UNIVERSITY OF LIVERPOOL

EFFECT OF IMMUNOMODULATORY AGENTS ON CRITICAL PATHWAYS IN CHRONIC LYMPHOCYTIC LEUKAEMIA

Thesis submitted in accordance with the requirements of

the University of Liverpool for the degree of

Doctor in Philosophy

By

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Dedication

To my Lord and Saviour, **Jesus Christ,** who has given me life, salvation, strength, comfort and hope to continue in the face of seemingly impossible odds.

Contents

Abstract

Despite recent advances in the therapy of Chronic lymphocytic leukaemia (CLL) the disease remains incurable and relapse remains the norm. There remains a clear need to develop novel therapeutic drugs and regimens to improve responses to frontline treatments. In recent years, immunomodulatory agents (IMiDs) have shown promising therapeutic activity in CLL. Here we investigated and compared the effects of Lenalidomide and a structurally related novel analogue, CC-122, on the proliferation of B-CLL cell lines and primary CLL cells. Both drugs promote down regulation of critical transcriptional factors, and direct targets, Ikaros and Aiolos but do not induce apoptosis of either B-CLL cell lines or primary CLL cells. In the present study, we show, both drugs differentially inhibit proliferation and the induction of p21 in B-CLL cell lines and primary CLL cells. Unlike Multiple myeloma (MM) and Diffuse large B-cell lymphoma (DLBCL), both drugs did not induce apoptosis, however ratios of both anti and pro-apoptotic proteins may predict different drugs combinations with IMiDs. Furthermore, we confirm that the expression of proteins involved in the B cell receptor (BCR) signalling pathway, which is critical to CLL cell survival, are inhibited by Lenalidomide and CC-122 treatment. Interestingly, the expression of the p-ERK and its downstream target c-Myc was suppressed. This, in conjunction with an upregulation of p21 likely contributes to the inhibition of proliferation in primary CLL cells. To enhance the activity of these compounds, pre-treatment with epigenetic priming drugs was investigated. We show that sensitisation with 5-Azacytidine or Romidepsin followed by either Lenalidomide or CC-122 treatment leads to greater inhibition of proliferation than the single agent IMiD in all examined B-CLL cell lines and primary CLL cells. Importantly, CC-122 has superior efficacy when compared to Lenalidomide in all aspects of its activity in CLL. Taken together, our results provide a new mechanistic understanding of the anti-proliferative effects of Lenalidomide and CC-122 and suggest that combination with epigenetic drugs, may potentiate the therapeutic efficacy of IMiDs in the treatment of CLL.

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Declaration

I declare that the entire data presented in this thesis is a result of my own work and effort and was generated from the experiments that I have performed during my work in this project. This was carried out in the laboratory of Dr Nagesh Kalakonda, Department of Molecular and Clinical Cancer Medicine, Institute of Translational Medicine, University of Liverpool, Liverpool, UK.

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Abbreviations

µg	**:**	Microgram
mA	**:**	Milliamperes
mL	**:**	Millilitre
mM	**:**	Millimetre
nM	**:**	Nanometre
^{o}C	**:**	Centigrade
5-Aza	**:**	5-Azacytidine
Ab	**:**	Antibody
AML	**:**	Acute myeloid leukaemia
ANOVA	**:**	Analysis of Variance
APRIL	**:**	A proliferation-inducing ligand
APS	**:**	Ammonium persulfate
ATL	**:**	Adult T-cell leukaemia or lymphoma
BAFF	**:**	B-cell activating factor
BCR	**:**	B-cell receptor
BH	**:**	Bcl-2 homology
BLNK	**:**	B-cell linker
BrdU	**:**	Bromodeoxyuridine
BSA	**:**	Bovine serum albumin
BTK	**:**	Bruton’s tyrosine kinase
CC	**:**	CC-122
CD-40L	**:**	Cluster of differentiation-40 ligand
CDK	**:**	Cyclin dependent kinase
CDKi	**:**	Cyclin dependent kinase inhibitor
CLL	**:**	Chronic lymphocytic leukaemia
CLLU1	**:**	CLL up-regulated 1
CO_2	**:**	Carbon dioxide

