Since JQ1 exhibited potent anti-tumour activity in xenograft models of AML and NUT midline carcinoma, and I observed JQ1 cytotoxic effects against ALL *in vitro*, I decided it was appropriate to begin testing the efficacy of JQ1 *in vivo* using the ALL xenograft mouse model our group has developed.

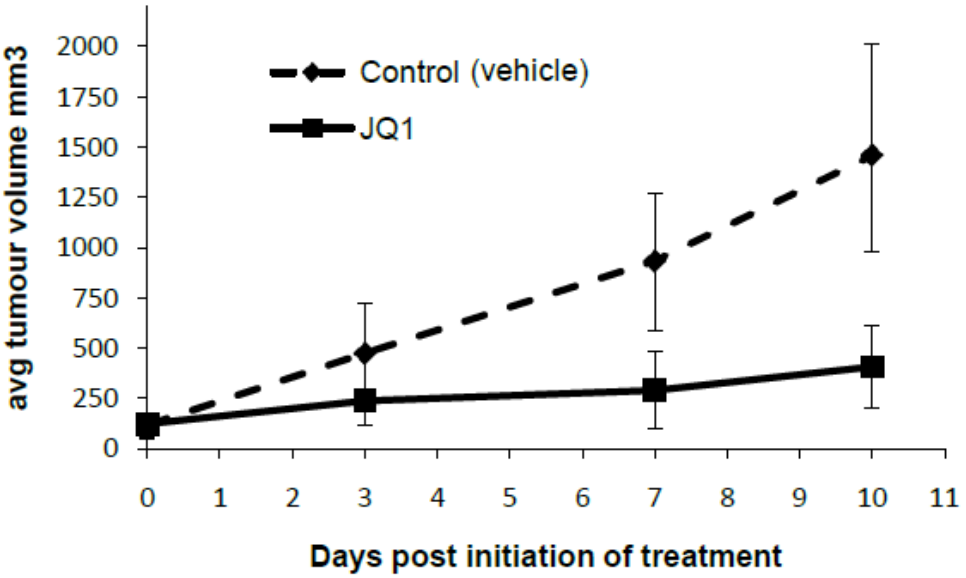
To address JQ1 activity *in vivo*, I performed two separate experiments using ALL xenograft models; one using an *in vitro* JQ1-sensitive ALL cell line, and the other using a JQ1-sensitive primary ALL tumour.

3.4.1 Investigating JQ1 activity in a NALM-6 xenotransplant model

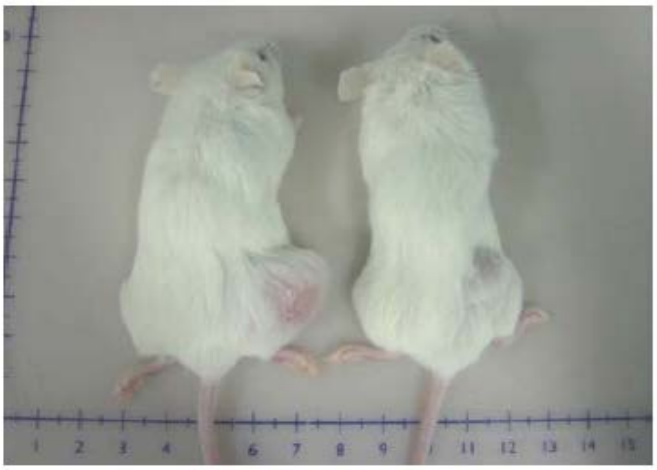
The first experiment involved the subcutaneous injection of NALM-6 cells into a cohort of 16 NOG mice. Once tumours were visible, with the help of a colleague from our group (Tracey Perry), animals received either 50mgkg^{-1} JQ1 (n=8) or vehicle alone (n=8) via intra-peritoneal (IP) injection for 5 days per week, for a period of 2 weeks. The tumour volume was then measured manually three times a week using callipers.

I found that animals receiving JQ1 showed a significant reduction in tumour growth over a 10-week period compared to animals treated with vehicle over the same period of time (Figure 3.27) ($p<0.05$)

a)



b)



vehicle (control) JQ1

Figure 3.27: JQ1 prevents proliferation of ALL tumour cells *in vivo*

An unpaired, 2-tailed t-test was used to compare the differences between vehicle (n=8) and JQ1 treated animals (n=8), with error bars representing the standard deviation.

a) Subcutaneous NALM-6 tumours show a significant impairment in growth when treated with 50mg/kg JQ1 over a 2 week time period compared to tumours treated with vehicle. This was significant at $p<0.05$
b) The difference in tumour size is shown in the photograph of two representative animals, treated with vehicle (left) and 50mg/kg JQ1 (right).

3.4.2 Investigating JQ1 activity in ALL-105 xenotransplant model

The second experiment involved the development of a xenograft model of an apoptotic-resistant, MRD high-risk, but JQ1-sensitive primary B precursor ALL (ALL-105), in a cohort of 14 NOG mice. Once animals had been injected with ALL-105 primary tumour cells (again with the kind help of Tracey Perry), the level of engraftment was monitored by measuring the percentage of human CD45 (hCD45) cells by weekly blood sampling and flow cytometric analysis. Once the levels of hCD45 reached 1%, treatment commenced. After 4 weeks of treatment with either JQ1 or vehicle, flow cytometric analysis was used to measure the overall tumour load in the spleens of engrafted animals, as well as the frequency of specific ALL progenitor populations.

Furthermore, I found that engrafted spleens from animals treated with JQ1 showed a significant reduction in tumour load compared to spleens from animals treated with vehicle ($p<0.001$). This was represented by a reduction in mean spleen weight, and reduction in spleen size in JQ1-treated animals (Figure 3.29).

I found that the level of hCD45-positive cells in all animals treated with vehicle exceeded the recommended experimental endpoint of 25% hCD45 by 28 days, whereas levels of hCD45 in samples taken from animals receiving JQ1 treatment were maintained below 25% throughout a period of 49 days (the end of the experiment) (Figure 3.28).

flow cytometric analysis revealed three ALL subpopulations in the engrafted leukaemia, CD34+CD19+CD10-, CD34+CD19+CD10+ and CD34-CD19+CD10+, that had the capacity to proliferate *in vivo*. Although the malignant origin was uncertain, a small percentage of CD34-CD19+CD10- cells were also detected in engrafted spleens. I observed a JQ1-induced reduction in all three ALL progenitor subpopulations, with the greatest reduction being exerted on CD34+CD19+CD10+ subset of ALL cells (Figure 3.30). I observed a slight increase in the frequency of CD34-CD19+CD10- cells in JQ1-treated animals in comparison to vehicle-treated animals, suggesting either differentially lower sensitivity of mature progenitors or differentiation of blast cells induced by JQ1.

Finally, I found that the mean body weight of animals treated with JQ1 did not significantly differ from those receiving vehicle during a 28 day period and did not exceed the recommended experimental endpoint of 15% loss in body weight (Figure 3.31). This indicated that the JQ1 compound was well tolerated by the animals and did not cause a significant deterioration in their health.

3.4.3 Summary

In conclusion, the data obtained from these animal studies confirmed that JQ1 confers anti-tumour activity *in vivo.* This was demonstrated by a clear reduction of tumour growth and leukaemia burden in JQ1-treated animals compared to those treated with vehicle. Data analysis also revealed that JQ1 eliminates all ALL progenitor subpopulations, with the greatest effect seen in CD34+CD19+CD10+ cells.

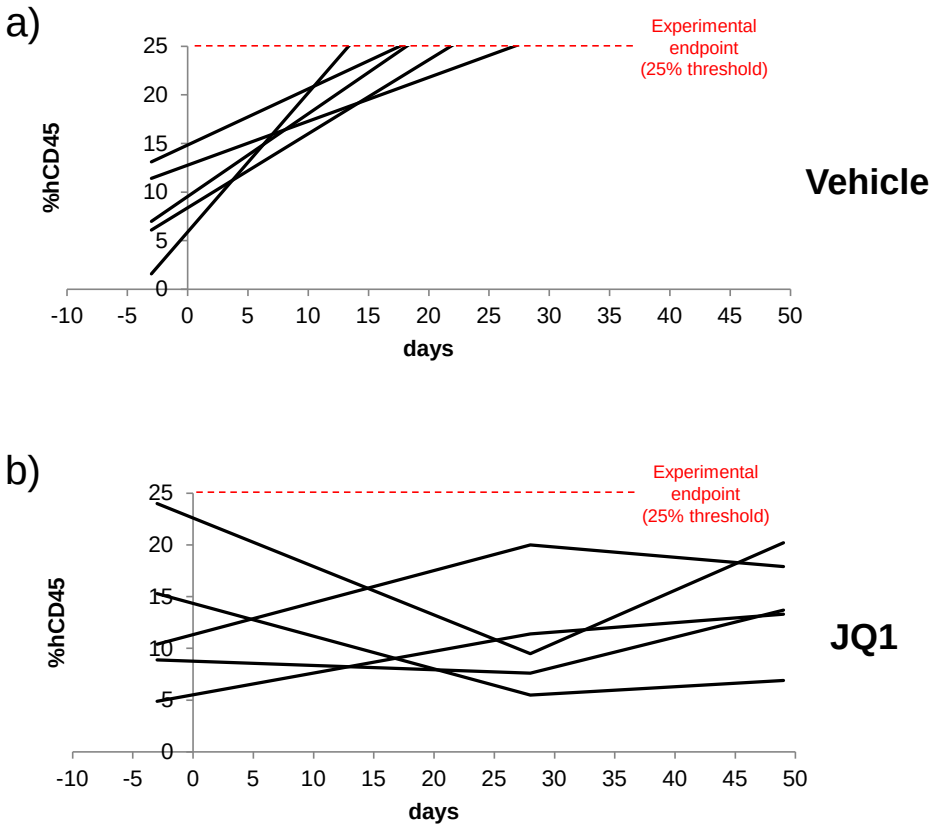


Figure 3.28: JQ1 prevents proliferation of apoptosis-resistant primary ALL tumour cells *in vivo*

a) Graph shows %hCD45 in vehicle-treated ALL-105 engrafted NOG mice. All vehicle-treated mice exceeded the experimental endpoint of 25% hCD45 by 28 days.

b) Graph shows %hCD45 in ALL-105 engrafted NOG mice treated with JQ1. The level of %hCD45 was maintained below 25% throughout a period of 49 days.

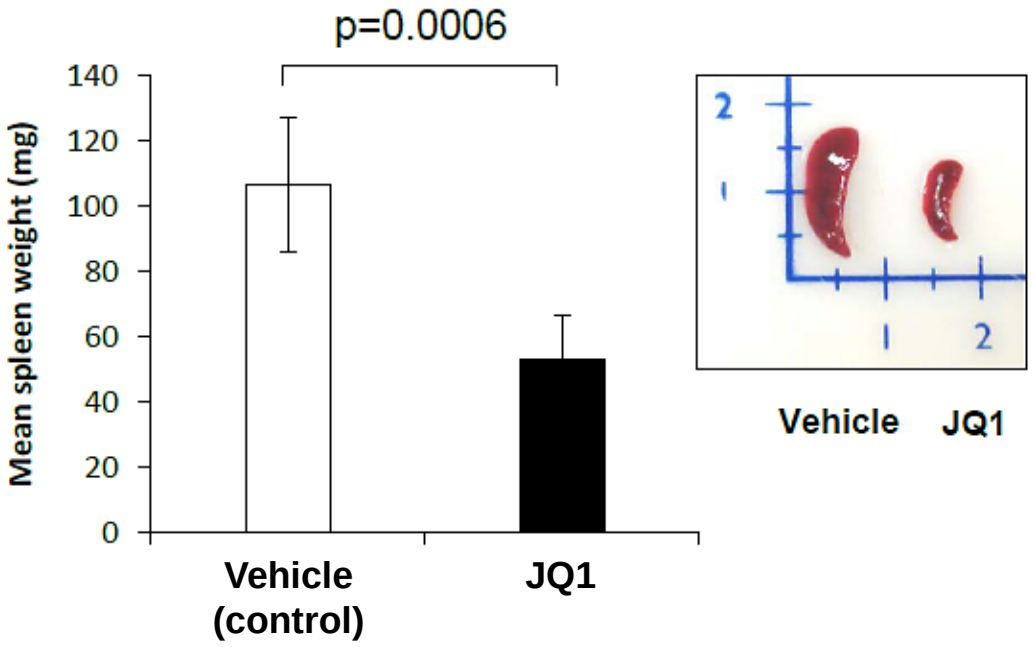


Figure 3.29: JQ1 treatment leads to reduced leukaemic burden in an ALL xenograft mouse model (ALL-105)

In a xenograft of a representative apoptosis-resistant primary ALL-105, animals treated with JQ1 (n=7) showed a significant reduction in tumour load compared to spleens from animals treated with vehicle (n=7).

This was represented by a reduction in mean spleen weight ($p<0.001$) (left), and reduction in spleen size (right) in JQ1-treated animals. The spleen size of the animals treated with JQ1 was representative of the spleen size seen in healthy control mice.

An unpaired, 2-tailed t-test was used to compare the differences between vehicle and JQ1 treated animals, with error bars representing the standard deviation.

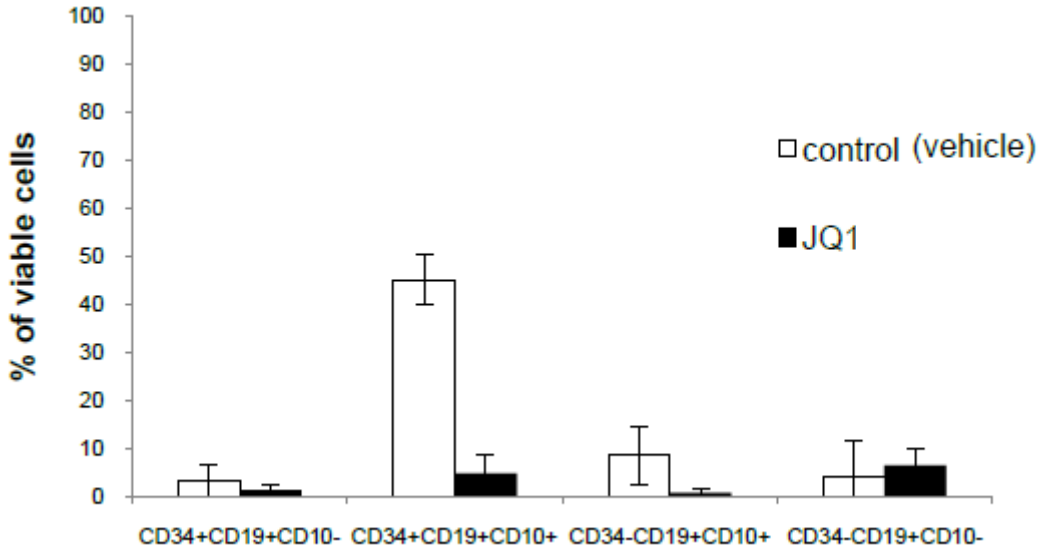


Figure 3.30: JQ1 eliminates multiple ALL subpopulations *in vivo*

Flow cytometric analysis of murine spleens engrafted with ALL-105 reveals that JQ1-treated animals show reduction in ALL subpopulations: CD34+CD19+CD10-, CD34+CD19+CD10+ and CD34-CD19+CD10+, and a relative increase in the CD34-CD19+CD10- population.

An unpaired, 2-tailed t-test was used to compare the differences between vehicle and JQ1 treated animals, with error bars representing the standard deviation.

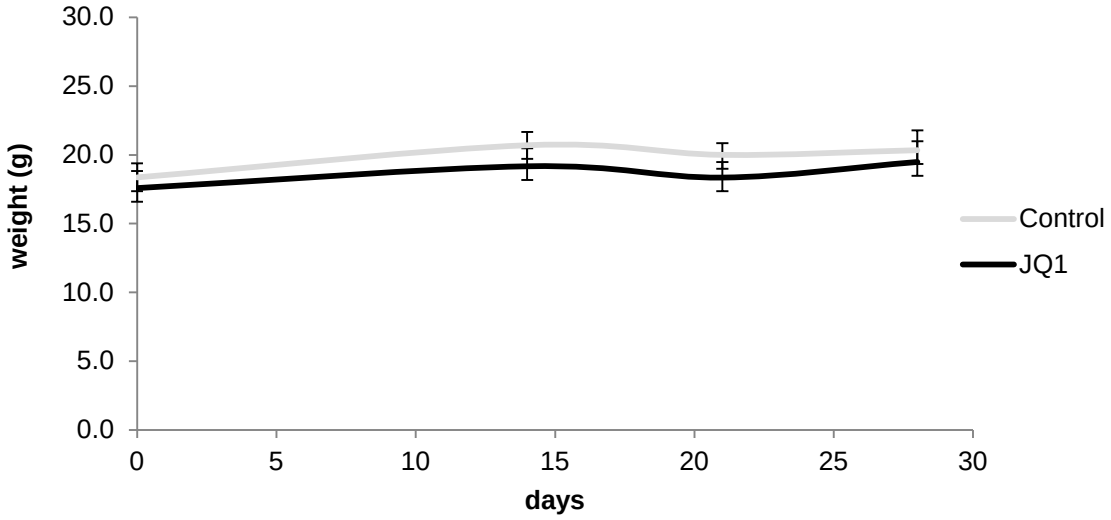


Figure 3.31: JQ1 is well tolerated in an ALL xenograft mouse model

NOG mice engrafted with ALL-105 and treated with either vehicle or JQ1 were weighed on days 0, 14, 21 and 28.

Total body weight of mice receiving either treatment did not fluctuate significantly or reduce by 15% (experimental endpoint). This suggests JQ1 is well tolerated *in vivo*.

CHAPTER 4

Results II: The PIM2 kinase as a candidate for further study

4. RESULTS II: THE PIM2 KINASE AS A CANDIDATE FOR FURTHER STUDY

4.1. Investigation of PIM kinases as potential targets in the treatment of precursor B-cell ALL

PIM kinases are expressed in a wide range of leukaemia and lymphomas, and have been positively associated with a poor treatment prognosis [481-484]. However their role in precursor B-cell ALL has not yet been explored.

As described in section 1.10.3, recent studies have demonstrated that the PIM kinases play an important role in the regulation of gene transcription by promoting specific histone acetylation events required for the docking of BET proteins, such as BRD4, to histones within transcriptionally active chromatin [386, 387]. This in turn allows for the recruitment of the P-TEFb complex, which is essential for the release of RNA polymerase II from promoter-proximal pause sites and subsequent transcript elongation [388].

In light of my results described in Chapter 3 that show BET proteins are associated with the transcriptional upregulation of multiple prosurvival pathways in ALL, I hypothesised that PIM kinases serve as an alternative means by which prosurvival signalling pathways are kept switched on and that consequently, targeting PIM kinases can induce apoptosis in ALL tumour cells.

Indeed, in support of this hypothesis, further analysis of the microarray results presented in this study, in which I ranked the fold-change in gene expression (as opposed to basal expression levels) in JQ1-treated primary ALL tumours according to EC_{50} values derived from *in vitro* cytotoxicity experiments, showed that upregulation of the *PIM2* gene in response to JQ1 treatment was strongly associated with JQ1-resistant tumours (Appendix 3). This suggests that PIM proteins might compensate for the pharmacological inhibition of BET protein activity, and confer resistance to JQ1.

I therefore proceeded to investigate whether PIM kinases were important in the pathogenesis of B-ALL, and posed as a viable therapeutic target for future studies.

4.1.1. Method validation for the screening of primary ALL tumours for PIM expression

I first wanted to identify the most practical method by which to measure the frequency of PIM kinase expression in primary pre-B ALL tumours, whilst using a limited amount of primary tumour material.

Since I had access to RNA samples extracted from a cohort of 68 primary ALL tumours, and PIM expression is reported to be regulated at the transcriptional level [385, 485], I decided to validate the qRT-PCR technique for the indirect screening of primary ALL tumours for PIM protein expression.

I began by testing the primers I designed to detect *PIM1* and *PIM2* transcripts on a panel of 7 primary ALL RNA samples (ALL-201, ALL-204, ALL-210, ALL-213, ALL-217, ALL-232 and ALL-240). I did not measure *PIM3* transcript levels as this gene is not associated with haematopoiesis.

I found that for each RNA test sample, the primer sets for both PIM genes amplified a single product at the expected molecular weight (Figure 4.1 a & b). Sequencing of the amplified PCR products also confirmed that they aligned to *PIM1* and *PIM2* mRNA sequences. These results showed that the PIM primer sets were specific and could be used in further experiments.