CRBN	**:**	Cereblon
CSB	**:**	Clear sample buffer
CUL4	**:**	Cullin-RING ubiquitin ligase 4
CXCR	**:**	C-X-C chemokine receptor
CYP450	**:**	Cytochrome P450
DDB1	**:**	DNA damage-binding protein 1
ddH_20	**:**	Double distilled water
DiOC6	**:**	Dihexyloxacarbocyanine iodide
DLBCL	**:**	Diffuse large B-cell lymphoma
DMEM	**:**	Dulbecco's Modified Eagle's medium
DMSO	**:**	Dimethyl sulfoxide
DNMT	**:**	DNA methyltransferase
DSMZ	**:**	Leibniz Institute DSMZ
ECL	**:**	Enhanced Chemiluminescence
EDTA	**:**	Ethylenediamine tetra-acetic acid
ELISA	**:**	Enzyme-linked immunosorbent assay
ERK	**:**	Extracellular signal regulated kinase
FACS	**:**	Fluorescence-activated cell sorting
FBS	**:**	Fetal bovine serum
FCR	**:**	Fludarabine, Cyclophosphamide, Rituximab
FDA	**:**	Food and drug administration
FDC	**:**	Follicular dendritic cell
FISH	**:**	Fluorescence in situ Hybridization
FL	**:**	Follicular lymphoma
HDAC	**:**	Histone deacetylase
HLA	**:**	Human leukocyte antigen
H_2SO_4	**:**	Sulphuric acid
HRP	**:**	Horseradish peroxidase
IC_{50}	**:**	Inhibitory concentration 50

Ig	**:**	Immunoglobulin
IgVH	**:**	Immunoglobulin variable region heavy chain
IKZ1	**:**	Ikaros
IKZ2	**:**	Helios
IKZ3	**:**	Aiolos
IKZ4	**:**	Eos
IKZ5	**:**	Pegasus
IMDM	**:**	Iscove's Modified Dulbecco's Media
IMiDs	**:**	Immunomodulatory drugs
IRF	**:**	Interferon
ITAM	**:**	Immunoreceptor tyrosine-based activation motif
LEN	**:**	Lenalidomide
mAb	**:**	monoclonal antibody
MBR	**:**	Major breakpoint cluster protein
MFI	**:**	Mean florescence intensity
M-CLL	**:**	Mutated-CLL
MCL	**:**	Mantle cell lymphoma
MDM2	**:**	Mouse double minute 2
MDS	**:**	Myelodysplastic syndrome
MHC-1	**:**	Major histocompatibility complex 1
MM	**:**	Multiple myeloma
MRD	**:**	Minimal residual disease
mRNA	**:**	Micro RNA
NF-κB	**:**	Nuclear factor-*k*B
NLC	**:**	Nurse-like cells
PAGE	**:**	Polyacrylamide gel electrophoresis
PBS	**:**	Phosphate buffer saline
PI	**:**	Propidium iodide
PI3K	**:**	Phosphatidylinositol-3-phosphate

PLC	**:**	Phospholipase-C
PVDF	**:**	Polyvinylidine difluoride
rhIL-21	**:**	Recombinant human interleukin-21
ROMI	**:**	Romidepsin
RPMI-1640	**:**	Roswell Park Memorial Institute
SCT	**:**	Stem cell transplantation
SDS	**:**	Sodium dodecyl sulphate
SEM	**:**	Standard error mean
sIg	**:**	Surface immunoglobulin
SYK	**:**	Spleen tyrosine kinase
TBS-T	**:**	Tris buffered saline with Tween 20
TEMED	**:**	Tetramethylethylenediamine
TP53	**:**	Tumor protein 53
TME	**:**	Tumor microenvironment
TNF	**:**	Tumor necrosis factor
UK	**:**	United Kingdom
UM-CLL	**:**	Unmutated-CLL
USA	**:**	United States of America
UT	**:**	Untreated
VEGF	**:**	Vascular endothelial growth factor
ZAP-70	**:**	Zeta-chain-associated protein kinase-70

Chapter 1 : General Introduction

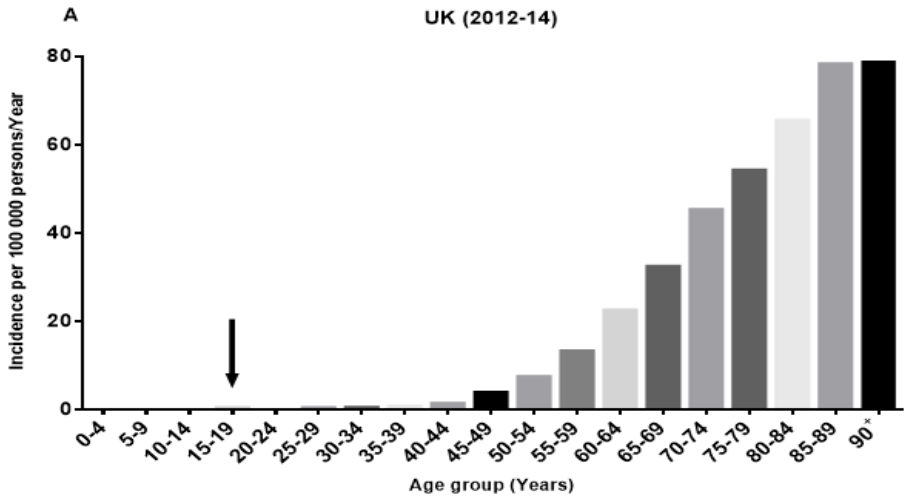
1.1. Chronic lymphocytic leukaemia (CLL)