Using qRT-PCR and Western blotting, I then decided to test whether *PIM* gene expression correlated with PIM protein expression levels in a panel of 7 leukaemia cell lines (REH, NALM-17, SD1, Kasumi, Jurkat, CEMC1 and K562). In contrast to the REH, NALM-17, Jurkat and CEMC1 cell lines that did not express PIM, I found that both PIM1 and PIM2 were expressed at the RNA and

protein level in Kasumi, SD1 and K562 cell lines (Figure 4.2 a & b). I detected two PIM1 protein isoforms (33 kDa and 44 kDa) and three PIM2 isoforms (34, 38 and 40 kDa), with the 38 and 40 kDa PIM2 isoforms being expressed at higher levels to that of the 34 kDa isoform. Although studies have shown that both PIM1 isoforms retain kinase activity, the kinase activity of PIM2 isoforms has been shown to negatively correlate with molecular weight, with the largest 40 kDa isoform showing the least kinase activity [380]. Since each of the PIM isoforms retains kinase activity, I wanted each isoform to be detected in the qRT-PCR PIM expression screen; thus I made sure to design primers that amplified regions of the PIM genes that were common to all PIM1 and PIM2 isoforms.

Importantly, I observed a correlation between PIM2 mRNA and protein expression in leukaemia cell lines (Figure 4.2 a & b). In contrast, *PIM1* mRNA expression did not correlate with PIM1 protein expression, since the difference in PIM1 protein expression in SD1 and K562 cell lines was not proportionate to the difference observed at the mRNA level in the same cell lines. This suggests that post-translational modification of PIM1 and modulation of its stability may be occurring, as is described in the literature [361].

Taken together, these results confirm that measurement of *PIM2* mRNA by qRT-PCR is a suitable method by which to indirectly and efficiently measure PIM2 protein expression, as opposed to the measurement of PIM1, which may prove to be inaccurate given the regulatory post-translational mechanisms of PIM1 protein expression.

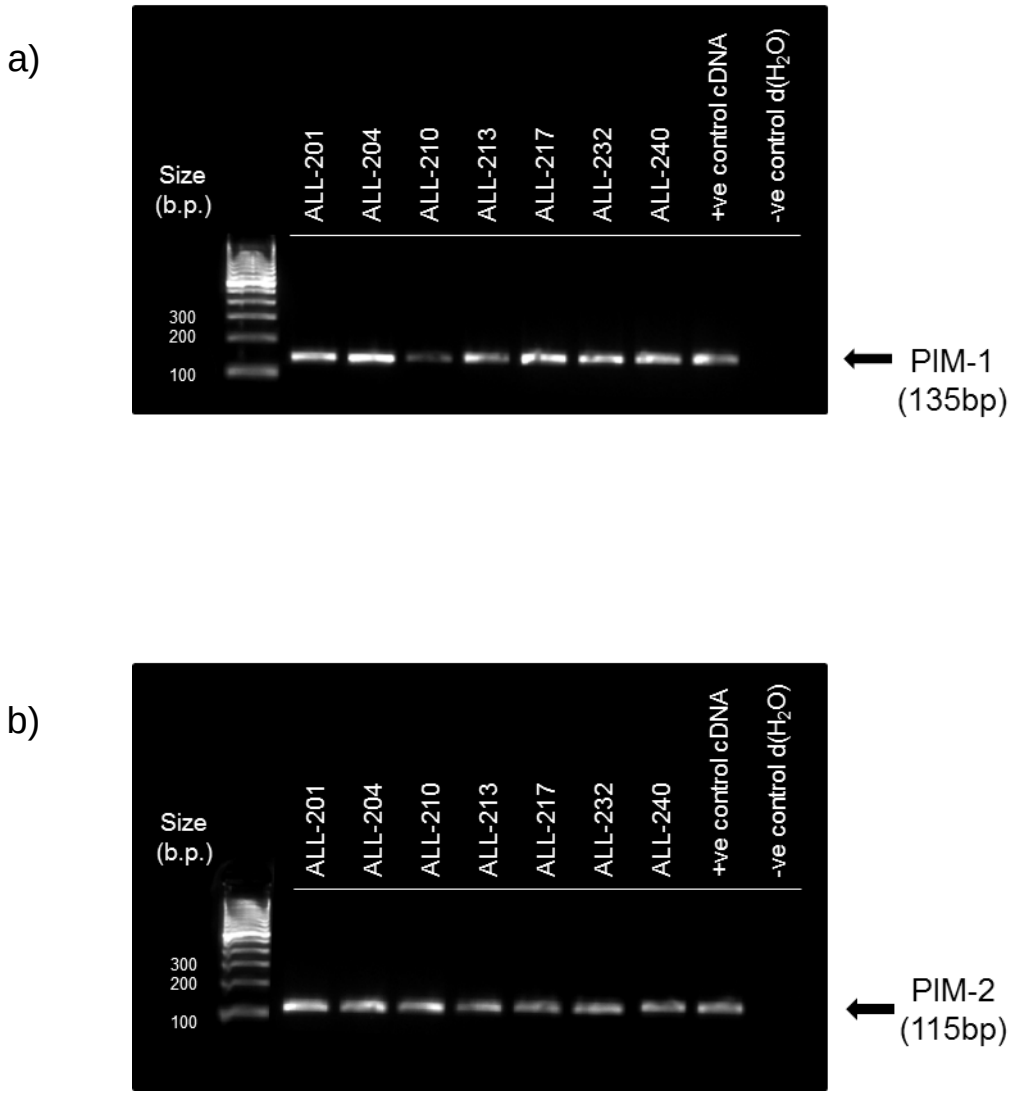
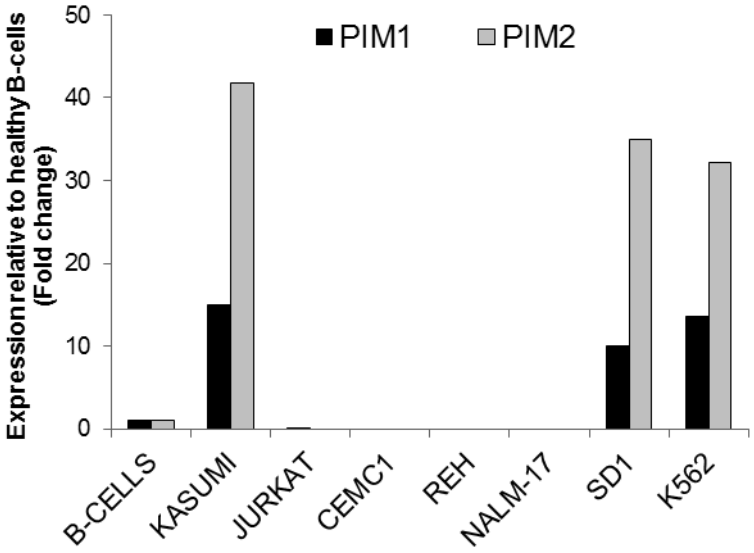


Figure 4.1: Confirmed specificity of PIM1 and PIM2 primer sets

PCR reactions were set up to test the a) PIM1 and b) PIM2 primer sets. cDNA synthesised from total RNA samples from a panel of 7 primary ALL tumours (ALL-201, ALL-204, ALL-210, ALL-213, ALL-217, ALL-232 and ALL-240) was used to prepare the PCR reactions. Reactions were prepared using the FastStart PCR system (Roche) and were set to 45 amplification cycles, with an annealing temperature of 60°C.

The primer sets used to amplify the PIM1 and PIM2 target sequence produced a single product in all the RNA samples tested, with products being of the expected size.

a)



b)

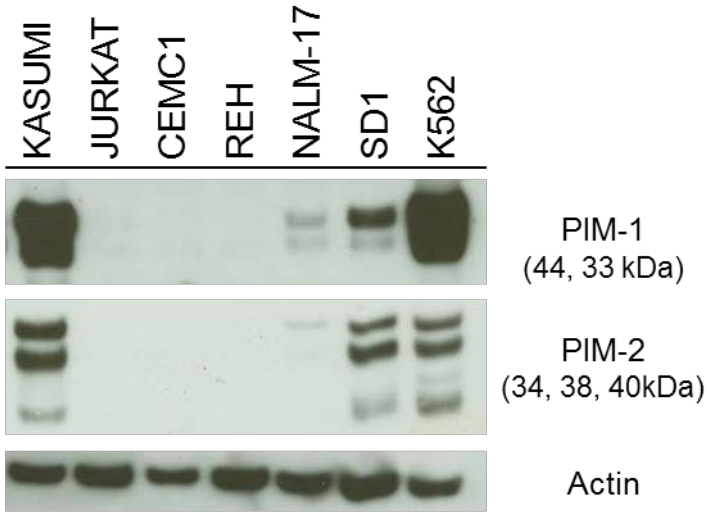


Figure 4.2: Correlation between PIM mRNA expression and protein expression

a) cDNA was synthesised from total RNA extracted from a panel of 7 leukaemia cell lines (Kasumi, Jurkat, CEMC1, REH, NALM-17, SD1 and K562). Graph represents PIM1 and PIM2 mRNA expression relative to the expression levels in normal B-cells as quantified by qRT-PCR. Ct values were normalised to β-actin mRNA

b) Graph represents PIM1 and PIM2 protein expression levels in the same panel of cell lines as above, detected by Western blotting. PIM kinase isoforms were also detected. Actin was used as a loading control.

A correlation between mRNA and protein expression exists for PIM2 but not PIM1.

4.1.2. PIM expression screen of a cohort of primary ALL tumour samples

I showed in section 4.1.1. that measurement of *PIM2* RNA expression by qRT-PCR can be used to indirectly measure PIM2 protein levels, given that PIM2 protein is constitutively active once expressed and is not regulated by post-translational modification [380]. I therefore proceeded to screening a cohort of 68 primary ALL tumour RNA samples for PIM2 expression.

Using the level of *PIM2* RNA expression in that of the PIM-expressing cell line K562 as a marker for high expression, I found that *PIM2* was highly expressed in 17/68 (25%) RNA tumour samples (Figure 4.3). In addition, I screened a small independent cohort of primary ALL tumours for PIM2 protein by Western blotting and confirmed that PIM2 was highly expressed in 2/7 (28.6%) (Figure 4.4), which corroborates the calculated frequency of PIM2 overexpression in B-precursor ALL derived from qRT-PCR data. However, it is likely that more tumours express significant levels of PIM2 protein, since the calculated frequencies of high PIM2 expression are based on a comparison to K562 that expresses high levels of this kinase.

I next wanted to see if PIM2 expression could be associated with clinical features in pre-B ALL. I decided to designate ALL tumours as PIM-2 positive and PIM2-negative if they expressed $\geq$50% or $<$50% PIM relative to K562, respectively. Using the available clinical information corresponding to the cohort of 68 RNA samples analysed, I found that there was no association between PIM2 expression and patient age or gender. I also found no difference in the

cytogenetic repertoire between PIM2-positive and PIM2-negative tumours, suggesting that PIM2 expression was not associated with cytogenetic features conferring a poor treatment prognosis.

To determine whether PIM2 expression influenced patient response to treatment, I checked whether PIM2 expression influenced patients' responses at Day 8 of treatment with respect to blast clearance. I found that there was a positive trend between PIM-2 positive tumours and poor blast clearance (Figure 4.5, top)

I then looked at whether PIM2 expression influenced MRD at Day 28 of treatment and found that PIM2-positive tumours were associated with positive MRD (Figure 4.5, bottom), suggesting that PIM2 expression may be associated with chemoresistance.

Overall, PIM2 appears to be overexpressed in over a quarter of primary ALL tumours and may influence chemoresistance *in vivo*.

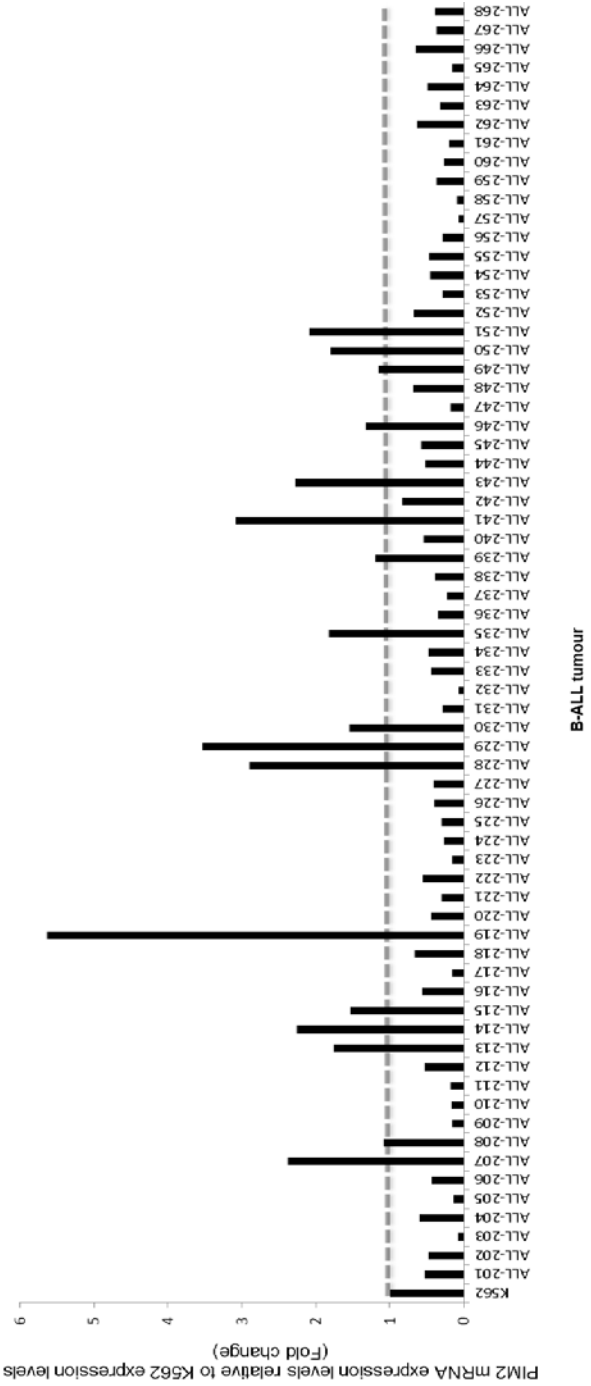


Figure 4.3: qRT-PCR screen for PIM2 expression in a large cohort of pre-B ALL tumours

A large cohort of primary ALL tumour RNA samples (n=68) were screened for PIM2 expression by qRT-PCR. Graph represents PIM2 mRNA expression relative the expression K562.

This shows PIM2 is overexpressed in 17/68 (25%) of B-precursor ALL tumours.

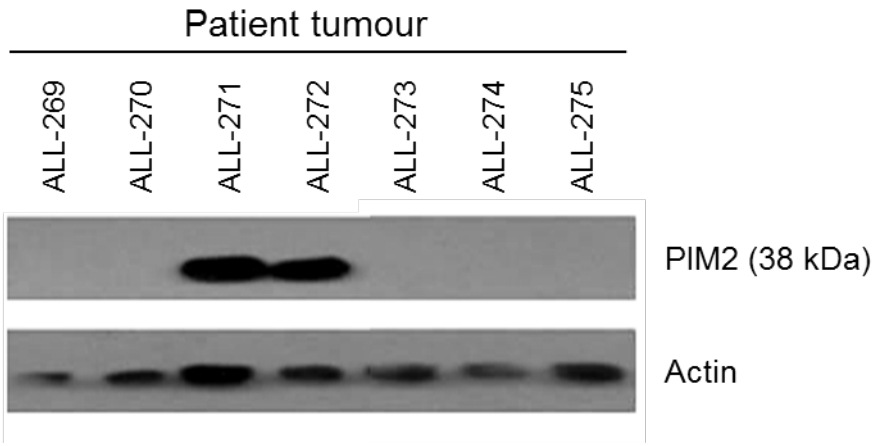


Figure 4.4: PIM2 is expressed at the protein level in primary pre-B ALL tumours

Western blotting was used to screen for PIM2 protein in a panel of primary ALL tumours (n=7). Actin was used as a loading control.

PIM2 protein is expressed in 2/7 (28.6%) of primary ALL tumours.

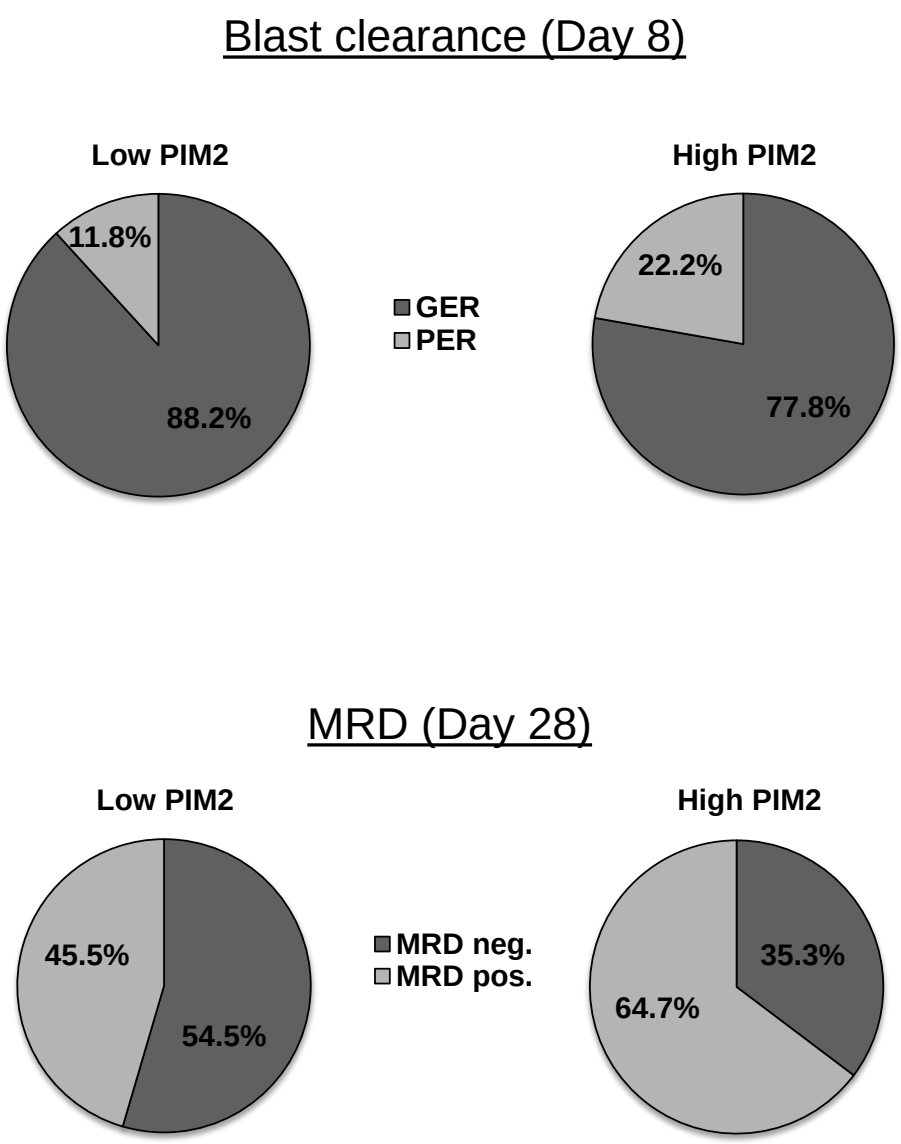


Figure 4.5: Influence of PIM2 expression on patient response to treatment

A positive trend towards an association between high-PIM2 expression and poor early response to treatment at day 8 is shown (top) (11.8 vs. 22.2%; p=0.5906).

A positive trend towards an association between high-PIM2 expression and MRD positivity at day 28 is shown (bottom) (45.5% vs. 64.7%; p=0.3340).

Due to the small number of tumour sample for which clinical information was available, statistical significance was not observed. However, this may be improved by extending this cohort in further studies.

(GER, good early response; PER, poor early response; MRD, minimal residual disease)

4.1.3 *In vitro* cytotoxic effect of K00135-mediated inhibition of PIM kinases in ALL cell lines and primary tumours

Since a significant proportion of primary ALL tumours overexpressed PIM2, I decided to use a luminescent ATP-based cytotoxicity assay to investigate the effect of PIM kinase inhibition on the cell viability of a panel of leukaemia cell lines (SD1, REH, Jurkat and K562) using the PIM inhibitor K00135 [391]; an imidazo[1,2-b]pyridazine derivative kindly provided to me by our collaborator Prof. Stefan Knapp. Cell viability was measured 72 hours following exposure to increasing concentrations of the K00135 inhibitor alone.

I also addressed K00135-mediated cytotoxicity in a panel of 11 primary B-precursor ALL tumours that were identical to some of the tumour samples tested in JQ1 experiments (Section 3.1.2), but independent of the primary ALL tumour cohort screened for PIM2 expression by qRT-PCR in section 4.1.2. Additionally, I tested for cytotoxic effects of K00135 on healthy non-tumour PBMCs, to determine whether any observed cytotoxic effects were tumour-specific.

I found that the treatment of the PIM1- and PIM2-expressing SD1 ALL cell line with K00135 induced cytotoxic effects in a dose-dependent manner (EC_{50} 5.4 µM) (Figure 4.6; Table 4.1). Similarly, the induction of cytotoxicity was observed in K562 cells (EC_{50} 19.3 µM), which were much less sensitive to the effects of K00135 treatment when compared to SD1 cells, despite the K562 cell line exhibiting the greatest PIM kinase overexpression of the leukaemia cell lines I tested (Figure 4.2b). As expected, the NALM-17 cell line that expressed very

low levels of PIM protein, was not sensitised following K00135 treatment (EC_{50} >100 µM). However, REH and Jurkat cell lines, which did not express any detectable PIM proteins (Figure 4.2a & b), appeared to be sensitised to K00135 treatment (REH EC_{50} 10.2 µM and Jurkat EC_{50} 4.6 µM). This suggests that K00135-induced cytotoxicity observed in the leukaemia cell lines not expressing PIM protein may be due to off-target effects, despite the K00135 inhibitor having been shown to exert selective antileukaemic activity in several AML cell lines and primary AML tumours [391].

With respect to primary ALL tumours, I found that treatment of tumour cells with K00135 over a 48 hour incubation period led to dose-dependent induction of cytotoxicity, with a range of sensitivity to K00135 being observed (Figure 4.7; Table 4.2). Three tumours (ALL-103, ALL-104 and ALL-111) demonstrated increased sensitivity to K00135 compared to the rest of the tumours tested in this panel, with 30% (3/10) tumours showing EC_{50} values <5 µM, compared to 70% (7/10) tumours that had EC_{50} values >5 µM. Importantly, similar treatment of healthy PBMCs did not demonstrate sensitivity to K00135 (EC_{50} >10 µM).

As primary tumour material was limited, I aimed to make good use of the information I had already obtained from previously analysed tumours. Thus, I referred to the microarray data from my studies involving JQ1 to derive PIM2 basal expression levels in the primary tumours I had treated with K00135 (Figure 4.8), since K00135-treated tumours were largely the same primary ALL tumours I had used to test for JQ1 cytotoxicity and I had also confirmed in section 4.1.1. that PIM2 transcript levels correlate with PIM2 protein levels.

Intriguingly, I found that at a clinically achievable concentration of 1 μM K00135, ALL-103 tumour cells, which expressed one of the highest levels of PIM2 in this panel of ALL tumours (table 4.2), was the most sensitive to the cytotoxic effects of K00135. In contrast, the primary ALL tumour with the lowest expression of PIM2 (ALL-106) was the least K00135-sensitive tumour of all the tumours analysed. A further observation was that ALL-105 expressing high levels of PIM2 was not sensitised to K00135, suggesting some tumours may be inherently resistant to the effects of PIM kinase inhibition.

Overall, the specificity with which K00135 targets PIM kinases in the leukaemia cell lines I tested remains largely unknown, since I observed cytotoxic effects of K00135 in two ALL leukaemia cells lines not expressing PIM kinases. This may be due to cross-reactivity of K00135 with one other kinase (CLK1), as has been reported [486]. However, similar to BET inhibition, Pim kinase inhibition by K00135 induces differential cytotoxicity in primary ALL tumour cells compared to normal healthy PBMCs. Importantly, some primary tumours also appear to be resistant to K00135 treatment, despite expressing high levels of PIM2.

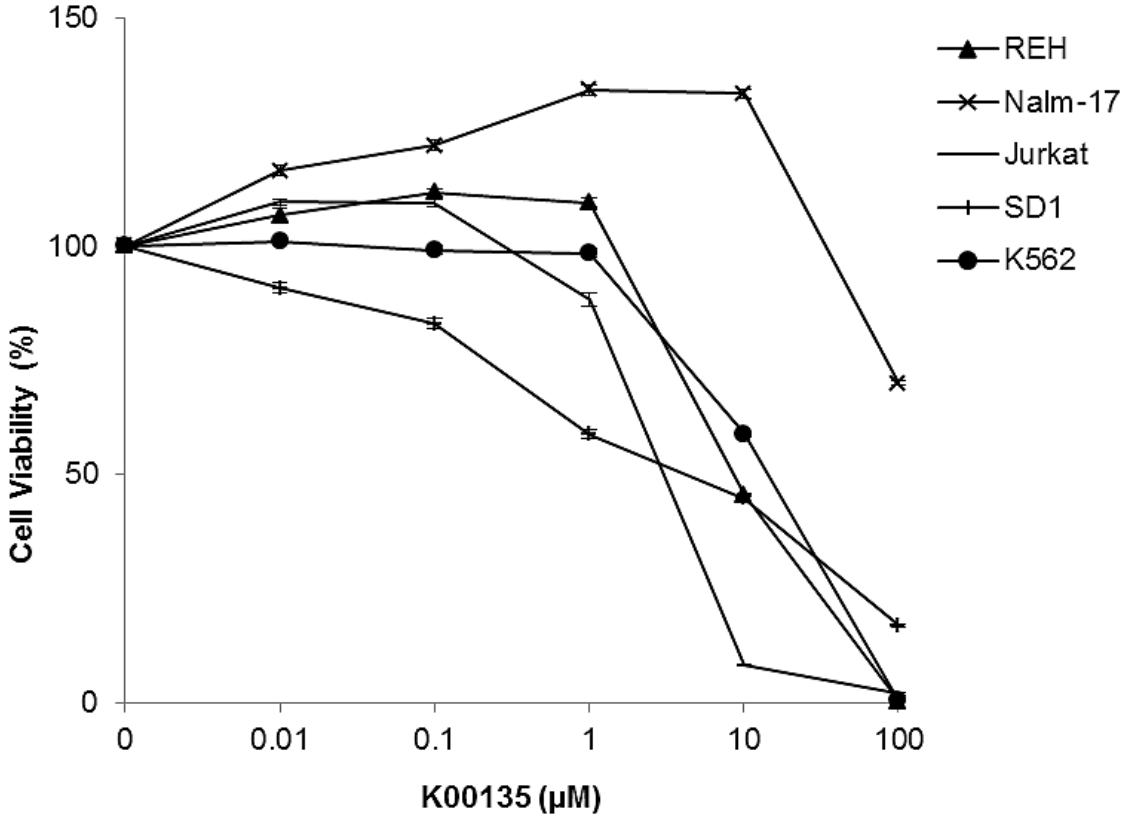


Figure 4.6: K00135-induced cytotoxicity in a panel of leukaemia cell lines

Effects of K00135 treatment on the cell viability of B-precursor ALL cell lines REH, NALM-17, Jurkat, and SD1 were measured at 72hr using a luminescent ATP-based cytotoxicity assay. The CML cell line K562 is confirmed as being sensitive to the cytotoxic effects of PIM-inhibition [487], and was therefore included as a positive control. Assays were performed in triplicate. Data are presented as mean ± SEM; n=3.

Apart from the NALM-17 cell line, which shows resistance to the cytotoxic effects of K00135, ALL cell lines (REH, Jurkat, SD1) show increased sensitisation to K00135 compared to the CML K562 cell line.

Cell line	K00135 EC_{50} (μM)
REH	10.2
NALM-17	398.4
Jurkat	4.6
SD1	5.4
K562	19.3

Table 4.1: Half-maximum-effect concentrations (EC_{50}) calculated for a panel of leukaemia cell lines treated with K00135 inhibitor

JQ1 EC_{50} values were calculated for REH, NALM-17, Jurkat, SD1, and K562 using the Prism 4 software package (GraphPad Inc.), which were derived from data values obtained for each cell line in cytotoxicity experiments following 72hr incubation of cells with a log-scale range of K00135 concentrations.

With the exception of NALM-17 cells, which show overt resistance to K00135 (EC_{50} 398.4), ALL cell lines are sensitised to the cytotoxic effects of K00135 EC_{50} 4.6-10.2 μM), with K562 positive control cells being less sensitive to K00135-induced cytotoxicity (EC_{50} 19.3 μM).

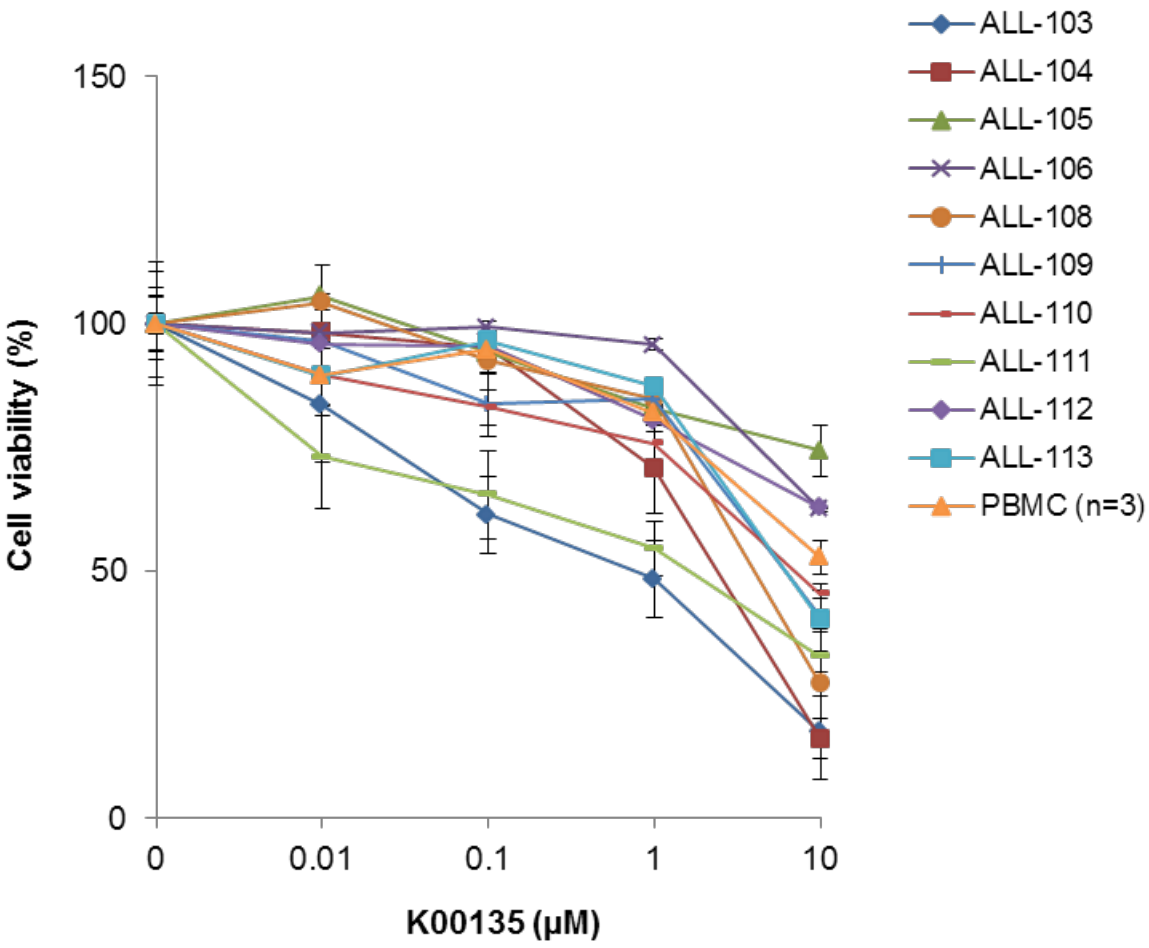


Figure 4.7: K00135-induced cytotoxicity in a panel of primary ALL tumours

Effects of K00135 treatment on the cell viability of B-precursor ALL cell lines REH, NALM-17, Jurkat, and SD1 were measured using a luminescent ATP-based cytotoxicity assay.

Compared to PBMCs obtained from healthy individuals (n=3), representative primary ALL tumours show a differential loss of viability upon 48hr incubation with 0.1 and 1 µM JQ1. Data are presented as mean ± SD.

Primary tumour	K00135 EC_{50} (µM)
ALL-103	0.9
ALL-104	3.8
ALL-105	4892.0
ALL-106	42.0
ALL-108	6.8
ALL-109	7.7
ALL-110	8.5
ALL-111	2.4
ALL-112	72.6
ALL-113	7.8
PBMC	14.0

Table 4.2: Half-maximum-effect concentrations (EC_{50}) calculated for a panel of primary ALL tumours treated with K00135 inhibitor.

Calculated median EC_{50} values were derived from cytotoxicity experiment data, where primary tumours were treated with a range of log-scale K00135 concentrations for 48hr.

Compared to PBMCs obtained from healthy individuals (n=3), a total of 7 representative primary ALL tumours (ALL-103, ALL-104, ALL-108, ALL-109, ALL-110, ALL-111 and ALL-113) show a differential loss of viability following 48hr exposure to K00135 (EC_{50} values <10 µM).

A total of 3 primary ALL tumours (ALL-105, ALL-106 and ALL-112) exhibit resistance to the cytotoxic effects of K00135 (EC_{50} >40 µM), with ALL-105 showing the greatest resistance (EC_{50} 4892 µM).

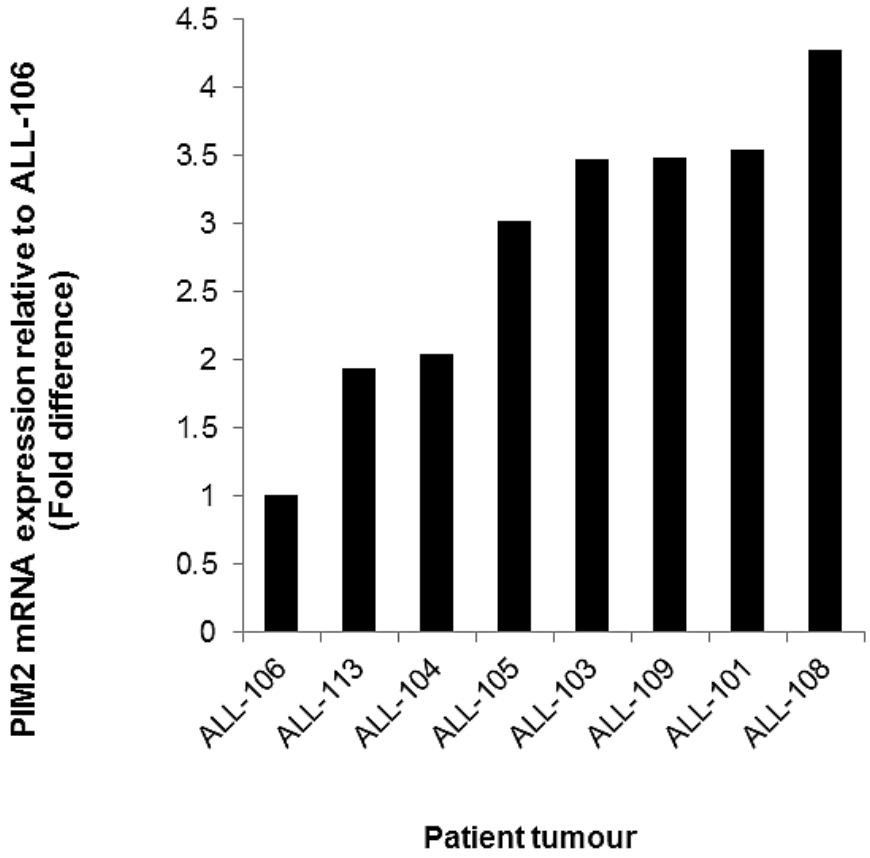


Figure 4.8: PIM2 expression in primary ALL tumours analysed in cytotoxicity experiments involving the K00135 inhibitor

PIM2 expression levels in tumours tested for sensitivity to K00135-induced cytotoxicity (**section 4.1.3**) were derived from JQ1 microarray data presented in **section 3.2.2,** as these were the same tumours.

The graph represents PIM2 mRNA expression relative to expression levels in ALL-106. Tumours are arranged from low to high PIM2 expression.

4.2. Identification of gene expression patterns associated with *PIM2* expression in primary B-precursor ALL tumours

As previously described in section 1.10.3, studies have shown that the PIM1 kinase promotes transcriptional pause release of stalled RNA Pol II, through a mechanism involving the BET protein BRD4 [488]. The role of other PIM kinase family members in regulating transcription has not yet been elucidated. However, given the high degree of amino acid sequence homology between PIM1 and PIM2 [362], it is possible that PIM2 may influence the expression of a distinct subset of genes.

Furthermore, results from cytotoxicity experiments (section 4.1.3.) indicate that the PIM inhibitor K00135 leads to differential sensitisation of a subset of primary ALL tumours, and that in the majority of tumours this effect depends on PIM2 expression. Thus, I decided to investigate the transcriptional profiles of primary ALL tumours associated with high and low levels of PIM2 expression to identify potential PIM2-regulated genes and pathways.

I did this by reanalysing microarray data previously obtained from our lab from 22 individual primary ALL tumours, which harboured a range of cytogenetic features and included several tumours derived from patients in the high-risk stratification group. With the help of a statistician (Dr. Wenbin Wei), I ordered tumours according to their baseline level of *PIM2* gene expression, and then selected the top 100 genes most significantly associated with PIM2 expression using the Spearman's rank correlation coefficient [489].

Using this approach, I found that high *PIM2* expression was associated with the upregulation of 33 genes, including multiple NFκB transcription factor target genes (*XIAP, TNFAIP3*, *NLRP3* and *CD48*), as well as *TIMP1*, *HYOU1* and *UBA1* (Figure 4.9).

The NFκB target gene *XIAP* encodes a member of the IAP family of apoptotic inhibitors [490], and is frequently overexpressed in a variety of malignancies, including leukaemias and lymphomas [491, 492]. It has been shown to be one of the most potent suppressors of apoptosis induced by a wide range of agents, such as TNF, TRAIL, Fas-L, staurosporine, paclitaxel and etoposide [493, 494]. Furthermore, the expression of *XIAP* has been shown to correlate with a poor clinical outcome in DLBCL [491].

Another gene associated with the overexpression of PIM2 was the tissue inhibitor of metalloproteinase 1 (*TIMP1*) gene, which is a STAT3 target gene that has been shown to be overexpressed in a spectrum of tumour types, and is frequently associated with an aggressive clinical phenotype [495-500].

The hypoxia up-regulated 1 (*HYOU1)* gene was also positively associated with PIM2 expression and has previously been shown to be overexpressed in prostate cancer and confer chemotherapeutic resistance via a mechanism involving the inhibition of caspase-dependent apoptosis [501, 502].

Upregulation of the *UBA1* gene encoding the ubiquitin-activating enzyme UBA1 (E1) was another gene I found to be associated with high PIM2 expression. The E1 enzyme initiates the multi-step proteasomal pathway of protein degradation by conjugating a single ubiquitin molecule to proteins, which are then further

ubiquitinated by other ubiquitin-conjugating enzymes, and consequently degraded [503, 504]. Whereas polyubiquitinated proteins are destined for proteasomal degradation, monoubiquitination of proteins has been shown to regulate gene transcription and DNA repair [505, 506]. Interestingly, recent *in vivo* studies using an AML primary xenograft mouse model have demonstrated that E1 activity is significantly upregulated and that its inhibition leads to targeted malignant cell death and delayed tumour growth [507].

I also identified a significant association between the downregulation of 67 genes and high PIM2 expression (Figure 4.10). These included DNA repair genes such as *MLH3* and *XRCC2*, which encode protein key to the regulation of mismatch repair (MMR) and homologous recombination (HR), respectively. Studies have shown that downregulation of the *MLH3* gene is observed in hereditary nonpolyposis colorectal cancer (HNPCC), ALL, CLL and malignant lymphoma [508-514], and is associated with an increased rate of spontaneous mutation and instability of microsatellites [515]. With respect to the *XRCC2*, a lack in *XRCC2* gene expression is associated with fragmentation of the centrosome and increased genomic instability [516]. Furthermore, decreased expression of this gene has been shown to promote tumourigenesis following DNA damage, as other less accurate DNA repair mechanisms are activated instead, introducing a higher rate of misrepaired DNA lesions [517]. Thus, *XRCC2* is recognised as a tumour suppressor gene.

Interestingly, I found that high PIM2 expression was associated with downregulation of the *POLH* gene encoding DNA polymerase eta (Pol η) that is also involved in the accurate repair of DNA, particularly translesion synthesis of

DNA damage caused by ultraviolet radiation [518]. Subsequently, decreased Pol η expression is linked to an increased risk of malignant melanoma [519]. Intriguingly, studies have also confirmed an association between deficiencies in Pol η and hypermutation occurring during immunoglobulin class switch recombination [520]. In addition, Pol η is targeted to stalled replication machinery and forms a complex with proliferating cell nuclear antigen (PCNA) and replication factor C (RFC) [521]; the latter of which is known to interact with the BET protein BRD4, as mentioned in section 1.10.3.

In addition, I found that downregulation of the RPL5 gene correlated with high PIM2 expression, of which the ribosomal protein product has been shown to block cell growth by enhancing tumour suppressor p53 activity in response to ribosomal stress during protein synthesis [522].

Next, I decided to use GSEA on the same microarray data to identify subtle associations between biological signalling pathways and PIM2 expression in B-precursor ALL tumours [403]. I identified 38 gene sets upregulated in PIM2-expressing ALL tumours that were significantly enriched at a false discovery rate (FDR) <25%.