1.1.1. Overview

CLL is a haematological malignancy characterised by the accumulation of antigen-experienced mature clonal B-lymphocytes in the peripheral blood with involvement of bone marrow, spleen and other lymphoid organs. CLL cells are characterised by strong expression of the surface protein markers CD5, CD19 and CD23, and weak expression of CD20, CD79b and monoclonal surface immunoglobulin (sIgM) [1]. The disease is characterised by variable clinical behaviour ranging from an indolent to aggressive phenotype with frequent relapses eventually culminating in drug resistance. To date, no single mutation or pathogenic translocation has been identified that is pathognomonic of the disease, but several karyotypic abnormalities including 11q-, 13q-, 17p-, trisomy 12, tumour protein 53 (TP53) mutations, and deregulation of microRNAs (miRNA) are commonly described. CLL cells receive pro-survival signals from growth factors and cytokines within the tumour microenvironment (TME) leading to prolonged survival that results in accumulation of clonal cells in the periphery and lymphoid organs [2, 3]. The disease is the most common form of leukaemia effecting adults in the UK, Europe, and the USA (**Figure 1. 1).**

1.1.2. Epidemiology

CLL predominantly affects elderly individuals with a median onset age of 64 - 70 years. The survival rates for people with CLL vary depending upon the disease stage [4] with an average of 5 years (83%). CLL is characterised by > $5x10^9$/L absolute clonal B-lymphocytes in the peripheral blood [5]. Several studies have identified risk factors that impact disease behaviour. Genetic familial studies have suggested that the risk of developing CLL is mostly in persons who are the immediate relatives of CLL patients or who have a family history of leukaemia or lymphoma [6, 7]. Several studies have been carried out to correlate the incidence rates of CLL with environmental and work-related factors such as exposure to pesticides, benzene, ionizing radiation, carcinogens, diet, alkylating agents, Hepatitis C, and Epstein- Barr virus. None of these factors, however, have reliable association with the disease [8]. Despite numerous studies, the specific cellular origin and the exact mechanisms that result in development of CLL remain unknown [9].



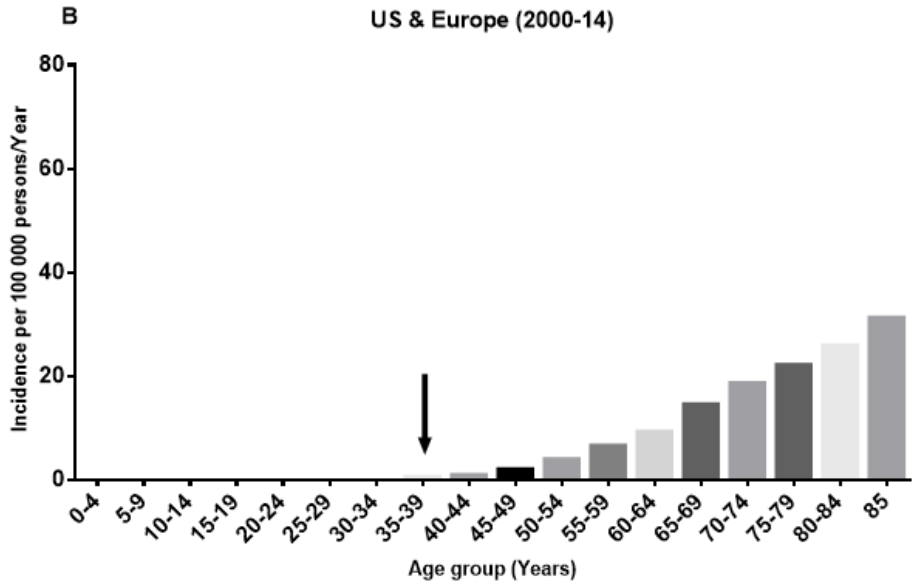


Figure 1. 1: Incidence rate of chronic lymphocytic leukaemia in the UK (A), Europe, and the US (B). Data available in the CRUK and cancer.org online database updated as of December 2017.

1.1.3 Diagnosis

The diagnosis is typically made by detection of a clonal B-cell population in the peripheral blood or bone marrow or by lymph node biopsy. B-CLL cells show characteristic morphology and immunophenotype that can be distinguished from those of other types of leukaemia. CLL cells express the cell surface markers CD19, CD5, CD23 with weak expression of CD20 and surface immunoglobulins IgM and IgD.

Several studies have demonstrated that surrogate markers such as Zeta-chain-associated protein kinase 70 (ZAP70), CD38, CD23, serum β2-microglobulin, and thymidine kinase levels can help in CLL prognostication, impact disease behaviour and therapeutic responses [10]. In addition, the mutational burden of immunoglobulin variable region heavy chain (IgVH)

genes, and in particular unmutated status correlate with poor clinical outcomes [1]. Patient derived IgVH sequences that are similar to the germline are termed as unmutated CLL (UM-CLL), whereas those with significant deviation are termed as mutated CLL (M-CLL) [11]. Lymphadenopathy, splenomegaly, soluble CD23, and lymphocyte doubling time are also useful parameters for evaluating disease behaviour and progression.

1.1.3.1 Clinical markers

Clinical classification of CLL is based on the Rai [12] and Binet [13] staging systems (summarised in **Tables 1 and 2).**

Table 1: Rai Classification system for CLL.

Stage	**Clinical Diagnosis**	**Median survival time**
0	Blood and marrow lymphocytosis	≥ 10 years
I	Lymphocytosis and lymphadenopathy	9 years
II	Lymphocytosis and hepatomegaly	7 years
III	Lymphocytosis and anaemia	5 years
IV	Lymphocytosis and thrombocytopenia	5 years

Table 2: Binet Classification system for CLL.

Stage	Clinical Diagnosis	Median survival time
A	Blood and marrow lymphocytosis and lymphoid involvement	> 7 - 10 years
B	Palpable lymphoid involvement	5 - 7 years
C	Anaemia or thrombocytopenia	< 2 - 5 years

1.1.3.2 Biological markers

The Immunoglobulin (Ig) molecule contains two heavy and two light chains. These heavy and light chains are formed with antigen specific selection and recombination of Variable (V), Diversity (D), and Joining (J) gene segments that occurs in B-cells. In response to the antigen encounter, B-cell Ig genes undergo somatic hypermutation within germinal centres in lymphoid tissues to produce several Immunoglobulin heavy chains [4, 14, 15]. These cells undergo differentiation and a high rate of clonal selection and expansion such that they express a unique B-cell receptor (BCR) that can be used as a molecular biomarker in normal and malignant B-cells. In the case of CLL, the degree of IgVH mutation can predict survival outcomes [16]. Thus, patients with unmutated IgVH genes have a shorter survival whereas mutated IgVH is associated with better prognosis [4]. The mutational state is also an indication of the degree of differentiation of CLL cells.

An independent biological marker of CLL cell behaviour is ZAP70 protein status. ZAP70 is a protein tyrosine kinase associated with enhanced signalling of BCR in CLL cells. ZAP70 is shown to enrich the downstream targets of NF-κB such as IL6 and IL8. It is a surrogate biomarker that discriminates IgVH mutated and unmutated CLL cases [17]. Increased levels of ZAP70 correlate with unmutated status of CLL cells.

CD38 is a single chain transmembrane glycoprotein involved in controlling stimulation and proliferation of T-cells [18]. Interactions of CD38 and CD31 induce proliferation in CLL cells [19] and increased expression of the former is associated with poor prognosis [20]. In addition, $CD38^{+}$ CLL cells are reported to be more resistant to apoptosis in *in vitro* cultures. CLL cells show high genetic homogeneity because of the co-expression of CD38 and CD31 independent of mutational status of CLL and their expression results in autocrine B-cell activation [21, 22].

1.1.3.3 Novel markers

Several novel biomarkers have been identified by exome sequencing of CLL cells. Genes identified as biomarkers in CLL include NOTCH1, SF3B1, FBXW7, SHIP-1 and BIRC3 [23-26]. Genetic aberrations in these genes are useful in disease prognosis, determining clinical course, and outcomes. Deletions in NOTCH1 are associated with Fludarabine resistance in CLL, whilst mutations in SF3B1 can potentiate cell growth and survival. FBXW7 gene mutations target activation of NOTCH receptors that are a component of the SCF ubiquitin protein complex [23, 24]. In addition NOTCH1

aberrations, such as the amino acid mutation of K212, are associated with transformed CLL [27, 28]. BIRC3 deletions are associated with activation of the NF-κB pathway that targets several anti-apoptotic genes. Changes in genes associated with poor prognosis in CLL [29] and frequently mutated genes are listed in **Table 3.**

Table 3: Summary of the most frequently mutated genes in CLL.