This included gene sets representing pathways that regulate gene transcription, the cell cycle, DNA replication, ribosome assembly, protein translation, proteasomal degradation and PI3K/Akt prosurvival signaling (Table 4.3).

Intriguingly, with regards to gene transcription, the expression of PIM2 was significantly associated with the upregulation of several basal transcription factor genes. The basal transcription factors (TFIIA, TFIIB, TFIID, TFIIE, TFIIF

and TFIIH) are proteins essential to RNA Pol II-mediated gene transcription and form protein complexes that constitute the pre-initiation complexes at gene promoters [523-527]. The basal transcription factors then promote the binding of RNA Pol II to DNA, and also ensure that promoter escape and entry into the transcript elongation phase takes place [523, 527, 528].

Furthermore, GSEA highlighted multiple pathways encompassing the DNA replication process. For example, tumours expressing high levels of PIM2 were associated with the upregulation of pathways that regulate interaction of the origin recognition complex (ORC) with replication origins, which are where DNA replication initiates from during the S-phase of the cell cycle [529, 530]. I will therefore be interesting to confirm whether PIM2 has a direct impact on DNA replication in future studies.

In contrast, I did not identify any statistically significant gene sets that negatively correlated with PIM2 expression.

Overall, the results I obtained from reanalysis of microarray data previously obtained in our lab suggest a link between PIM2 expression and the upregulation of prosurvival (NFκB and PI3K signaling) and anti-apoptotic pathways. Furthermore, results indicate PIM2 is positively associated with the expression of genes involved in the regulation of gene transcription, cell proliferation, DNA repair, DNA replication and protein translation. However, at this stage it is not possible to specify whether these pathways are up- or downstream of PIM2 activity.

Particularly interesting, is the observation that many of the pathways and biological processes associated with PIM2 expression, such as upregulated NFκB pathway activity and anti-apoptotic pathway signaling, gene transcription and DNA replication, overlap with those downregulated following the inhibition of BET proteins, suggesting this overlap may be contributing to the resistance to K00135 and JQ1 observed in some tumours.

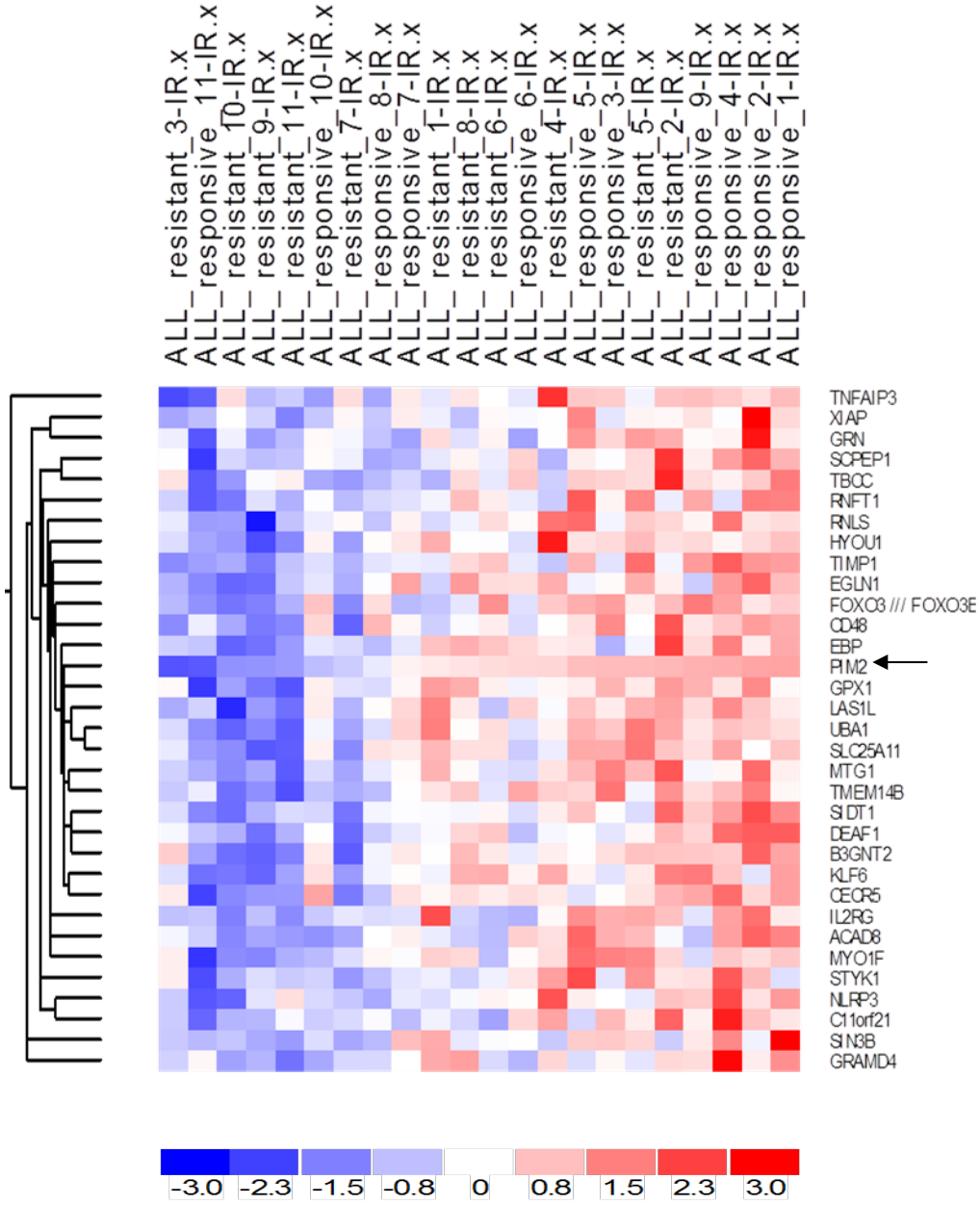


Figure 4.9: Re-analysis of global gene expression data identifies a positive correlation between the expression of a specific subset of genes and PIM2 expression in primary ALL tumours

Microarray data previously obtained in our lab [247] was re-analysed to look for associations between PIM2 expression and specific genes in pre-B ALL. A total of 22 tumours were ordered from low to high PIM2 gene expression (PIM2 gene indicated by arrow).

Transcript expression heat-map was generated using dChip (http://www.dchip.org/) with the default settings, and shows a subset of genes with the strongest positive correlation with PIM2 expression, determined using Spearman's rank correlation coefficient statistical analysis (coefficient ≥ 0.6; $p<0.05$).

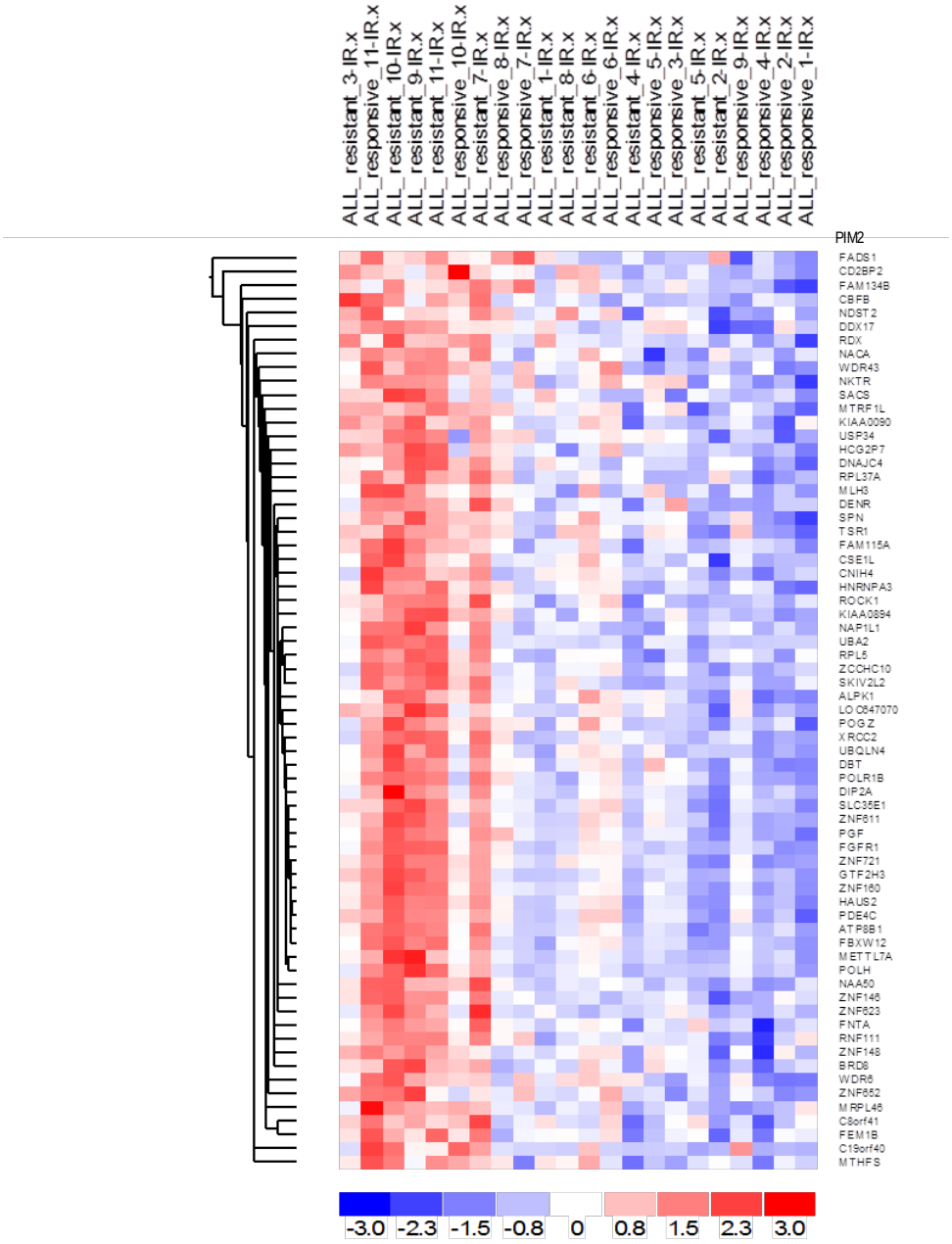


Figure 4.10: Re-analysis of global gene expression data identifies a negative correlation between the expression of a specific subset of genes and PIM2 gene expression in primary ALL tumours

Microarray data previously obtained in our lab [247] was re-analysed to look for associations between PIM2 expression and specific genes in pre-B ALL. Tumours were ordered from low to high PIM2 gene expression (PIM2 gene indicated by arrow).

Transcript expression heat-map was generated using dChip (http://www.dchip.org/) with the default settings, and shows a subset of genes with the strongest negative correlation with PIM2 expression, determined using Spearman's rank correlation coefficient statistical analysis (coefficient ≥ 0.6; $p<0.05$).

Gene Set	Size	FDR q-val
ORC1 REMOVAL FROM CHROMATIN	62	0.02
CELL CYCLE CHECKPOINTS	102	0.03
CDT1 ASSOCIATION WITH THE CDC6 ORC ORIGIN COMPLEX	51	0.04
CDC20 PHOSPHO APC MEDIATED DEGRADATION OF CYCLIN A	59	0.06
AUTODEGRADATION OF CDH1 BY CDH1 APC	53	0.07
CELL CYCLE MITOTIC	283	0.09
CD28 DEPENDENT PI3K AKT SIGNALING	17	0.11
PROTEASOME PATHWAY	19	0.11
p53-INDEPENDENT DNA DAMAGE RESPONSE	43	0.11
EIF PATHWAY	15	0.17
BASAL TRANSCRIPTION FACTORS	31	0.21
PEPTIDE CHAIN ELONGATION	83	0.23
GTP HYDROLYSIS AND JOINING OF THE 60S RIBOSOMAL SUBUNIT	103	0.23
RIBOSOME	85	0.24
VIRAL MRNA TRANSLATION	83	0.25
PROTEIN TRANSLATION	117	0.25

Table 4.3: GSEA pathways positively associated with PIM2 gene expression in primary ALL tumours

GSEA results were obtained from the re-analysis of gene expression data previously obtained in our lab [247], and derived from the analysis of 22 DMSO-treated primary ALL tumours. Tumours were ranked in order of low to high PIM2 expression.

Spearman's rank correlation coefficient was used to identify pathways and biological processes most strongly associated with PIM2 expression (coefficient ≥ 0.6; $p<0.05$).

The pathways listed above are significant at FDR <0.25 (<25%).

4.3. Cellular effects of K00135 on ALL tumour cells

4.3.1. Investigating the effect of K00135 on c-Myc expression

As mentioned in Chapter 1, the c-Myc transcription factor plays a major role in the regulation of cell proliferation, growth and differentiation, and is deregulated in a wide range of leukaemias and lymphomas [343]. Importantly, studies using bitransgenic mice overexpressing either *PIM1* or *PIM2* together with *MYC* led to mice succumbing to precursor B-cell lymphoma in a very short period of time, demonstrating the strong synergy between these two oncogenes [379]. Further studies showed that PIM2 greatly enhances c-Myc transcriptional activity by stabilising c-Myc protein via a mechanism involving phosphorylation of c-Myc Ser 329 [531].

Taking the above into consideration, together with the fact that PIM kinases have been shown to enhance BRD4-mediated gene transcription by forming a complex with c-Myc [346], I decided to use Western blotting to measure the level of c-Myc protein in K00135-sensitive ALL tumour cells and determine the effect of K00135-mediated PIM kinase inhibition on c-Myc expression. Using the K562 CML cell line as a positive control, I found that treatment of the PIM-expressing pre-B ALL cell line SD1 and K562 with 1 µM K00135 led to complete downregulation of c-Myc protein by 72 hours post-treatment in both cell lines (Figure 4.11a).

I next proceeded to test primary ALL tumour cells treated with K00135 for the effect on c-Myc expression, as was observed in cell lines. Thus, I selected primary ALL tumour ALL-103, which overexpressed PIM2 as shown in Figure

4.8. On this occasion, I used a range of increasing concentrations of K00135 inhibitor and a fixed 48 hour timepoint at which to harvest the cells, since primary tumour cells are less resilient than cell lines and exhibit a significant loss of viability at timepoints beyond 48 hours of *in vitro* culture. I found that ALL-103 primary tumour cells also overexpressed c-Myc protein, which was completely downregulated in tumour cells treated with 1 µM K00135 (Figure 4.11b).

In summary, similar to BET protein inhibition, treatment of PIM-expressing ALL tumour cells with the PIM inhibitor K00135 leads to a loss of c-Myc protein expression.

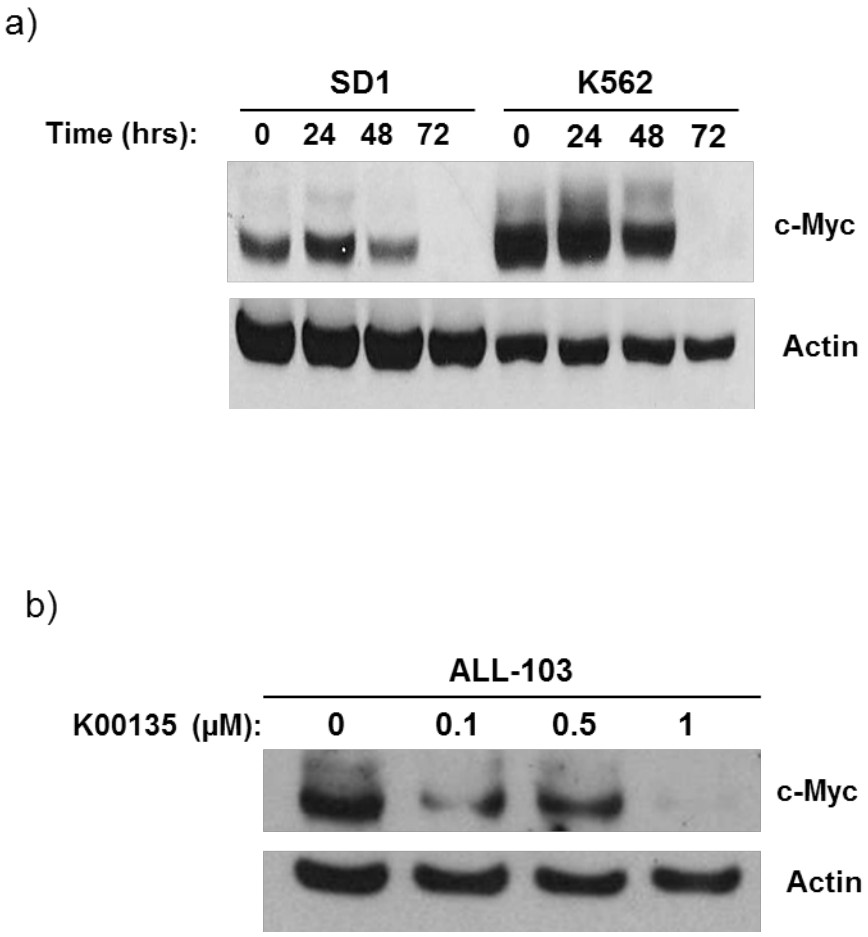


Figure 4.11: Myc downregulation in ALL tumour cells following K00135 treatment

Protein lysates were analysed by Western blotting, where samples were probed for c-Myc and β-actin (loading control).

a) SD1 and K562 cells were treated with 1μM K00135 for 0 (DMSO control), 24, 48 and 72hrs.

Treatment with 1μM K00135 leads to complete downregulation of c-Myc protein by 72hr in both leukaemia cell lines analysed. The K562 cell line was used as a positive control for PIM kinase inhibitor activity.

b) PIM2-expressing ALL-103 primary tumour cells were treated with increasing concentrations of K00135 inhibitor (0, 0.1, 0.5 and 1 μM).

Treatment of ALL-103 tumour cells with 1 μM K00135 leads to complete downregulation of c-Myc protein.

4.3.2. Effect of K00135 on apoptosis

As previously mentioned, PIM2 has been shown to inhibit apoptosis and caspase activation by phosphorylating the pro-apoptotic protein BAD on Ser112, confirming that PIM2 is a prosurvival kinase [380]. Since PIM kinases are upregulated by JAK/STAT and NFκB pathway signaling, and our group has previously shown that the NFκB pathway is frequently upregulated in apoptotic-resistant, I decided to test B-precursor ALL tumour cells (ALL-103) for an effect on apoptosis following treatment with the PIM kinase inhibitor K00135.

I did this by treating ALL-103 tumour cells with increasing concentrations of K00135 (0, 0.1, 0.5 and 1 μM) for 24 hours, and then using the Western blotting technique to probe for cleaved procaspase-7 and PARP1 protein as an indicator of apoptosis induction. I found that the degree of cleavage of these proteins increased in a dose-dependent manner, where treatment with 1 μM K00135 led to an almost complete cleavage of procaspase-7 and PARP1 proteins (Figure 4.12a).

Interestingly, I found that K00135 treatment of SD1 cells led to the cleavage of PARP1, which also coincided with the downregulation of proapoptotic BAD phosphorylation on Ser112 (Figure 4.12b). This observation was in line with a study that showed this effect in the FDC-P1 mouse myeloid cell line [380], but was an effect not yet reported within the context of ALL.

In summary, the above observations indicate that pharmacological inhibition of PIM2 leads to the induction of caspase-dependent apoptosis in primary ALL

tumour cells, and that this might involve the downregulation of Bad Ser112 phosphorylation.

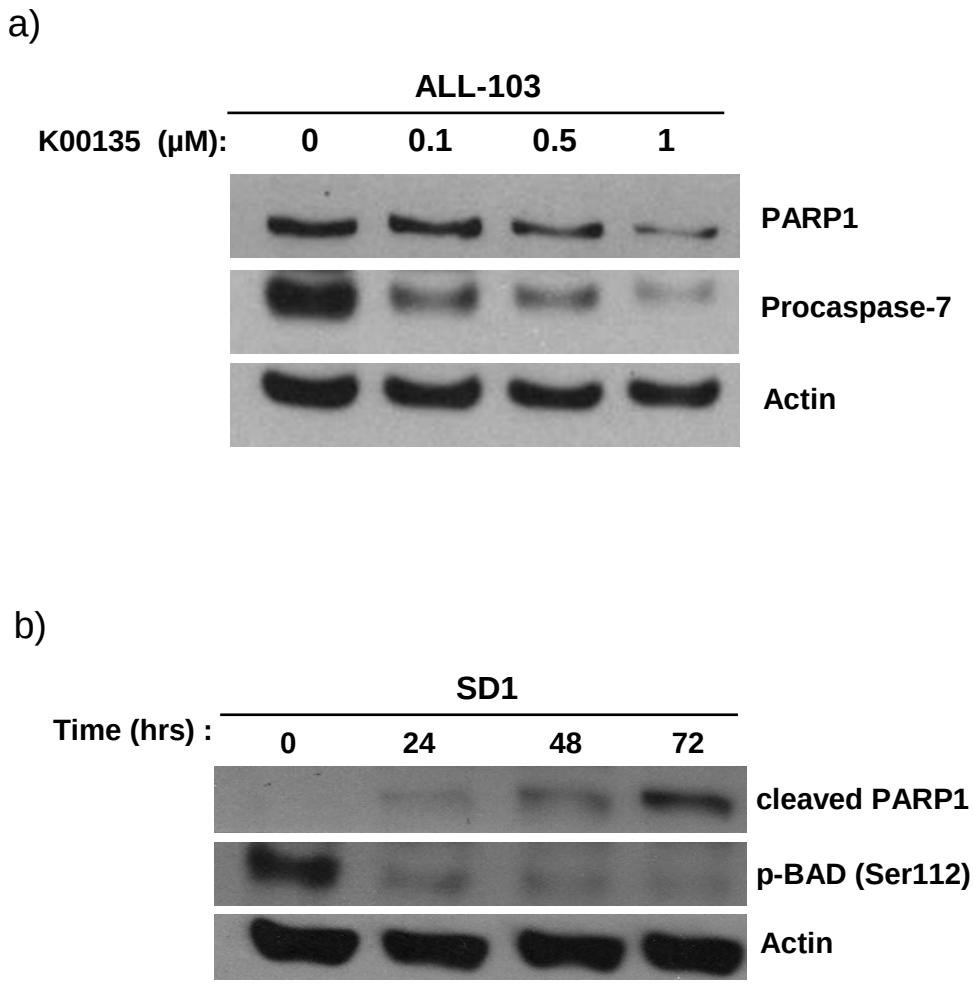


Figure 4.12: K00135 induces caspase-dependent apoptosis in ALL tumour cells

a) Western blotting shows dose-dependent induction of apoptosis in ALL-103 primary tumour cells treated with K00135 as measured by the cleavage of PARP1 and procaspase-7 proteins. Actin was probed for as a loading control.

b) Western blotting shows induction of apoptosis over a period of 72hr in SD1 cells exposed to 1 μM K00135 as measured by the cleavage of PARP1 and downregulation of proapoptotic BAD phosphorylation on Ser112. Actin was probed for as a loading control.

CHAPTER 5

DISCUSSION I:

Inhibition of BET proteins as an alternative strategy for the sensitisation of high-risk B-precursor ALL

5. DISCUSSION I: INHIBITION OF BET PROTEINS AS AN ALTERNATIVE STRATEGY FOR THE SENSITISATION OF HIGH-RISK B-PRECURSOR ALL

5.1 Treatment resistance in B precursor ALL and the requirement for more targeted treatments

As pointed out in the introduction, paediatric B precursor ALL is currently a highly curable disease, with long-term disease-free survival rates of over 80% being achieved in children [69]. However, despite major improvements in the treatment of this disease in previous years, treatment resistance in a significant number of patients together with general long-term toxic effects from currently used therapies are still observed [532]. Thus, alternative targeted therapeutic approaches are needed if we are to overcome these problems within the clinic.

In my thesis I have addressed regulators of transcription and epigenetic readers as therapeutic targets. The therapeutic potential of pharmacologic inhibition of bromodomains has only recently been explored in haematological malignancies such as AML, demonstrating striking anti-tumour effects [344]. In addition, although recently cytotoxic effects were demonstrated in ALL cell lines, this approach has not been tested in a wide range of primary ALL tumours and little is known of the cellular effects following bromodomain inhibition [345].

In this study, I investigated the inhibition of bromodomains in B-precursor ALL as a potential novel strategy for the sensitisation of primary ALL tumours,

including high-risk ALL tumours that are frequently associated with resistance to therapy. I utilised the thieno-triazolo-1,4-diazepine, JQ1, which binds specifically and competitively to the acetyllysine binding pocket of the conserved bromodomain and extra-terminal domain (BET) protein family (BRD2, BRD3, BRD4, BRDT). Data published by our collaborator Prof. Stefan Knapp shows that, of the BET protein family members, JQ1 binds to the bromodomains of BRD4 with the greatest affinity [334]. I investigated JQ1-mediated cytotoxicity and also attempted to characterise transcriptional changes and cellular effects in ALL tumour cells in response to JQ1 treatment. Finally, I addressed the efficacy of JQ1 in eliminating leukaemia cells using the *in vivo* ALL xenograft developed by our group.

5.2 A role for JQ1-mediated BET bromodomains inhibition in B precursor ALL

Previous findings have shown that BET proteins are frequently expressed in aggressive diseases such as AML and NUT midline carcinoma, and that treatment of these malignancies with the BET protein inhibitor JQ1 successfully leads to their elimination [334, 344]. Data presented in this study suggests that this may also be a promising avenue in the treatment of B-ALL. Results presented in Chapter 3 show that a wide range of ALL cell lines and primary tumours express the BET protein family member BRD4 and that tumour cells treated with JQ1 can be successfully sensitised irrespective of the cellular phenotype or patient risk stratification. Furthermore, all primary ALL tumours express BRD4 and the level of BRD4 expression does not appear to influence JQ1 sensitivity. Furthermore, BRD4 is not downregulated upon treatment with

JQ1. This latter observation is in keeping with the fact JQ1 does not alter transcriptional regulation of BRD4 but instead blocks any protein activity downstream of BRD4 [334]. Data in Chapter 3 also demonstrate that several high-risk primary ALL tumours are highly sensitive to JQ1 *in vitro* activity at submicromolar concentrations, as are ALL cell lines harbouring cytogenetic features associated with poor prognosis and aggressive disease such as BCR-ABL1. Indeed positive results were obtained testing JQ1 *in vivo* using a xenograft model of high-risk ALL, as is further discussed in section 3.4. Together these findings address the current complications associated with therapy-resistant high-risk ALL, and instil confidence in the further development of JQ1 as a compound with potential inclusion in clinical trials. At this point, it is not possible to infer whether or not the cytotoxic effects of JQ1 treatment observed in this study are a consequence of the sole inhibition of BRD4 since BET bromodomains exhibit high sequence conservation, and all inhibitors developed to date exhibit some degree of inhibitory activity toward other BET protein family members [334]. However, published data highlights the structural basis for potent and specific activity of JQ1 towards BRD4 in particular [334], and an RNA interference (RNAi) screen targeting multiple chromatin regulators in an AML mouse model also identified BRD4 as being key to disease maintenance [344]. This provides indirect evidence that the cytotoxic effects observed in JQ1-treated ALL tumour cells are primarily due to inhibition of BRD4 activity. Additionally, some crossover in inhibition may have a desirable effect on tumour cells, since other members of the BET family of proteins are also implicated in tumorigenesis [340, 533].

Results from *in vitro* cytotoxicity experiments show that ALL cell lines are all sensitive to the cytotoxic effects induced by JQ1. In contrast, primary ALL tumours treated with JQ1 showed a wide range of responses. Cell lines are immortal and continually proliferating unlike isolated primary tumour cells, and so it is possible that this is reflected in the degree of sensitivity to JQ1, since the cytotoxic effects of JQ1 have been shown to be partly due to its impact on cell cycle progression [340]. Primary ALL tumour cells obtained from patients' bone marrow do not proliferate in standard culture. Thus, it may be possible that those primary ALL tumours sensitised by JQ1 *in vitro*, respond as a result of alternative cellular effects exerted by JQ1 other than on the regulation of the cell cycle, which may not be as prominent. Therefore, a possibility remains that, if primary ALL tumour cells were induced to proliferate *in vitro,* they would show similar degrees of JQ1 sensitivity to the ALL cell lines. Another alternative explanation may be that tumour cells showing a lack of response to JQ1 are not dependent upon BET protein-regulated pathways for their survival. For example, primary AML tumour cells I tested were highly sensitive to JQ1 treatment, whereas healthy peripheral blood mononuclear cells (PBMCs) were not, further supporting the tumour-specificity of the JQ1 inhibitor and the unlikelihood of off-target effects leading to undesirable toxicity.

Treatment of ALL cell lines with a combination of JQ1 together with conventional chemotherapeutic agents dexamethasone, vincristine, or daunorubicin produced variable results, where the combination of JQ1 with dexamethasone led to greatly enhanced sensitivity in cell lines TOM-1 and NALM-6, which was in contrast to the response observed following treatment of

these cell lines with either JQ1/vincristine or JQ1/daunorubicin combinations. These synergistic effects of combined JQ1/dexamethasone treatment were also observed in several representative primary ALL tumours.

Microarray results presented in Chapter 3 demonstrate that JQ1-mediated inhibition of BET proteins leads to the downregulation of prosurvival pathways, including the NFκB pathway (section 3.2.4) previously reported by our group to be frequently upregulated in treatment-resistant preB-ALL [248]. Studies using a human lung carcinoma cell line have also reported that BRD4 binds to acetylated RelA, and coactivates the transcriptional activity of NFκB [338]. In addition, the mechanism of apoptosis-induction by dexamethasone could be partly explained by its ability to upregulate IκB; an inhibitory protein that sequesters the NFκB transcription factor in the cytoplasm, preventing the expression of NFκB target genes [534, 535]. It is therefore possible that the synergistic cytotoxic effects observed in ALL tumour cells treated with the JQ1/dexamethasone combination are due to modulation of the NFκB pathway by each of these drugs, in a disease where NFκB activity is frequently upregulated.

It may be possible that the lack of synergistic effects observed in ALL cell lines treated with JQ1 in combination with either vincristine or daunorubicin was due to the mechanism of cytotoxicity induction by both vincristine and daunorubicin being largely dependent upon proliferation, and may therefore be counteracted by JQ1 inhibitory activity on the cell cycle. However, this does not explain the similar absence of synergism observed in non-cycling primary tumours. Nevertheless, the JQ1/dexamethasone combination may have positive

implications in the clinic if the synergistic effects translate *in vivo*, since it may be possible to eliminate ALL blasts more effectively, as well as lower the dose of dexamethasone given to a patient, and therefore the risk of toxic side-effects.

At this point it is worth noting that response to glucocorticoids remains one of the most powerful predictors of outcome in ALL [536] and therefore, development of compounds such as JQ1 that can manipulate this effect is highly desirable.

Interestingly, dexamethasone failed to induce cytotoxicity in the REH cell line. Unsurprisingly, combined treatment with JQ1 did not exhibit synergistic cytotoxicity. This can be explained by the fact that REH does not express GR [407], which is required for dexamethasone to bind to in order for cytotoxicity to be induced. Limited evidence exists to suggest a GR-independent mechanism of action exists for dexamethasone, but this aspect remains largely inconclusive [537].

5.3 Microarray analysis reveals differentially expressed genes belonging to apoptotic, prosurvival, and protein translation pathways in JQ1-treated primary ALL tumours

Since results presented in section 3.2 show that ALL tumour cells respond well to the cytotoxic effects of JQ1, and JQ1 inhibits BET proteins that play a crucial role in the epigenetic control of gene expression, I decided genome-wide expression analysis using microarray technology would be the best approach to investigate transcriptional responses in a panel of primary ALL tumours following JQ1 treatment. Furthermore, the full set of genes which BRD4 regulates has not been thoroughly investigated, and has not been extensively looked at in primary ALL. A panel of primary tumours with a range of cytogenetic abnormalities and sensitivity to JQ1 were selected for inclusion in the microarray experiment, so that the transcriptional responses observed were not just characteristic of a particular cellular phenotype, but instead factored in the biological heterogeneity of ALL. Stringent statistical parameters were set to filter for the most significant and uniform differentials in gene expression across the ALL tumour panel. This, together with rigorous quality control of RNA and the experimental procedure, ensured that observed JQ1-induced expression changes were most likely to be genuine and reproducible.

Microarray data show that the *MYC* gene is significantly downregulated in primary ALL following JQ1 treatment (section 3.2). This is a very important result, since *MYC* plays a central role in cell cycle progression and survival [343, 412] and is one of the most deregulated oncogenes in cancer, including

several different haematological malignancies [343]. For these reasons, a drug that downregulates *MYC* expression has long been sought after in the treatment of *MYC*-expressing tumours. JQ1 appears to be a promising candidate to fulfil this task, since it not only downregulates MYC at the transcript level, but also shows striking downregulation of c-Myc protein (section 3.3).

Other genes found to be consistently downregulated by JQ1 activity include *SENP1, ALKBH8,* and *CARD6,* which are all associated with a role in tumour cell survival [420-422].

Results in Chapter 3 also show that 127 genes were significantly upregulated in primary ALL tumours following JQ1 treatment. These included genes with a role in the inhibition of the mTor growth-promoting signaling pathways (*EIF4EBP2*) [427] and mRNA translation (*PAIP2B)* [538], as well as enhancement of the apoptotic signaling (*PPP1R13B)* [539]. The JQ1-mediated upregulation of the *PPP1R13B* gene is particularly interesting from a clinical standpoint, since this gene encodes ASPP1 protein capable of stimulating the apoptotic function of the p53 tumour suppressor, but also triggering apoptosis independently of p53 via interactions with both p63 and p73 [539]; a feature of ASPP1 that could potentially be of use in the targeting of tumours harbouring mutant p53. Furthermore, decreased levels of ASPP1 have been shown to confer a poor prognosis in patients with ALL [424].

From the microarray data in this study, it is evident that the inhibition of BET proteins has great impact on the expression of genes involved in multiple cellular processes and signaling pathways. Since JQ1 has such a profound

cytotoxic effect on ALL cell lines and primary tumours *in vitro*, it is likely that tumour cell death is induced following JQ1-mediated modulation of transcription of genes involved in apoptosis, proliferation, survival and cellular growth. This is not surprising given that BRD4 recruits the positive transcription elongation factor b (P-TEFb) complex to gene promoters, leading to the phosphorylation of RNA polymerase II and stimulating transcriptional elongation [442, 540]. The fact JQ1 has such a broad effect on gene transcription in primary ALL may account for the multiple JQ1-induced cellular effects observed in this study (section 3.3).

GSEA results in (section 3.2.4) show that JQ1 activity causes significant downregulation of multiple prosurvival signaling pathways, including the NFκB pathway that is capable of exerting prosurvival effects and countering apoptotic signaling. NFkB target genes in this gene set that were differentially expressed in primary ALL tumours following JQ1 treatment included *IL7R*, *IL6, IL8,* and *STAT5A.* The impact JQ1 had on this pathway was of particular interest, since our group had previously reported that the NFκB pathway was upregulated in primary ALL tumours that were resistant to DNA damage-induced apoptosis *in vitro* [248].

GSEA results also indicate that JQ1 activity leads to the downregulation of the JAK-STAT pathway in primary ALL. This holds great potential in the treatment of particular subsets of ALL, such as BCR-ABL-positive ALL and BCR-ABL-like ALL, which are known to exhibit increased JAK-STAT pathway activation and

demonstrate poor treatment outcome [191]. Although the BCR-ABL1 oncogene itself activates the JAK-STAT pathway, almost half of all BCR-ABL-like ALL tumours that do not express BCR-ABL1 harbour rearrangements of the *CRLF2* gene that are frequently associated with activating mutations within *JAK* genes [190]; again demonstrating a poor response to treatment. Furthermore, approximately 10% of all cases of ALL harbour *JAK* mutations, some of which harbour *IKZF1* gene alterations that are associated with a poor clinical outcome [190].

Interestingly, upon consideration of GSEA results, it is possible that the observed downregulation of JAK-STAT and NFκB pathways might be largely attributable to the significant impact JQ1 has on the IL-7R gene, since the IL-7R belongs to the same signalling axis, and is an important upstream regulator of JAK-STAT and NFκB activity.

The Myc pathway was also shown to be downregulated by JQ1 activity, which coincided with the observation that the *MYC* gene itself is downregulated in JQ1-treated primary ALL tumours in this study. Indeed, results support the downregulation of the Myc pathway, since c-Myc protein is also completely downregulated. Since *MYC* is central to cell proliferation, an impact on the cell cycle of ALL tumour cells was predicted and confirmed in cell cycle analysis experiments (section 3.3.1).

Given that GSEA shows the ability of JQ1 to downregulate major prosurvival pathways in primary ALL tumours (section 3.2.4), a possibility remains that the primary tumours that failed to respond to JQ1 treatment in cytotoxicity

experiments were not driven by the same prosurvival pathways downregulated by JQ1.

Importantly, the fact that our group has previously reported the upregulation of prosurvival pathways in high-risk primary ALL tumours following DNA damage-induction [248], and that JQ1 is able to downregulate multiple prosurvival pathways in primary ALL tumours, including high-risk tumours, suggests BET protein inhibition may provide a solution to targeting these pathways, at least in MRD high-risk leukaemias. Additionally, crosstalk between prosurvival pathways that may confer resistance to chemotherapy in tumour cells [541], may be circumvented with this alternative approach of targeting proteins such as the BET family of proteins on a level at which many signaling pathways converge. This may also explain why JQ1-mediated inhibition of BET proteins leads to such broad cellular effects and sensitises ALL tumours with a range of phenotypes in this study. Importantly however, despite potent anti-tumour activity, some tumours still demonstrate resistance to JQ1. Although two potential biomarker genes of JQ1 sensitivity were identified (*PLAUR* and *REL*), further studies are required to confirm this in a wider range of paediatric leukaemias.

5.4 JQ1 activity induces multiple cellular effects on ALL tumour cells

Since Myc expression plays a major role in driving tumour growth in a wide range of cancers including leukaemias and lymphomas [343], a clinically viable

strategy to downregulate its expression has long been sought after. Previous studies have reported that downregulation of Myc and disruption of the c-Myc-induced transcriptional program constitutes one of the main mechanisms of cell-killing following the inhibition of BRD4 [333, 334, 340, 341]. In line with this, malignancies driven by c-Myc such as AML, NMC and multiple myeloma, are sensitised by JQ1-mediated BRD4 inhibition through displacement of BRD4 protein from the *MYC* gene promoter [334, 344, 488].

Similar to previous reports, results in Chapter 3 showed that JQ1 led to uniform downregulation of c-Myc protein in all ALL tumour cells, as well as the downregulation of c-Myc target genes and cell cycle arrest. However, in contrast to previous findings, only transient reductions in *MYC* mRNA were observed by qRT-PCR in this study. This suggests that BRD4 influences c-Myc protein stability, which is in keeping with reports that implicate BRD4 in the regulation of protein stability of the papillomavirus-encoded E2 protein [448]. Indeed, this was later confirmed in experiments where ALL tumour cells were exposed to JQ1 in the presence or absence of the 28S proteasome inhibitor MG132, with MG132 coincubation completely abolishing JQ1-induced loss of c-Myc expression (section 3.3.1). This observation highlights a potential role for BRD4 in the regulation of cellular proteins at the post-translational level, as well as the transcriptional level.

The question of whether or not the cytotoxic effects induced by JQ1 can be entirely attributed to c-Myc downregulation remains debateable. Several studies suggest that this is not the case, since ectopic expression of *MYC* was shown to

be unable to prevent JQ1-induced cell death [344], and resistance to JQ1 can occur despite c-Myc downregulation [333].

Results presented in Chapter 3 support the notion that BRD4 inhibition exerts an anti-tumour effect by different mechanisms and that the cytotoxic effects following BRD4 inhibition expand beyond c-Myc downregulation. Apart from results that showed JQ1 leads to substantial downregulation of prosurvival signaling pathways in non-cycling primary ALL tumour cells (an effect not previously reported in other malignancies), JQ1-induced cellular effects other than c-Myc downregulation included the perturbation of the DNA replication process.

In section 3.3.2 results showed that replication fork progression rates were reduced in S phase ALL tumours cells following treatment with JQ1. This coincided with a dramatic increase in stalled replication forks. Taken together, these observations suggest that JQ1-mediated BRD4 inhibition has a direct role in the induction of replicative stress.

Reduced fork progression rates observed after 24 hour exposure of ALL tumour cells to JQ1, were also observed at the shorter exposure of just 1 hour. This suggests that the reduction in fork progression rates is a direct effect induced by BRD4 inhibition and not an indirect effect caused by inhibition of the cell cycle. This was also supported by BrdU incorporation experiments (section 3.3.2) that showed that JQ1 led to a decrease in the proportion of S phase cells and did not affect S phase entry, suggesting a direct effect on the DNA replication process.

In section 3.3.2 JQ1 treatment led to an increase in the proportion of first label termination structures and not second label terminations. This confirms that the replication inhibitory effect induced by JQ1 is in fact due to fork-stalling and not an overall increase in terminations (i.e. successfully completed replication of DNA). This effect is supported by the fact that other drugs, such as etoposide, also induce fork-stalling (Peterman, unpublished observation).

A role for BET proteins in replication is not unlikely, especially since BRD4 has been reported to interact with the RFC complex essential for DNA replication [443], and inhibition of BRD4 led to changes in expression of the gene encoding another key regulator of DNA replication, Claspin, as observed in the microarray experiment (section 3.2.2).

Although further investigation is warranted, a possible explanation for the defective replication induced by JQ1 is that loaded transcription complexes are not completely processed once BRD4 is inhibited, thus preventing transcript elongation and presenting a physical obstruction to faster proceeding replication machinery. Although little is known regarding collisions between transcription and replication machinery, there is evidence in the literature to suggest this phenomenon is not uncommon [542].