Gene name	**Mutational frequency in CLL**
SF3B1	13%
NOTCH1	11%
ATM	11%
TP53	10%
MYD88	4%
POT1	3%
XPO1	2%
CDKN2A	2%
BCL11B	2%

1.1.4 Chromosomal aberrations in CLL

Chromosomal aberrations in CLL can be characterised with fluorescent in situ hybridisation (FISH) [20]. The most frequent abnormalities identified are deletions of 17p (17p-), 11q (11q-) 13q (13q-), and trisomy 12 [30]. **Table 4** lists the frequency of these chromosomal abnormalities and the affected genes. However, there is no common single genetic aberration or mutation that is implicated in CLL [31]. Presence of one or more chromosomal abnormalities or genetic mutations is used to determine the prognosis of the disease.

Deletion of 17p is associated with an aggressive form of CLL and is stereotypically associated with loss of p53 function [32]. The tumour suppressor gene p53 plays a major role in regulating the cell cycle, DNA damage repair, and apoptosis when DNA repair fails [9]. In addition, loss-of-function mutations of the p53 gene lead to drug resistance, high incidence of relapsed or refractory disease and short survival in CLL [4, 33, 34].

Deletion of 11q results in the loss of the ATM gene and associates with lymphadenopathy, poor treatment response, and shorter survival in CLL patients [25]. Deletions or mutations of the ATM gene alter the expression of p53 protein in response to DNA damage/repair and also impact the NF-κB pathway. Frameshift mutations and deletions of BIRC3 are also reported in CLL. These changes affect MAP3K14, a serine threonine kinase in the NF-κB signalling pathway, which downregulates p53 through MDM2 expression leading to disease progression [35, 36].

Trisomy 12 occurs as a result of the duplication of 12q13 and is associated with early disease progression in CLL. It reportedly results in the overexpression of the oncogene MDM2 [37] although some reports suggest a lack of such correlation [38]. More recently, it is thought likely that NOTCH1 aberrations are associated with trisomy 12 [39]. Deletion of 13q is the most common chromosomal abnormality observed in CLL, and is shown to be associated with mutated IgVH [34, 40]. Deletion of 13q results in the loss of expression of the linked microRNAs 15a and 16-1 (miR15a and miR16-1) with resultant up-regulation of Bcl-2 expression leading to prolonged cell survival [41].

Table 4: Chromosomal aberrations and their location in CLL [42].

Chromosomal aberration	**Genes located on the chromosome**	**Cases of CLL patients**	**Clinical course**
17p13del	TP53	5-10%	Aggressive
11qdel22-23	ATM	15-20%	Poor survival
Trisomy 12q	MDM2 CLLU1	7-30%	Early progression
13qdel14	miR15/16	40-60%	Progression

1.1.5 Apoptosis

Cells that are damaged or infected are eradicated by a process called programmed cell death or apoptosis [43]. Apoptosis also occurs in cells during the immune response and as a result of cell mediated cytotoxicity [44]. Activation of the apoptotic pathway leads to chromatin condensation, shrinkage and fragmentation of the nucleus leading to cell death [45]. The cell death programme has two pathways, an intrinsic pathway (death receptor dependent) and the extrinsic pathway (mitochondria dependent). The intrinsic death receptor pathway is triggered by engagement of cell surface receptors such as Fas and TRAIL whereas the extrinsic mitochondrial pathway is activated by cytokines and caspases [46, 47]. Defects in apoptosis may cause the accumulation of genetic alterations and tumorigenesis within the cell [48, 49]. Mutations of p53, over expression of the anti-apoptotic protein Bcl-2, and inactivation of Bcl-2 homology (BH) 3 containing pro-apoptotic family members are frequently observed in several B-cell malignancies including CLL [48].

The Bcl-2 family proteins predominantly reside on the mitochondrial outer membrane and regulate cell death and survival [50]. They are characterised by the presence of sequences known as BH motifs that consist of four conserved domains (BH 1 - 4). The anti-apoptotic members share all four BH domains whereas the pro-apoptotic members can be divided into two sub categories, one of which has several BH domains whilst the other group only harbours a BH3 domain [50, 51]. This division of Bcl-2 family on the basis of

BH domains provides a balance between cell survival and death. The members of the Bcl-2 family are listed in **Table 5**.

Table 5: Classification of Bcl-2 family proteins.