Results in (section 3.3.3) show that JQ1 induces the cleavage of proteins characteristic of apoptosis. Additionally, the onset of apoptosis appeared to mirror the degree of JQ1 sensitivity of ALL tumour cells in cytotoxicity experiments. This suggests that the mechanism of cell killing induced by JQ1 is indeed via programmed cell death, which is corroborated by results in previous

studies showing the induction of apoptosis following BRD4 inhibition in AML [344] and NMC [334].

In section 3.3.3 I showed that JQ1 treatment of ALL tumour cells did not induce the upregulation of p53, unlike treatment with the chemotherapeutic agent daunorubicin. This indicates that JQ1 does not cause the activation of the DNA damage response pathways, and that the induction of apoptosis is via a p53-independent mechanism. To test this hypothesis, I probed for common anti-apoptotic proteins associated with many different types of cancer and checked to see if JQ1 induced any changes in expression. Results in showed a dramatic loss in expression of the anti-apoptotic protein survivin, suggesting that the mechanism of apoptosis induction by JQ1 involves the downregulation of survivin. The fact that an anti-apoptotic protein such as survivin plays a role in JQ1-induced apoptosis is not surprising since anti-apoptotic proteins are commonly overexpressed in Myc-activated tumour cells, to inhibit the proapoptotic functions of Myc [447]. However, since microarray results did not highlight the *BIRC5* gene that encodes survivin protein as being differentially expressed after JQ1 treatment, I decided to check whether or not survivin was being regulated by JQ1 at the post-translational level. Indeed results in section 3.3.3 showed that this was the case, since co-incubation of ALL tumour cells with JQ1 together with the 28S proteasome inhibitor MG132 led to complete abrogation of the ability of JQ1 to downregulate survivin protein expression; an effect also observed for c-Myc protein. These observations suggest that BET proteins regulate the stability of oncogenic proteins.

Additionally, results in this study show that JQ1 induces apoptosis independent of p53. Although mutations of the p53 gene in precursor-B-cell ALL are rare and occur at a frequency of approximately 2% [248, 543], p53 mutant ALL still holds significance since alterations of the p53 gene can confer resistance to treatment and a poor outcome [248, 544]. Thus, it is possible that JQ1 may prove to be beneficial in the treatment of patients with p53 mutant ALL.

5.5 JQ1 prevents tumour growth in an ALL xenograft model and targets different ALL progenitors

Results in section 3.4, involving the use of a murine xenograft model of an apoptotic-resistant B precursor ALL with high-risk MRD (ALL-105), showed that JQ1 potently inhibited the growth of xenografted primary leukaemia cells *in vivo,* when compared to vehicle-treated animals. Results also show a dramatic reduction in tumour growth in JQ1-treated mice with a subcutaneous engraftment of NALM-6 cells, when compared to those treated with vehicle. This suggests that JQ1 exhibits cytotoxic effects on ALL tumour cells both *in vitro* and *in vivo*, which is in agreement with the *in vivo* JQ1-mediated anti-tumour effects observed in other studies using xenograft models of AML [344] and NMC [334]. Additionally, JQ1 targeted all the progenitor subpopulations that constituted the engrafted leukaemia and had the capacity to proliferate *in vivo*, with a slight increase being observed in the proportion of the CD34-CD19+CD10- ALL subset. This suggests that JQ1 is able to target ALL progenitor cells regardless of their stage of differentiation, and potentially cause a shift towards more mature cells. The ability for JQ1 to induce differentiation of leukaemia progenitors has been confirmed in studies that show JQ1-treatment

of AML blasts leads to maturation via a JQ1-mediated impact on the transcriptional programme that controls myeloid differentiation [340, 344].

5.6. Summary I: BET protein inhibition as a novel therapeutic strategy to sensitise apoptosis-resistant B-precursor ALL

- In this study, I have confirmed that the BET protein BRD4 is widely expressed in ALL tumour cells, and that the pharmacological inhibitor JQ1 can successfully target BET proteins to induce potent cytotoxic effects *in vitro,* irrespective of the cellular phenotype.

- The tumour-specificity of JQ1 was supported by the fact that primary AML tumours were highly sensitive to JQ1 in this study (an observation in line with previous reports [344]), and that healthy PBMCs did not exhibit significant cytotoxic effects following JQ1 treatment.

- Furthermore, I showed that JQ1 acts synergistically with dexamethasone, suggesting this drug combination may allow for the lowering of dexamethasone doses administered to ALL patients, and therefore reduce the toxic side-effects associated with its use.

- Using microarray technology, I have elucidated the transcriptional effects of JQ1-mediated BET protein inhibition in a cohort of ALL primary tumours.

- I have shown that JQ1 activity leads to the downregulation of major prosurvival signaling pathways that include the NFκB pathway, which our lab previously confirmed to be upregulated in high-risk ALL tumours

[248], and the JAK-STAT pathway, which is a prosurvival pathway commonly activated in the tumour cells of high-risk ALL patients with a poor treatment outcome [190, 191].

- I have also shown that JQ1 activity leads to downregulated expression of genes belonging to multiple cytokine signaling pathways that are often deregulated in haematological malignancies and are known to drive tumour growth and proliferation [545-548].

- I confirmed that c-Myc and its associated target genes are also downregulated by JQ1. However, I have shown that this was an effect caused primarily by Myc downregulation at the post-translational level, rather than at the transcriptional level, highlighting a role for BET proteins in the modulation of c-Myc protein stability that has not previously been reported.

- The impact of JQ1 on c-Myc expression is of great importance, since c-Myc potently drives tumorigenesis and is frequently overexpressed in many cancers, including haematological malignancies [343]. Thus, an effective therapeutic approach towards the downregulation of c-Myc has long been sought after, and I show that BET protein inhibition using the JQ1 inhibitor provides a means by which to achieve this.

- I have also shown that downregulation of c-Myc coincides with the induction of G1 arrest in actively proliferating ALL tumour cells treated with JQ1, together with a substantial reduction of the proportion of S phase cells.

- I have subsequently confirmed that inhibition of BET proteins has a direct impact on DNA replication, seen as a reduction in fork progression rates and an increase in replication fork stalling.

- I have confirmed that the mechanism of JQ1-induced cell killing is mediated via a p53-independent mechanism that involves the downregulation of the anti-apoptotic protein survivin, and that like c-Myc, survivin is regulated by BET proteins at the post-translational level; most likely by BRD4.

- The most striking results in this study were those obtained from animal studies. I showed that JQ1 was capable of inhibiting the proliferation of ALL xenografts *in vivo,* including a xenograft model of a high-risk ALL patient our lab had previously shown to be resistant to DNA damage-induced apoptosis. Importantly, JQ1 targets both immature and mature ALL subpopulations, reducing the likelihood of treatment resistance as a result of the re-expansion of stem-like populations. Furthermore, during the writing of this thesis, with the assistance of Tracey Perry, additional results have been obtained by our group using our ALL mouse xenograft model, which show that high-risk primary ALL tumours are highly sensitised to dexamethasone when co-treated with JQ1 (personal communication).

5.7. Conclusion I

Overall, results from this study support the rationale for the targeting the BET proteins, including BRD4, in B-precursor ALL using JQ1, which is capable of sensitising high-risk ALL tumours both *in vitro* and *in vivo.* This suggests that treatment involving JQ1 may be a clinically viable option for the treatment of high-risk ALL patients that respond poorly to conventional therapies. The fact that this study and several others have successfully demonstrated potent JQ1-mediated anti-tumour activity *in vivo*, in which the JQ1 inhibitor was well tolerated by its recipient host and targeted tumours with great specificity, holds promise for the further development of this compound. Importantly, it should be relatively straightforward to include JQ1 into the current UK ALL clinical trial by further stratifying enrolled dexamethasone-resistant patients into two subgroups; one group receiving dexamethasone only, and the other receiving a JQ1/dexamethasone drug combination.

Altogether, this study justifies further work in this area of research to aid the design of the first clinical trial based on BET inhibition in paediatric ALL.

CHAPTER 6

DISCUSSION II:

The PIM-2 kinase as a suitable candidate for further study

6. DISCUSSION II: THE PIM-2 KINASE AS A SUITABLE CANDIDATE FOR FURTHER STUDY

6.1. Evidence that the prosurvival PIM2 kinase is overexpressed in B-precursor ALL and is an ideal candidate for further study

As described in section 1.10.3 the PIM kinases are overexpressed in a wide range of haematological malignancies and solid tumours, and have been shown to confer prosurvival activity by counteracting the increased sensitivity to the induction of apoptosis that is observed in tumours overexpressing c-Myc [356]. Furthermore, previous studies have confirmed that PIM1, and possibly other PIM proteins, play a key role in gene transcription by both regulating Myc-transcriptional activity, and promoting the binding of the BET protein BRD4 to histone acetyllysine residues, which in turn recruits the P-TEFb complex required for transcript elongation [386, 388].

In an attempt to prove my main hypothesis that primary B-precursor ALL can be sensitised following inhibition of oncogenic transcriptional activity, I previously showed that targeting BET proteins is an effective approach by which to do so.

Here, I discuss an additional approach involving the targeting of PIM2, and evaluate preliminary evidence to support PIM2 as a promising additional target for the sensitisation of primary ALL, and therefore as an ideal candidate for further study.

The first step in investigating the feasibility of this strategy was to determine whether PIM kinases were overexpressed in B-precursor ALL. I needed to establish a suitable method by which to measure PIM expression, and proceeded to indirectly quantify PIM protein levels by measuring PIM mRNA using qRT-PCR. The qRT-PCR technique was an ideal method by which to determine the frequency of PIM overexpression, since I had access to a large cohort of 68 primary ALL RNA samples. I considered the use of this method on the basis that PIM2, and in some cases PIM1, is constitutively active once expressed [385] and no post-translational modifications of the PIM2 kinase have been reported.

I found that PIM2 was significantly overexpressed in primary ALL tumours at a frequency of 25% relative to the expression levels in the K562 leukaemia cell line, whilst observing a similar proportion of tumours expressing PIM2 protein in a smaller cohort of primary ALL tumours I had analysed by Western blotting. This was unsurprising, since PIM genes are confirmed to be upregulated by activated STAT transcription factors that are downstream of prosurvival signaling pathways, such as the JAK-STAT and NFκB pathways [369], which our group has previously shown to be upregulated in primary ALL [248].

In addition, I observed a trend towards a positive association between PIM2 expression in primary ALL tumours and the presence of MRD at Day 28 in the corresponding patients from which the tumours were obtained. However, although this result may suggest a role for PIM2 in chemoresistance *in vivo,* this has to be considered with caution, as I did not have access to MRD clinical data for all the primary ALL tumours I screened. Thus, it would be necessary to

increase the number of ALL tumours in the PIM screen for which we have full clinical information in future studies.

Overall, my observations that PIM2 is significantly overexpressed in ALL and might play a role in chemoresistance *in vivo*, together with the already reported prosurvival functions of PIM2 in other haematological malignancies, support my rationale for proceeding with investigations into the role of PIM2 in B-precursor ALL.

6.2. Evidence to support PIM kinases can be targeted in B-precursor ALL

I previously provided evidence to support that PIM2 is overexpressed in pre-B ALL and so I next wanted to determine whether PIM2 could be specifically targeted in order to sensitise primary ALL tumours.

Since PIM kinases are also involved in normal haematopoiesis, it was reassuring to find data in the literature from studies using compound PIM1, PIM2 and PIM3 knockout mice that showed these animals were still viable and fertile [362]. Despite exhibiting a reduced body size at birth, the results from this study suggest that PIM kinases are important to mammalian development but are kinases mediating growth factor signaling that can be inhibited and not cause cytotoxicity to healthy developing lymphocytes [362].

I therefore proceeded to test ALL tumour cells with a pharmacological inhibitor of PIM kinases, K00135, which was obtained from our collaborator Prof. Stefan Knapp and had shown encouraging anti-tumour activity in PIM-expressing primary AML tumours [391]. In K00135-treated leukaemia cell lines, I failed to

see a clear correlation between PIM expression and induction of cytotoxicity, since cell lines not expressing any detectable PIM kinase protein (for example, REH and Jurkat) were sensitised in addition to the PIM-expressing ALL cell line SD1.

This suggests that K00135-induced cytotoxicity observed in the leukaemia cell lines not expressing PIM protein may be due to off-target effects, despite the K00135 inhibitor having been shown to exert selective antileukaemic activity in other studies involving the use of several AML cell lines and primary AML tumours [391]. However, published structural data, involving a screen of K00135 against a panel of 50 kinase catalytic domains, confirms that this inhibitor cross-reacts with one additional kinase, namely Cdc-like kinase 1 (CLK1) [391].

The possibility that CLK1 inhibition leads to cytotoxic effects in the ALL cell lines I tested that do not express PIM kinases remains to be investigated. Interestingly, although the cellular functions of CLK1 are not yet fully understood, studies have shown that CLK1 plays an important, evolutionarily conserved role in pre-mRNA splicing; a process associated with increased protein expression and often deregulated in cancers to promote growth and survival [549].

However, K00135-mediated cytotoxic effects in primary ALL tumours appeared to positively correlate with PIM2 expression with no significant cytotoxic effects being observed in healthy PBMCs.

I also noticed that there were some instances where tumours expressed PIM2 and did not respond to K00135 treatment, as opposed to PIM2 negative tumours that were sensitised by K00135 treatment. This suggests that treatment with K00135 targets PIM-expressing ALL tumours with some degree of specificity, and that some tumours may be resistant to the effects of K00135-mediated PIM inhibition.

6.3. Cellular effects of K00135 on PIM-expressing B-precursor ALL tumour cells involves downregulation of c-Myc protein and induction of apoptosis

The PIM2 kinase has been show to efficiently stabilise c-Myc protein in a range of cell lines derived from human solid tumours by phosphorylating c-Myc at Ser329, thus decreasing c-Myc transcriptional activity [531]. Given that c-Myc has been reported to regulate transcription by forming a complex with PIM1, and possibly other PIM family members, to promote BRD4-mediated transcript elongation [386-388], I decided to investigate whether c-Myc would be downregulated in PIM-expressing ALL tumour cells following treatment with K00135, as the PIM2/c-Myc interaction may be an important mechanism through which PIM2 is able to regulate gene transcription in ALL.

Indeed, I found that K00135 treatment of both leukaemia cell lines and primary ALL tumours expressing PIM2 led to complete downregulation of c-Myc protein. Further investigation, perhaps in experiments involving the use of the proteasomal inhibitor MG132, as well as measurement of MYC expression before and after K00135 treatment, will be required to confirm that regulation of

c-Myc by PIM in ALL is at the post-translational level. Since my GSEA data shows that upregulation of the MYC signaling pathway was not associated with ALL tumours expressing PIM2, it is possible that c-Myc is regulated at the protein level.

I found that K00135 induced caspase-dependent apoptosis in primary ALL tumours (ALL-103) and that this coincided with the induction of cytotoxicity observed in previous experiments. As studies have suggested that the oncogenic properties of PIM2 are partly dependent upon its regulation of prosurvival signaling [359], and PIM2 is reported to predominantly phosphorylate (and therefore inactivate) the Ser112 residue of proapoptotic BAD [380], I decided to test the effect of K00135 treatment on BAD phosphorylation in SD1 cells. Subsequently, I found that K00135 led to a decrease in BAD Ser112, suggesting this may be a mechanism by which PIM kinases are able to promote survival in ALL.

Intriguingly, studies have confirmed that the Akt kinase of the PI3K prosurvival signaling pathway shares several overlapping substrates with PIM2, including proapoptotic BAD (both kinases phosphorylate BAD Ser112), and the translational repressor 4EBP1 [359]. However, PIM2-mediated survival was shown to be distinctly independent of Akt signaling, since the overexpression of PIM2 in haematopoietic cell lines conferred growth factor-independent survival that could not be inhibited by rapamycin; an inhibitor of the mammalian target of rapamycin (mTOR) kinase found downstream of Akt [385].

Taken together, the effects of K00135 on c-Myc protein expression, and the phosphorylation of BAD in ALL tumour cells, indicate that the K00135 inhibitor is likely to be targeting PIM protein and that doing so sensitises ALL. Furthermore, it is possible that the observed complete downregulation of the c-Myc transcription factor has an impact on gene expression as a consequence.

6.4. Patterns of gene transcription associated with PIM2 expression in B-precursor ALL

As mentioned, PIM kinases regulate gene transcription by forming a complex with c-Myc and promoting the binding of BRD4 to histones, which in turn recruits the P-TEFb complex [388]. The P-TEFb complex then phosphorylates RNA pol II at Ser2 leading to the elongation and subsequent termination of transcripts at promoter-proximal pause sites [337, 388]. Furthermore, I showed that treatment of SD1 cells with K00135 led to a decrease in RNA Pol II Ser2 phosphorylation, hinting at a possible role for PIM kinases regulating gene transcription in ALL.

To gain some insight into the genes and pathways PIM2 may regulate, I reanalysed microarray data previously obtained in our lab, which was derived from an independent cohort of 22 primary ALL tumours. I arranged the tumours in order of low to high basal PIM2 expression, and then identified the most significant genes associated with PIM2 expression.

I took this approach as the K00135 inhibitor was not sufficiently specific to produce reliable microarray data, and an alternative, more specific PIM kinase inhibitor was not available. Furthermore I had also previously made multiple

attempts to generate stable PIM2 knock-down (KD) ALL cell lines using both lipid-based transfection and lentiviral transduction methods to introduce a short hairpin RNA (shRNA) to silence the PIM2 gene in ALL tumour cells. However, I was unsuccessful in doing so, despite having successfully cloned the PIM2 gene into a lentiviral expression construct with the help of Dr. Phil Byrd.

For the above reasons I proceeded with reanalysis of existing microarray data, which had its limitations as I was unable to confirm whether individual genes and pathways associated with PIM2 expression in primary ALL tumours were upstream or downstream of PIM2.

Nevertheless, my approach produced interesting results that in many cases I could relate to existing findings reported in the literature, as well as other novel roles can be further investigated and confirmed in future. For example, I found that multiple NFκB target genes (for example *XIAP, TNFAIP3*, *NLRP3* and *CD48*), which regulate NFκB-mediated survival and anti-apoptotic signaling, were associated with PIM2 expression.

Interestingly, limited evidence derived from studies using the FL5.12 murine pro-B cell line data exists to support PIM2 being an upstream activator of NFκB, where PIM2 was shown to phosphorylate the oncogenic serine/threonine kinase, Cot, which in turn phosphorylates IκB [369]. IκB then releases NFκB dimers, which localise to the nucleus and activate gene transcription [369]. Thus, it remains to be confirmed whether inhibition of PIM2 activity downregulates NFκB activation.

In addition, previous studies have shown that PIM2 is upregulated following the induction of DNA DSBs during V(D)J recombination in developing B-lymphocytes [550]. This supports my GSEA analysis data, where I observed an association between the upregulation of the DNA damage-response pathway and the expression of PIM2 in primary ALL tumours. The fact that this pathway is upregulated at basal levels in untreated primary tumours, could mean that tumours expressing high levels of PIM2 exhibit a greater level of endogenous DNA damage due to deregulated proliferation [551]. It would be interesting to test whether PIM-expressing ALL tumours, including PIM2-expressing tumours resistant to DSB-inducing chemotherapeutic drugs, can be sensitised by co-incubation of a PIM inhibitor with a cytotoxic agent such as doxorubicin.

Importantly, the most noticeable pathways and individual genes to be closely associated with high and low PIM2 expression were those belonging to cellular processes such as gene transcription (basal transcription factors), protein translation (mRNA translation, peptide chain elongation, ribosome metabolism), and DNA replication (regulation of origin recognition complexes). These processes were highly represented in GSEA analysis results, suggesting that PIM2 may play a key role in maintaining these processes in B-precursor ALL.

The above observations are supported by aforementioned previous studies that show PIM1 enhances BRD4-mediated gene transcription [386, 388]. Furthermore, my observations from GSEA results that PIM2 expression is associated with protein translation, is supported by studies showing that PIM2 activity leads to the phosphorylation and release of 4E-BP1 from EIF4E, which are key regulator proteins of the Cap-dependent translation pathway [346, 383,

385]. Ultimately, this allows for the recruitment of ribosomes and translation machinery to the ‘capped’ mRNA, and for the synthesis of oncogenic proteins such as c-Myc and Mcl-1 to take place [552].

However, very limited evidence exists in the literature to suggest PIM kinases regulate the DNA replication process. Intriguingly, PIM1 has been shown to bind and phosphorylate the latency-associated nuclear antigen 1(LANA-1), which is a protein essential for the maintenance of Kaposi's sarcoma herpes virus (KSHV) latency, resulting in the activation of viral replication [553]. Thus, it is possible that PIM2 regulates DNA replication in ALL, given that GSEA in this study showed its expression was also associated with pathways regulating DNA replication. As a side note, studies have shown that the BET proteins BRD4 and BRD2 also interact with LANA-1 to activate KSHV replication [554], suggesting overlapping activity between PIM and BET proteins; a feature I have noticed on various occasions throughout my studies.

6.5. Evidence to suggest overlapping functions between PIM and BET proteins

Previous studies have shown that PIM kinases enhance BRD4 activity, and thus promote transcript elongation [386, 388]. In this study I confirmed that inhibition of BET proteins leads to the transcriptional downregulation of genes involved in prosurvival signaling. Furthermore, I showed that the targeting of both these oncogenic proteins leads to the sensitisation of B-ALL tumour cells to apoptosis.

Taken together, it appears that a close relationship may exist between PIM proteins and BET proteins that promote tumorigenesis and chemoresistance in ALL.

Here, I summarise observations that lend additional support to the existence of overlapping activity between PIM and BET proteins.

Firstly, my observation that PIM2 expression is significantly and differentially upregulated in JQ1-resistant primary ALL tumours following treatment with JQ1, suggests that PIM2 may act as a compensatory pathway to help maintain survival and anti-apoptotic signaling. When highly specific PIM2 inhibitors become available, it will be interesting to determine whether JQ1-resisitant tumours also expressing PIM2 can be sensitised to JQ1 activity following co-incubation with a PIM kinase inhibitor.

However, although cytotoxicity could be induced in most primary ALL tumours following JQ1 treatment, I noticed that the JQ1-resistant primary ALL tumour ALL-103, expressing both PIM and BRD4, could be successfully sensitised to the cytotoxic effects of K00135 instead. In addition, primary tumours ALL-105 and ALL-113, which were resistant to K00135 treatment, were successfully sensitised following exposure to JQ1. Taken together, this suggests that in some cases JQ1-resistance and K00135-resistance can be circumvented by targeting PIM kinases or BET proteins, respectively, and thus demonstrates the benefits of being able to target both PIM kinases and BET proteins in B-precursor ALL.

In addition, I confirmed that treatment of ALL tumour cells with either PIM kinase inhibitor K00135 or the BET protein inhibitor JQ1 leads to complete downregulation of c-Myc protein. This highlights a further overlap in function between PIM and BET proteins that may promote tumorigenesis through the stabilisation of c-Myc; an oncogenic kinase known to be frequently overexpressed in haematological malignancies and potently drive cell proliferation [343].

With respect to gene expression, I also observed a similarity in the genes that appeared to be regulated by PIM kinases and BET proteins. For example, I found that BET protein activity was associated with the regulation of *TMEM71, TMEM156, IL2RA* and *XIAP* genes (which are all upregulated by JAK-STAT pathway activity), whereas PIM2 expression was associated with the upregulation of *TMEM14B, IL2RG,* and *XIAP* genes. This suggests PIM and BET proteins may co-operate in promoting tumorigenesis and may confer resistance to either K00135 or JQ1 by independently regulating similar genes.

Taken together, these observations and other previously reported findings lend support to suggest PIM kinase and BET proteins may be cooperating together to maintain ALL, and may be doing so via the regulation of proliferation, survival signaling, and inhibition of apoptosis.

6.6. Summary II: Selection of the PIM2 kinase as a candidate therapeutic target for further study

- In this study, I have validated the qRT-PCR technique as an efficient and reproducible method by which to indirectly screen ALL tumours for PIM2 protein expression, since I confirmed that PIM2 mRNA closely correlates with PIM2 protein expression levels.

- Subsequently, I screened a large cohort of primary ALL tumours (n=68) and determined the frequency of PIM2 overexpression in B-precursor ALL as being 25%.

- *In vitro* cytotoxicity data derived using leukaemia cell lines were largely inconclusive. However, in contrast to PBMCs, primary ALL tumours were sensitised to the cytotoxic effects of treatment with the PIM kinase inhibitor K00135. Furthermore, a subset of primary tumours appeared to be resistant to PIM-inhibition.

- Re-analysis of microarray data derived from an independent cohort of primary ALL tumours revealed that PIM2 expression was significantly associated with the upregulation of genes involved in NFKB and PI3K prosurvival signaling pathways, gene transcription, cell cycle progression, DNA replication, ribosome metabolism and protein translation. In addition, multiple DNA repair genes were negatively associated with PIM2 expression.

- I confirmed that K00135 treatment of ALL cell lines and primary tumours led to complete downregulation of c-Myc protein.

- I also confirmed that exposure of ALL tumour cells to K00135 led to the induction of caspase-dependent apoptosis, which coincided with a decrease in BAD Ser112 phosphorylation; a post-translational modification known to inactivate this pro-apoptotic protein [380].

- Finally, several observations in this study hint toward a close relationship between PIM2 and BET proteins that require further investigation.

6.7. Conclusion II

Overall, the preliminary results from experiments investigating PIM kinases confirm that PIM2 is significantly overexpressed in B-precursor ALL, and can be targeted to induce ALL tumour cell death. Ultimately, the data presented in this thesis have initiated several lines of investigation, and support the rationale for the selection of PIM2 as a candidate for further study, and for its validation as a novel therapeutic target in B-precursor ALL in addition to BET proteins.

CHAPTER 7

FUTURE WORK

7. FUTURE WORK

7.1 Extension of ALL tumour cohort

To further evaluate my hypothesis that targeting BET proteins, such as BRD4, is an alternative strategy to the sensitisation of primary ALL tumours, including apoptotic-resistant high-risk tumours, it will be important to extend the ALL tumour cohort analysed in this study. Our lab has previously reported an association between ALL apoptotic-resistance *in vitro* and high-risk or relapsed ALL [247]. Since the data presented in this thesis confirms that BRD4 is widely expressed in ALL, independent of the phenotype, and that JQ1-mediated inhibition of BET proteins effectively induces a cytotoxic response in both apoptosis-sensitive and apoptosis-resistant primary ALL tumours, it will be interesting to find out how genetically diverse, refractory ALL tumours respond to BET inhibition. This would be highly relevant work, since an estimated 30% of ALL patients treated at Birmingham Children's Hospital are diagnosed with either high-risk or relapsed disease (personal communication with Dr. Shaun Wilson).

Refractory ALL tumours can be tested *in vitro* using the luminescent ATP-based cytotoxicity assay described in this study, as it is efficient and produces consistent results. Refractory ALL tumours can be treated with JQ1, and other highly specific BET inhibitors currently under development by our collaborator Prof. Stefan Knapp, in order to further validate that targeting BET proteins is a highly effective, novel approach to sensitising chemoresistant ALL tumours.

Given the range of *in vitro* responses of primary ALL tumours to JQ1 it will be important to determine whether or not the different responses are in part due to different rates of cycling among individual tumours. Since primary tumours are predominantly non-cycling *in vitro* and results in section 3.1.2 indicate that tumours most resistant to JQ1 are those with upregulated expression of the *CDKN1A* and *GADD45A* genes involved in cell cycle arrest, it would be ideal to compare the cytotoxic effects in non-cycling primary ALL cells with primary ALL cells that have been induced to cycle. This would be possible using a culture system containing CD40L, IL-3, IL-7, IL-10 and Flt3L previously established in our lab [555].

7.2 Investigation of the synergy between JQ1 and dexamethasone *in vitro*

Results in this study show that JQ1 confers synergistic effects when combined with dexamethasone, as demonstrated by *in vitro* data that shows both ALL cell lines and primary tumours are sensitised to dexamethasone using this drug combination (section 3.1.3). Since dexamethasone is a key component of current ALL treatment protocols and sensitivity to glucocorticoid treatment *in vivo* is a major prognostic factor in B precursor ALL [556], it will be important to elucidate the mechanisms by which JQ1 is able to potentiate the cytotoxic effect of dexamethasone as this would allow for the identification of tumours that are most likely to benefit from addition of BET inhibitors to standard ALL protocols. The mechanisms behind such synergy can be investigated by first testing for *in vitro* synergistic cytotoxic effects of combined JQ1/dexamethasone in dexamethasone-resistant ALL tumours and then using genome-wide gene

expression profiling to compare the transcription profiles of dexamethasone-resistant ALL tumours that can be sensitised by JQ1 and that are either untreated, treated with JQ1, treated with dexamethasone, or treated with a combination of JQ1/dexamethasone.

It has been suggested that dexamethasone exerts its anti-tumour effect via downregulation of the NFκB and c-Myc pathways, and that the decision for a tumour cell to undergo either apoptotic death or cell cycle arrest is dependent upon the expression of the anti-apoptotic protein Bcl-2 and Bcl-2 protein family members [557-559]. It will therefore be important to monitor changes in expression of proteins such as Bcl-2, Bax, Bak and Bim, in response to different treatments using qRT-PCR and Western blotting techniques. Indeed, BIM expression has been shown to be essential to dexamethasone-induced apoptosis in ALL, and to be regulated mainly at the transcriptional level [560].

Interestingly, studies have shown that glucocorticoid resistance in primary B precursor ALL cells is not due to decreased GR expression, and instead occurs downstream of ligand-induced translocation of GR to the nucleus, and upstream of Bim induction [561], suggesting that elucidation of the exact signaling events between GR activation and Bim induction may aid attempts to overcome dexamethasone resistance in ALL. Furthermore, the mechanism of action of dexamethasone involves antagonism of Rel A (p65) activity by interfering with RNA Pol II-mediated gene transcription [562]. Since BET proteins such as BRD4 promote RNA Pol II transcription activity [442], it may be that sensitisation of ALL tumour cells to dexamethasone, when co-treated with JQ1, is in part due to favouring an overall inhibitory effect on RNA Pol II activity; this

will require further investigation. Should combined JQ1/dexamethasone treatment cause differential downregulation of NFκB target genes, chromatin immunoprecipitation (ChIP) may be used to determine whether or not exposure to both JQ1 and dexamethasone reduces the occupancy of NFκB at target gene promoters, compared to untreated cells, or cells treated with dexamethasone alone.

7.3 Investigation of the *in vivo* effects of BET protein inhibition in xenograft models of refractory and dexamethasone-resistant ALL

The high efficacy with which JQ1 sensitises apoptotic-resistant ALL cells derived from a high-risk ALL patients *in vivo* has been demonstrated in this study. To further support the concept of targeting BET proteins to sensitise chemoresistant ALL, it will be necessary to develop additional xenograft models of high-risk and refractory ALL, in order to test the anti-leukaemic effects of JQ1 and other available specific BET inhibitors. It will be interesting to see how effective BET inhibition will be in eliminating leukaemia *in vivo* in models of ALL tumours with a range of cytogenetic abnormalities that are commonly associated with a poor prognosis.

Additionally, results in this study indicate that treatment of ALL tumour cells with a JQ/dexamethasone combination leads to synergistic cytotoxic effects *in vitro.* To confirm that these synergistic effects are translatable in an animal model, it will be necessary to also develop primary xenograft models of dexamethasone-

resistant ALL, in order to measure the effects combined treatment has on tumour load and ALL progenitor populations.

7.4 Evaluation of transcriptional biomarkers

Despite uniform expression of BRD4, not all primary tumours are sensitive to BET protein inhibition, which is why I set out to identify transcriptional biomarkers of sensitivity to JQ1 (section 3.2.5). It will be important to validate the biomarker genes I identified in an independent cohort of ALL tumours with known sensitivity to JQ1. This work may hold crucial clinical implications in future, which would allow for the prediction of a patient's response to JQ1 based on biomarker gene expression levels, and avoid administering the JQ1 inhibitor to patients unnecessarily.

7.5 Investigation of the role of BET proteins in protein stability

Cellular proteins are targeted for proteasomal degradation following the activity of ubiquitin ligases (E1, E2 and E3), which transfer a chain of ubiquitin molecules to specific lysine residues on the target protein [563, 564]. Intriguingly, several studies have shown that the acetylation of lysine residues of key regulatory factors, such as p53, p73, Runx3, FOXO4 and E2F1, can lead to an increase in their protein stability [565-571]. As in the case of E2F1, acetylated lysine residues create a binding site for a binding partner, which when bound, masks the lysine residue and prevents its ubiquitination [565, 566].

Upon consideration of these observations, together with the results I present in this thesis showing that inhibition of acetyllysine-binding BET proteins leads to proteasomal degradation of both c-Myc and survivin, it would interesting to determine whether BET proteins regulate protein stability by binding and masking lysine residues of cellular proteins.

7.6 Investigation of the effects of BET protein inhibition on DNA replication and repair

Since monotherapies tend to lead to treatment resistance, combined treatment of drugs with different mechanisms of action is preferred. Thus, it will be important to elucidate the role of BET proteins in DNA replication and repair and provide rationale for combined treatments for ALL involving JQ1, above combinations with dexamethasone.

Results presented in this thesis suggest a JQ1-induced delay in replication fork progression and an increase in replication fork stalling. To further understand this effect, it will be necessary to verify whether replication fork stalling is associated with the induction of DNA damage and increased levels of single-strand DNA (ssDNA) breaks. This can be measured by Western blot analysis, as well as immunofluorescence and quantification of phosphorylated RPA foci, which are a marker of ssDNA. Furthermore, increased levels of spontaneous γH2AX foci, a frequently used marker of DSBs, in ALL tumour cells treated with JQ1 will also be indicative of replication-associated DNA damage.

Additionally, to test the hypothesis that the presence of incomplete transcription complexes in JQ1-treated cells has an impact on replication fork progression and stalling, it will be interesting to see if treatment of ALL tumour cells with the CDK9 inhibitor 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB) leads to similar effects in DNA replication to JQ1-treated cells, since DRB inhibits transcription through a similar mechanism to that suspected for JQ1-mediated BRD4 inhibition [339, 572].

It will also be important to investigate the effects of BET protein inhibition on DNA repair. BET proteins interact with histone deacetylases [573], and it is conceivable that their inhibition may affect the configuration of chromatin [574]. Since chromatin status is an important determinant of the DNA damage response, including homologous recombination repair (HRR) [575-577], it will be interesting to see if replicative stress observed in JQ1-treated tumour cells exhibit deregulated HRR. This can be tested in experiments using immunofluorescence to determine whether JQ1 causes delayed resolution of IR-induced foci of phosphorylated γH2AX, which would indicate reduced efficacy of DSB repair. If this is the case, further investigation looking at IR-induced foci of HRR proteins, such as RAD51, RNF168, BRCA1 and RAD18, will aid elucidation of changes to DSB repair kinetics.

7.7 Determine the role of other BET proteins in the regulation of ALL survival and proliferation

Recent evidence suggests that in addition to BRD4, other BET protein family members might also play a role in cancer pathogenesis. For example, BRDT is frequently expressed in non-small-cell lung carcinoma (NSCLC), whereas BRD2 interacts with the Kaposi sarcoma associated herpes virus (KSHV) latency associated nuclear antigen 1 (LANA-1) that is required for replication of this tumour-promoting virus [554, 578, 579].

Although evidence exists to show that JQ1 binds to the bromodomains of BRD4 with the greatest affinity, bromodomains of other BET proteins are also targeted by JQ1 to some extent [334]. Thus, it will be important to determine the biological significance of individual members of the BET family, by studying the expression levels of BRD2, BRD3 and BRDT, as well as the effect of their downregulation, in a number of primary low-risk, high-risk, and relapsed ALL tumour cells, as well as in ALL cell lines NALM-6 and REH, which are representative of apoptosis-sensitive and apoptosis-resistant phenotypes, respectively. The establishment of stable shRNA knock-down in ALL cell lines for the BET proteins that are expressed in ALL and demonstrate an impact on survival, will also be important future work to link cytotoxic effects to the downregulation of specific members of the BET family of proteins.

7.8 Validation of the PIM2 kinase as a viable therapeutic target in B-precursor ALL

The overexpression and oncogenic functions of PIM kinases have previously been demonstrated in a wide range of haematological malignancies [356]. Furthermore, the expression of PIM kinases is activated by JAK-STAT and NFκB prosurvival signaling pathways, both of which are frequently upregulated in high-risk ALL [67, 68, 189, 192, 248].

The results in this study indicate that PIM2 may play a role in maintaining pre-B ALL and so it will be important to further characterise its activity and confirm that pharmacological inhibition of PIM2 is an additional strategy by which to downregulate survival signaling and overcome apoptotic-resistance observed in a subset of chemoresistant primary ALL tumours [248].

Importantly, a next-generation PIM kinase inhibitor is currently being developed (personal communication with Prof. Stefan Knapp), which targets PIM2 with greater affinity and specificity than K00135, despite K00135 being found to cross-react with only one additional kinase, CLK1 [486]. Once available, it will be important to test the effects of the new PIM2 inhibitor in a larger cohort of primary ALL tumours, and to verify that targeting PIM2 in tumours exhibiting chemoresistance to clinically used cytotoxic agents leads to their sensitisation to apoptosis. This will need to be tested *in vitro,* as well as *in vivo* using our ALL xenograft mouse model.

In addition, it will be important to test the effects of the new PIM inhibitor in combination with chemotherapeutic agents such as doxorubicin, vincristine and dexamethasone, since synergistic effects may improve the efficacy with which these cytotoxic agents eliminate ALL tumour cells, and allow for a reduction in

the doses used to treat patients. It will also be important to test the effects of combined treatment with a PIM kinase inhibitor and JQ1 in ALL tumours that are resistant to either of these compounds alone, since results in this study suggest a significant degree of overlap exists between PIM kinase and BET protein activity.

Overall, further investigation into the targeting of PIM2 with specific pharmacological inhibitors will help fully evaluate this approach in the treatment of chemoresistant B-precursor ALL, and potentially provide scientific evidence to support inclusion of the first PIM kinase inhibitor in a B-ALL clinical trial.

CHAPTER 8

APPENDICES

8. APPENDICES

8.1. Appendix 1- Clinical information for the AML patients used as positive controls in JQ1 cytotoxicity experiments

Patient No.	Age at diagnosis (years.months)	WCC at diagnosis (x10^9/L)	Cytogenetic profile	*In vitro* response to 1 μM JQ1 (EC_{50})
AML-1	4.4	46.3	46XY, t(9;22)	0.118
AML-2	7.6	6.5	46XY, t(15;17)	3.720

Two primary AML tumours were used as positive controls for JQ1-induced cytotoxicity, since previous studies demonstrated potent anti-tumour effects of JQ1 *in vivo,* in a primary AML xenograft model [344]

The above table shows clinical information for the two primary AML control tumours along with corresponding JQ1 EC_{50} that were derived from cytotoxicity experiments in this study.