Anti-apoptotic	Multi-domain pro-apoptotic	BH3 only pro-apoptotic
Bcl-2	Bax	Bad
Bcl-xL	Bak	Bik
Bcl-w	Bok	Bid
Mcl-1	Bcl-xs	Bim
Bfl-1		Noxa
Bcl-2		Puma

Several studies have shown that high levels of the anti-apoptotic Bcl-2 protein are often observed in CLL patients and link with aggressive disease, drug resistance, and shorter survival [52, 53]. High expression of Bcl-2 affects cytokine mediated apoptosis in B-CLL [54]. Recent studies suggest that Bcl-2 over expression also results from hypomethylation at the gene promoter [55]. Additionally, rearrangements of the Bcl-2 gene involve translocation of a major breakpoint cluster region (MBR) that affects Bcl-2 protein expression, leading to poor prognosis and resistance to single agent

therapy in CLL [56]. Mcl-1, another anti-apoptotic Bcl2 family member, is also frequently over expressed in CLL. Similar to Bcl-2, the mirs-15a and16-1 target Mcl-1 in CLL and its dysregulation leads to distinct clinical and biological features. The loss of expression of miRs -15a and 16-1 also target the 3' untranslated region of TCL1A and affect the expression of Mcl-1. Overall the miRs, TCL1A, and Mcl-1 axis results in epigenetic changes in CLL leading to drug resistance [57]. Expression of proteins in the apoptotic pathway such as Bcl-xL, BAD, and BAX is higher in CLL. Specifically, increased expression of Bcl-xL along with Bcl-2 prevents apoptosis in CLL cells [58].

The p53 protein is a major regulator of apoptosis and has been shown to be frequently mutated in solid tumours and CLL [59]. In general, when a cell experiences stress or damage, the ATM protein becomes activated and subsequently leads to transcriptional initiation of p53 [60, 61]. Expression of p53 then leads to cell cycle arrest through the induction of p21 expression, promotes apoptosis, and cellular senescence [62, 63]. In CLL cases, TP53 gene mutations are detected in 50% of 17p- cases and 5% of CLL patients carry p53 mutations without 17p- [64-66].

1.1.6 B-Cell Receptor (BCR) Signalling Pathway

Key to understanding B-cell biology is the appreciation of the generation of antigen receptor diversity at all stages of B-cell development. The B-cell receptor (BCR) has been extensively studied in various *in vitro* and *in vivo*

models, and below is an attempt to summarize some of what is known about BCR signalling.

The B-cells in the immune system are able to recognise foreign and deleterious antigens through the BCR. The BCR is a complex composed of surface membrane bound immunoglobulin (sIg) molecules and co-receptors CD79A (Igα) and CD79B (Igβ). The sIg is a heterodimer that consists of 2 heavy chains and 2 light chains held together by disulphide bridges [57]. Each Ig chain has 2 components; an N-terminal variable (V) region for antigen binding, and a C-terminal constant (C) region for membrane insertion and additional functions. The C region is responsible for the Ig isotype variation (IgA, IgD, IgE, IgG and IgM) of the BCR or antibody [67]. Antigen binding to BCR leads to activation of immuno-receptor tyrosine based activation motifs (ITAMs) that phosphorylate the surface immunoglobulin, and lead to formation of the BCR signalosome, a complex of kinases that trigger subsequent phosphorylation in the C-terminal tail of Igα and Igβ and kinases downstream of the SRC family kinase LYN. Phosphorylated ITAMs also recruit and activate the tyrosine kinase protein SYK. Activation signals are propagated by SYK through the signalosome and by assembly of intracellular molecules such as Phospholipase Cγ2 (PLCγ2), Bruton tyrosine kinase (BTK), Phosphoinositide 3-kinase (PI3K), B-cell linker (BLNK) and VAV [68, 69]. This signalosome allows cascade of events such as calcium flux, antigen internalisation, and gene expression (**Figure 1.2).** BCR engagement has been shown to be crucial for B-cell and T-cell activation & B-cell anergy [70].

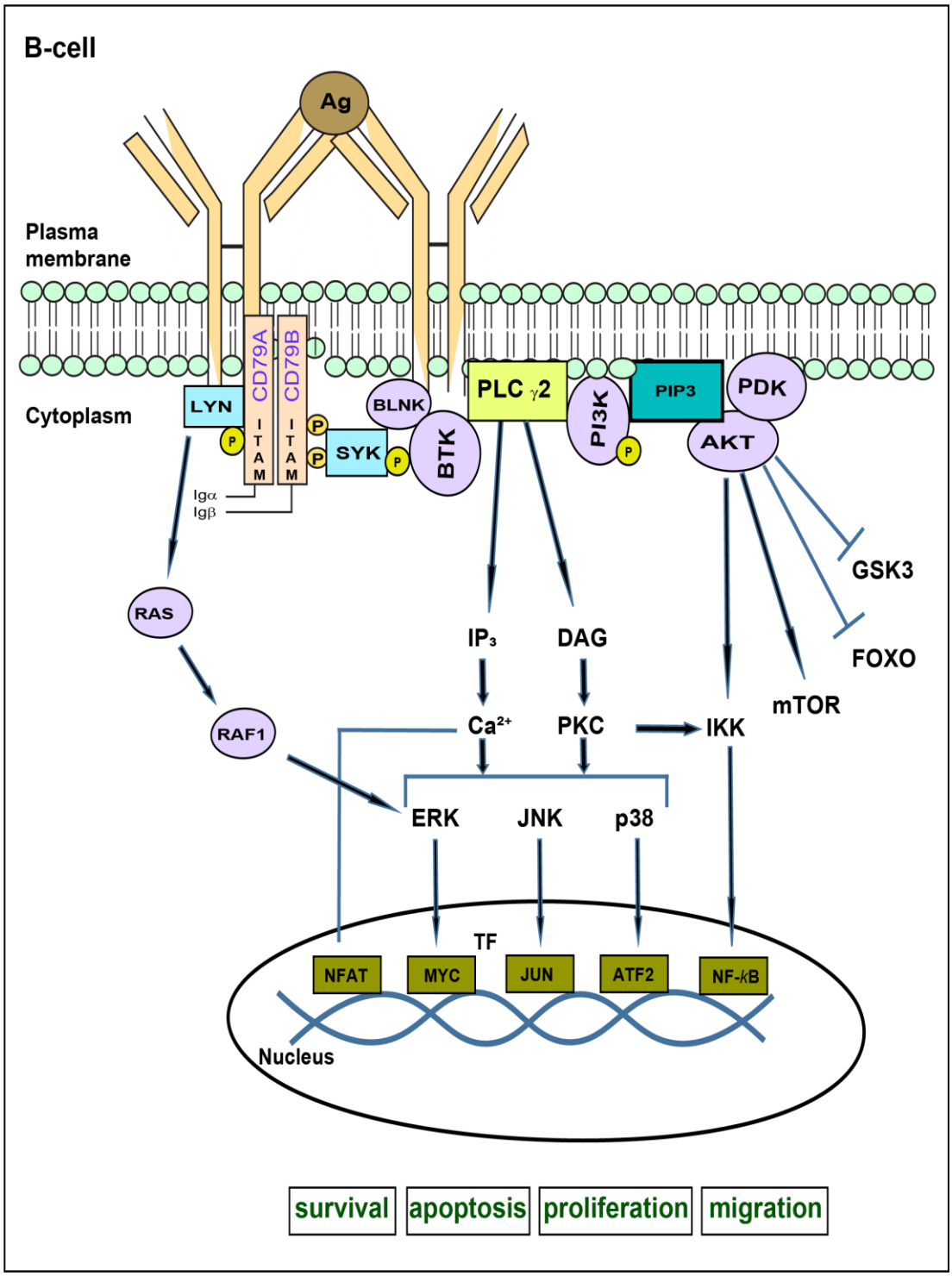


Figure 1.2: B-cell receptor signalling pathway. Antigen binding to the BCR leads to phosphorylation of ITAM regions of CD79A and CD79B by LYN kinase. Conformational changes then activate SYK and other downstream kinases leading to formation of the signalosome. This leads to a cascade of events such as calcium release, cytoskeletal changes, and gene expression.

1.1.7 Abnormalities of BCR in CLL

BCR signalling is a key feature in determining the clinical outcome of CLL patients. In CLL, BCR signalling significantly contributes to survival and proliferation of clonal B-cells [14]. IgM and IgD are the most commonly expressed immunoglobulin molecules in CLL, with fewer cases expressing IgG or A. However, their expression levels are low compared to normal B cells [57]. Recent studies suggest that enhanced B-cell activation observed in unmutated IgVH CLL cases may lead to B-cell anergy [71]. Other studies report that low levels of CD79A or CD79B expression result in poor signalling [72]. In CLL, LYN and SYK kinases are constitutively expressed resulting in survival and proliferation [73, 74]. Phosphorylated BTK, SYK and PLCγ2 are also constitutively active in CLL [75]. Small molecular inhibitors of SYK, PI3K, and BTK are demonstrating promising activity in patients with CLL. Pharmacological inhibition of BCR signalling can be achieved using kinase inhibitors such as Ibrutinib (BTK) or Idelalisib (PI3K) [76, 77]. Similarly, overexpression of p-ERK, in the Ras-MAPK pathway, is common in CLL and results in enhanced proliferation [78]. Signalling via the BCR pathway may also lead to activation of other cell surface receptors such as CD49d. Such activation may result in enhanced adhesion of CLL cells that express higher levels of α4β1, In contrast, activation of the CXC chemokine receptor 4 (CXCR4) inhibits BCR induced activation of α4β1 [79, 80]. Studies focused on BCR signalling mechanisms and abnormalities are enabling researchers to better understand B-cell malignancies such as CLL and are helping to identify novel targets for therapies.