8.2. Appendix 2- Clinical information for the B-precursor ALL patients used in the PIM2 qRT-PCR expression screen

Patient No.	Age	Sex	Karyotype	Blast clearance (Day 8)	Mol. MRD (Day 28)	K00135 EC_{50} µM
ALL-201	8.24	F	48,XX,+X,der(14)t(8;14)(q11;q32),+21c[8]/ 47,XX,+21c[2]	-	-	0.53
ALL-202	15.2	M	54,XY,+X,+4,+6,+10,+14,+18,+21,+21[10]	GER	LR	0.48
ALL-203	-	-	-	-	HR	0.08
ALL-204	-	-	-	-	-	0.60
ALL-205	-	-	-	GER	LR	0.14
ALL-206	9.68	M	47,XY,dup(1)(q32q12),t(8;14)(q24;q32),+22[3]/ 46,XY[14] IGH/MYC positive [33/93]	-	-	0.44
ALL-207	11.9	M	-	GER	HR	2.37
ALL-208	2.2	F	55,XX,+X,ins(1;?)(q2;?),+4,+6,+10,+14,+17,+18,+21,+21[9]/ 46,XX[1]	-	HR	1.09
ALL-209	10.5	M	46,XY,del(9)(p1),der(19)t(1;19)(q23;p13)[9]/ 46,XY[1]	-	HR	0.16
ALL-210	0.34	M	46,XY[20]	-	-	0.17
ALL-211	-	-	-	-	-	0.18
ALL-212	6.43	M	57~60,XY,+der(X)t(X;1)(q28;q2),+Y,+4,+5,+6,+8,+10,add (10)(q?), +14,+17,add(17)(p1),+18,+18,+21,+21,+1~2mar[cp8]/46,XY[2]	GER	HR	0.54
ALL-213	18.1	F	46,XX,add(3)(p12),inv(5)(p15.3p14)c,add(20)(q1)[6]/ 46,XX,inv(5)(p15.3p14)c[14]/	-	-	1.75
ALL-214	17.9	F	44,X,-X,-7,t(9;22)(q34;q11)[12]	-	-	2.26
ALL-215	20.6	M	46,XY,t(2;7)(p1;p1)[8]/ 46,XY[2]	-	-	1.53
ALL-216	14.7	M	47,XY,+X,t(4;11)(q21;q23)[10] MLL rearrangement positive [5/5] Metaphase FISH.	GER	HR	0.56
ALL-217	12.5	F	46,XX,t(3;10)(q25;p11.2),del(9)(p21p21),der(9)t(9;16)(p21;q22)del(9)(p21 p21), der(16)t(9;16)(p21;q22)[11]/47,idem,+8[3]	-	LR	0.16
ALL-218	0.78	F	46,XX,t(4;15)(q21;q15)[9]/ 46,XX[1]	-	-	0.66
ALL-219	4.71	F	52~54,XX,+X,+4,+6,+10,+14,+17,+18,+21,+mar,inc[cp10]	GER	-	5.63
ALL-220	3.38	M	2]	-	-	0.45
ALL-221	8.56	M	48,XY,t(2;12)(p1;q1),-13,-20,+21c,+21,+3~4mar,inc[cp7]	PER	HR	0.30
ALL-222	8.91	M	46,XY,inc[8]/ 47,XY,+12,?add(12)(p1)[7] p16 monoallelic deletion [40/113]/ p16 biallelic deletion [56/113] 12cen x3 [18/133] Interphase FISH analysis	-	LR	0.55
ALL-223	0.73	F	46,XX,t(4;11)(q21;q23)[9]/46,XX[1] MLL rearrangement positive [74/105] Interphase FISH analysis	-	-	0.16
ALL-224	0.53	M	46,XY,t(4;11)(q21;q23),t(14;18)(q32;q1)[10] MLL rearrangement positive [96/99] Interphase FISH analysis MLL rearrangement positive [5] Metaphase FISH analysis	-	-	0.27
ALL-225	10.1	F	57,XX,+X,+X,+4,+6,+8,+10,+14,+17,+18,+21,+21[8]/ 46,XX[2]	GER	HR	0.30
ALL-226	14.2	M	46,XY,add(7)(q2),add(12)(p13)[10]	GER	HR	0.41
ALL-227	7.43	M	-	GER	LR	0.42
ALL-228	14	M	46,XY,?del(9)(p2?1p2?1)[20] p16 partial deletion x1 [22/49] Interphase FISH	PER	HR	2.89
ALL-229	2.84	M	54,XY,+?X,+Y,+?4,+6,+10,+14,+18,+21,+21,inc[cp3]/ 46,XY[1]	GER	LR	3.53
ALL-230	1.87	M	TEL/AML1 positive [51/56] Interphase FISH	GER	LR	1.55

ALL-230	1.87	M	TEL/AML1 positive [51/56] Interphase FISH	GER	LR	1.55
ALL-231	2.59	M	0]	-	LR	0.30
ALL-232	4.22	F	46,XX,der(19)t(1;19)(q23;p13)[9]/ 46,XX[1] E2A gene rearrangement [101/109] Interphase FISH	GER	HR	0.07
ALL-233	3.51	M	46~47,XY,?add(12)(p1),?t(12;21)(p13;q22),?+21[cp8] TEL/AML1 fusion, TEL deleted [43/80] TEL/AML1 fusion, TEL deleted, gain of AML1 [12/80] Interphase FISH	GER	LR	0.44
ALL-234	22.9	M	46,XY[20]	-	-	0.48
ALL-235	5.48	M	47,XY,+X,-14,-16,add(17)(p1),+2mar,inc[4]/ 46,XY[6]	-	HR	1.83
ALL-236	18.2	M	46,XY[20]	-	-	0.34
ALL-237	11.6	F	46,XX[20]	-	-	0.22
ALL-238	3.96	M	46,Y,add(X)(q1),t(1;14)(q21;p1),der(6,)add(6)(p1)add(6)(q1),?t(12;21)(p13;q22), add(22)(p1)[9]/ ??,?idemx2,inc[?]/ 46,XY[1]	GER	LR	0.39
ALL-239	2.94	M	TEL/AML1 positive [83/87]	-	-	1.20
ALL-240	3.82	F	45,X,-X[7]/46,XX[3] Xcen x1[84/94] Interphase FISH	-		0.54
ALL-241	-	-	-	-	-	3.09
ALL-242	18.9	F	48,X,t(X;14)(p2;q32),+der(X)t(X;14)(p2;q32),+21c[7]/ 47,XX,+21c[16] IGH gene rearrangement positive [4/5] Metaphase FISH analysis	-	-	0.84
ALL-243	10.6	F	47,XX,?t(12;21)(p13;q22),+21[5]/ 47,XX,add(12)(p1),?t(12;21)(p13;q22),+21[4]/ 47,XX,add(6)(q1),-9,?t(12;21)(p13;q22),+21,+mar,inc[3] TEL/AML positive [101] TEL deleted x 1 [95/101] Interphase FISH	-	LR	2.28
ALL-244	8.11	M	46,XY,add(6)(q1),?t(12;21)(p13;q22)[4]/ 46,XY,?t(12;21)(p13;q22)[6] G-band metaphase TEL/AML1 positive [100/104] Interphase FISH	-	HR	0.52
ALL-245	7	F	58,XX,+4,+6,+7,+8,+10,t(10;12)(p1;q1),+11,+12,+14,+17,+18,+21,+21[7]/	-	-	0.58
ALL-246	-	-	-	-	-	1.33
ALL-247	18.2	M	46,XY[20]	-	-	0.18
ALL-248	10.2	M	46,XY,t(5;5)(q1;q31)[7]/ 46,XY,t(3;20)(p21;q13),t(5;5)(q1;q31)[7]/ 46,XY[1]	PER	HR	0.69
ALL-249	5.19	M	46,XY,?t(12;21)(p13;q22)[?6]/ 47,XY,+10,?t(12;21)[2]/ 46,XY[?3] TEL/AML1 fusion positive FISH analysis on previous sample	-	-	1.15
ALL-250	5.72	M	46,XY,- 6,add(7)(p22),?del(9)(p21p21),add(10)(q2),?t(12;21)(p13;q22),+r[7]/ 46,XY,-6,add(8)(p1),?del(9)(p21p21),?t(12;21),+r[5]/46,XY[18]	-	NR	1.80
ALL-251	2.46	M	46,XY,del(9)(p11)[20]	GER	HR	2.09
ALL-252	5.95	F	59~61,XX,+X,t(2;12)(q14;p13),+4,+5,+6,+8,+10,+11,+14,+15, +18,+21,+21,+mar[cp7]/ 46,XX[6]	-	LR	0.68
ALL-253	2.93	F	55,XX,+X,+X,ins(1;?)(q21;?),+6,+10,+14,+17,+18,+21,+21[10]	GER	LR	0.29
ALL-254	2.11	M	45,XY,der(22)?t(2;9;22;10)(p1;q34;q11;q2),add(3)(p2), - 7,der(9)?t(2;9;22;10)del(9)(q34q34),der(10)?t(2;9;22;10),der(22)?t(2;9;22;10)[9]/ 46,XY[1]	GER	HR	0.46
ALL-255	6.88	M	54,XY,+X,+4,+6,+9,+14,+20,+21,+21[11]/ 46,XY[1]	GER	HR	0.47
ALL-256	16.7	F	46,XX,+5,idic(5)(p1),t(14;19)(q32;q13)[10]	-	-	0.29
ALL-257	13.4	M	-	-		0.08
ALL-258	11	F	46,XX,der(19)t(1;19)(q23;p13.3)[11]/ 46,XX[1] TCF3 (E2A) rearrangement [45/60] Interphase FISH	GER	LR	0.09
ALL-259	8.28	M	46,XY,t(12;21)(p13;q22),add(12)(p1)[23]/ 46,XY,der(12)t(12;21),idicder(21)(p1)t(12;21)[9]/ 46,XY,der(12)t(12;21),idicder(21)t(12;21),add(12)(p1)[39]/ 46,XY[18] TEL/AML1 positive	-	-	0.37

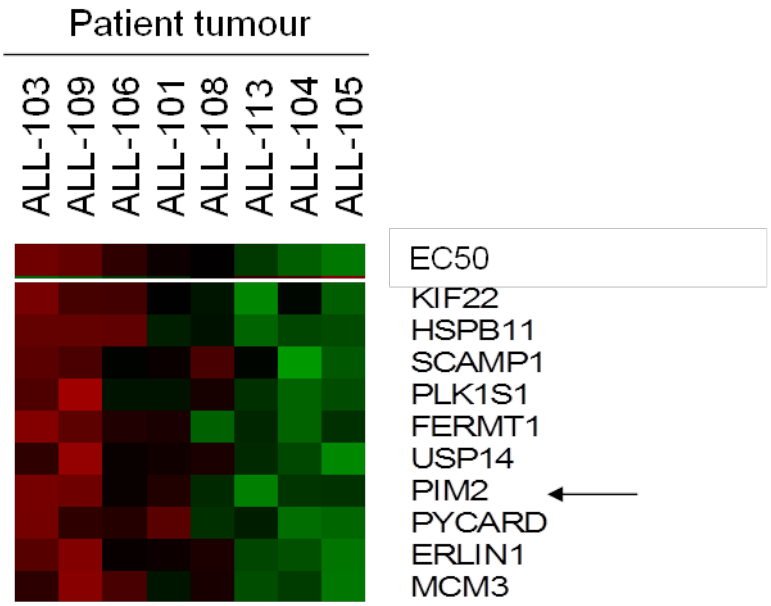
ALL-260	8.28	M	-	-	-	0.27
ALL-261	5.2	M	46,XY,?del(12)(p13p13),?t(12;21)(p13;q22)[20] G band analysis	-	-	0.20
ALL-262	3.06	F	46,XX,?del(9)(p21p21)[4]/46,XX[1] Metaphase G-banding and FISH	-	LR	0.64
ALL-263	10.1	F	51~53,XX,+4,+6,del(6)(q1q2),+8,+14,+21,+21,+mar[cp9]/ 46,XX[1]	GER	LR	0.32
ALL-264	8.76	F	45,X,-X,?del(12)(p1?p13),?t(12;21)(p13;q22),inc[6]/ 46,XX[2] TEL/AML1 rearrangement positive, partial TEL deletion [61/106]	GER	LR	0.49
ALL-265	4.44	M	46,XY,t(9;22)(q34;q11)[5]/ 46,XY[17] BCR/ABL positive [1/20] Metaphase FISH	GER	HR	0.16
ALL-266	3.85	F	45,XX,der(8;12)(q10;q10),?t(12;21)(p13;q22)[2]/ 46,XX,add(12)(p1),?t(12;21)(p13;q22),+mar,inc[cp9]/ 46,XX[4] TEL/AML1 positive, TEL deleted x1[93/102] Interphase FISH	-	HR	0.65
ALL-267	3.31	M	?46,XY,?del(9)(p?21p?21),?t(12;21)(p13;q22)[?]/46,XY[?] TEL/AML1 positive [25/91]; p16 deleted (monoallelic) [21/96]	PER	HR	0.37
ALL-268	3.63	F	53,XX,+X,+6,+14,+17,+18,+21,+21[12]/ 46,XX[3]	-	LR	0.39

Data includes age, gender, cytogenetics, blast clearance (Day 8), molecular MRD (Day 28).

K00135 EC_{50} values from cytotoxicity experiments are also included.

‘-‘ denotes where clinical data was not available. M/F = Male/Female, PER = poor early response, GER = good early response, LR/HR = low-risk/high-risk

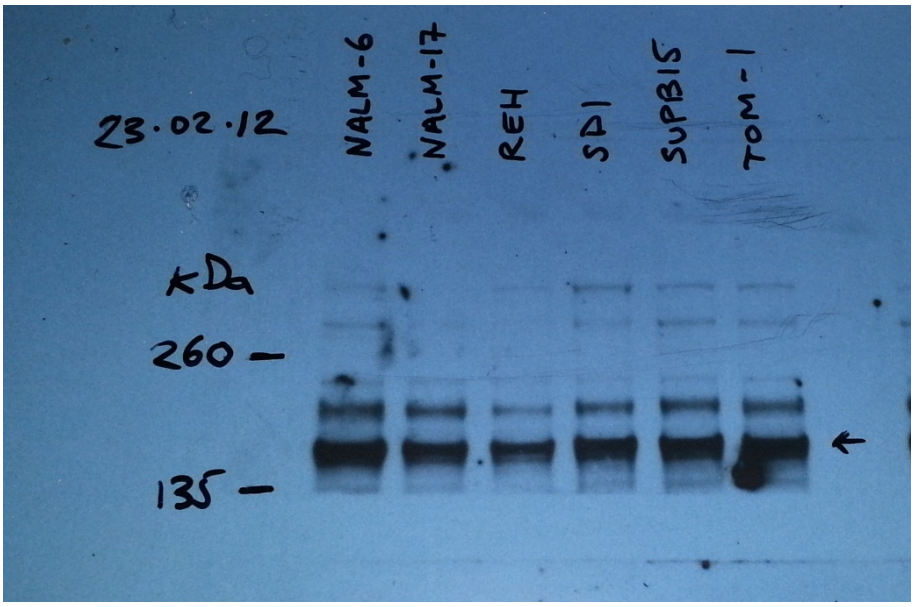
8.3. Appendix 3- The PIM2 gene is among the most differentially upregulated genes in tumours resistant to JQ1



Primary ALL tumours were ranked according to JQ1 $EC_{50,}$ as determined in previous cytotoxicity experiments. Fold-changes in gene expression showing the strongest positive correlation with JQ1 EC_{50} values were then identified using Spearman's rank coefficient correlation (coefficient ≥0.6; $p<0.05$).

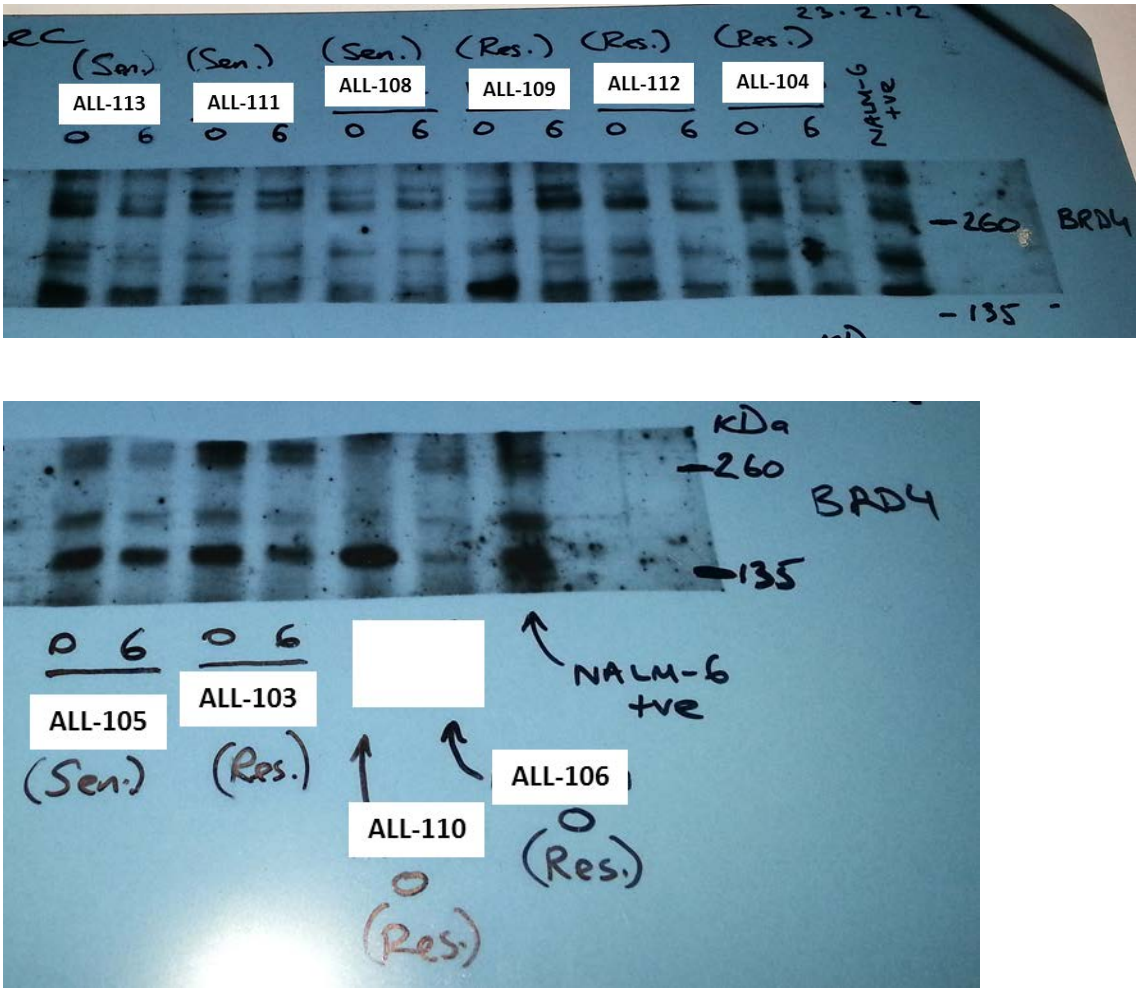
Among those genes selected (shown in the above heatmap) PIM2 was shown to be one of the most differentially upregulated genes in JQ1-resistant primary ALL tumours indicated by black arrow), suggesting PIM2 may confer resistance of ALL tumour cells to the cytotoxic effects of JQ1.

8.4. Appendix 4- Photograph of original blot showing BRD4 expression in ALL cell lines



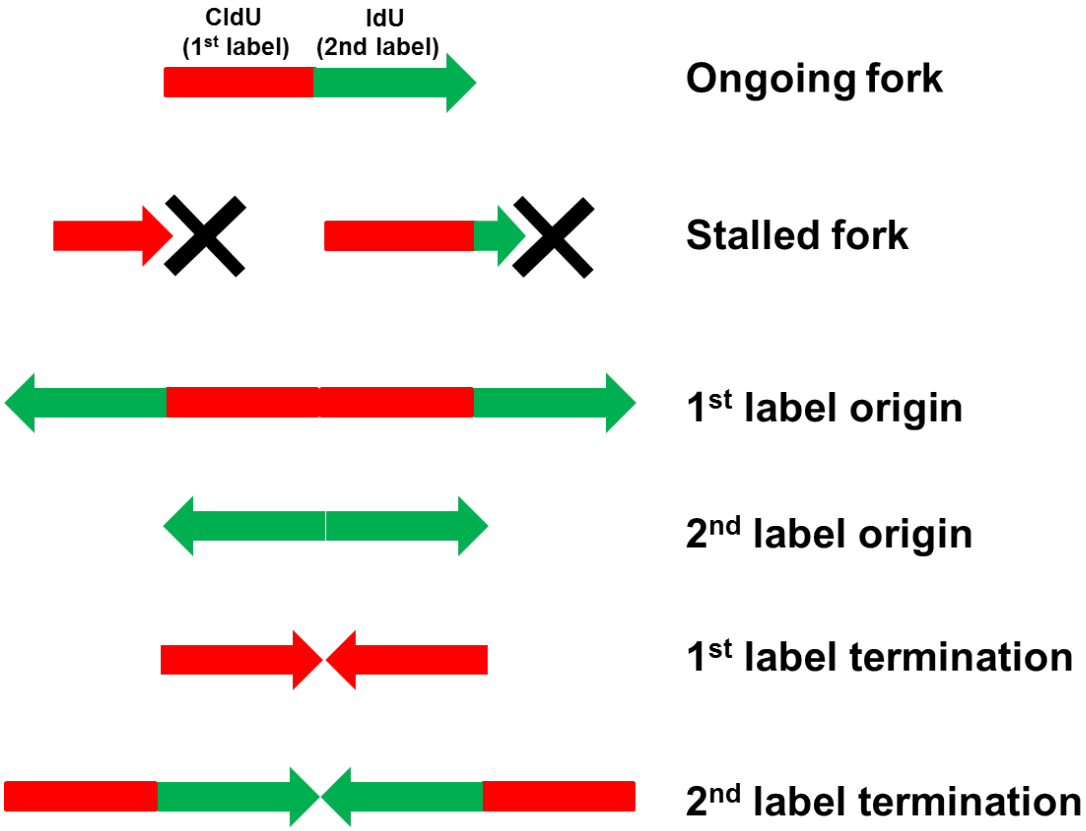
The above image is the original western blot produced in experiments investigating the expression of BRD4 in a panel of 6 ALL cell lines (NALM-6, NALM-17, REH, SD1, SUPB15, TOM-1), and is the same blot used to produce Figure 3.1a in Results Chapter 3. The arrow indicates the protein band representing BRD4 which was detected at the expected molecular weight of 152 kDa. BRD4 was expressed at high levels across all ALL cell lines.

8.5. Appendix 5- Photograph of original blot showing BRD4 expression in ALL primary tumours



The above images are the original western blots produced in experiments investigating the expression of BRD4 in a panel of 10 ALL primary tumours and are the same blots used to produce Figure 3.1b in Results Chapter 3. BRD4 protein was detected as a 152 kDa protein in all ALL primary tumours. The numbers ‘0’ and ‘6’ represent the time in hours that primary tumour cells were exposed to 1μM JQ1.

8.6. Appendix 6- Schematic showing the possible structures that can be identified using the fluorescent DNA replication fibre technique



The above replication structures can be visualised using the fluorescent DNA fibre-labelling technique, whereby replication tracts are first labelled with the nucleotide analogue CIdU and then IdU (20 minutes incubation each), followed by immunostaining with appropriate antibodies conjugated to two different fluorophores. After laser-excitation, the fluorphores emit red light (1st label) and green light (2nd label), allowing replication structures to be identified, as well as the direction of DNA replication and the rate of progression (by measuring the lengths of red and green fibres).

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