1.1.8 Microenvironment in CLL

In vitro, CLL cells purified from peripheral blood rapidly die as a result of spontaneous apoptosis. *In vivo*, interaction of CLL cells with the microenvironment is crucial in the development of the disease [81, 82]. CLL cells interact with stromal cells and other accessory cells in the microenvironment resulting in greater survival, proliferation, homing, and drug resistance [83-85]. Expression of CD62L, CXCR4, CD49d (on CLL cells), T lymphocytes, a variety of stromal cells, and Nurse-like cells (NLCs) play a key role in enhancing the proliferation and inhibition of apoptosis in CLL [86, 87]. Such interactions of CLL cells are critical for cell migration, chemotaxis, and infiltration of lymphoid tissues [88]. In addition, $CD4^+$, $CD40L^+$ and CCL22 expressing T-cells also help in migration and accumulation of CLL cells [89, 90].

1.1.9 *In Vitro* culture systems

The cells in the CLL microenvironment in lymph nodes and bone marrow are essential for proliferation and survival. Since CLL cells undergo spontaneous apoptosis if cultured alone *in vitro*, simulation of the microenvironment is essential to understand these different interactions and their role in CLL development. In addition, testing novel treatments in cultures that mimic the microenvironment is an important way to assess the potential and effectiveness of novel drugs. Several culture systems have been explored over the years that also provide insights into CLL biology [91].

1.1.9.1 BCR stimulation by Anti-IgM

As described above, B-cell activation is essential for the CLL cell survival and proliferation. Several studies describe that Anti-immunoglobulin antibodies and *Staphylococcus aureus* Cowan strain can be used to mimic BCR stimulation in normal B-cells [92]. CLL response to BCR stimulation is heterogeneous and studies have shown a correlation with IgVH status (21). Bernal and others revealed that Anti-human IgM F(ab')$_2$ promotes CLL cell survival by increasing the activities of NF-κB, PI3K, ERK-MAPK, and anti-apoptotic proteins [91, 93]. When CLL cells are co-cultured with NLCs they stimulate BCR pathway proteins by NF-κB activation and elevated levels of chemokines CCL3 and CCL4 [94]. In summary, addition of purified immunoglobulins to primary CLL cultures is a validated model to activate BCR and associated downstream proteins.

1.1.9.2 CD40L system

T-cells, which are an important part of the CLL microenvironment, express CD154. The interaction between CD154 and CD40 on the surface of CLL cells stimulates TRAF proteins [95], which activate several downstream pathways such as canonical and non-canonical NF-κB [96]. Ultimately, this interaction leads to protection from apoptosis through expression of pro-survival proteins such as BCL2, BCL-xL, and Mcl-1 [97]. *In vitro* the CD40L/CD40 can be mimicked by using either fibroblasts expressing anti-CD40 or soluble anti-CD40 antibodies in combination with exogenous co-

stimulants such as interleukins (IL-4, IL-2, IL-21) [98]. Several T-cell derived cytokines have been studied to induce proliferation. Interestingly recent studies report that IL-21 leads to potent induction of CLL proliferation. Using CD154 fibroblasts with IL-21 is a suitable model because it simulates both stroma and T-cells in the CLL microenvironment and enhances proliferation [99].

1.1.9.3 Stromal cell model

Co-culture of CLL cells with bone marrow-derived stromal cells can inhibit spontaneous apoptosis of leukemic cells. This model promotes cell survival by production of cytokines, promoting autocrine signalling, and also consistent activation of PI3-K/AKT and SYK [100]. Due to the presence of integrin, CLL cells adhere to stroma and avoid apoptosis [101]. Similarly, NLCs found in the lymph node and bone marrow protect CLL cells from spontaneous apoptosis through production of stroma-derived factor (SDF-1α) [102]. The above-mentioned *in vitro* models are widely used in CLL research to investigate disease biology and for identifying potential therapeutic options. Other factors that promote *in vitro* BCR activation include Toll like receptors (TLR), cytokines, chemokines, BAFF, and Integrins.

1.1.9.4 *In vivo* model

In vivo models are also increasingly used to screen novel compounds and to understand CLL pathogenesis. One such model is the transgenic TCL1 mouse which is specifically engineered to overexpress the human TCL1

gene under the control of IgVH promoter and immunoglobulin heavy chain enhancer to mirror CLL [103]. Peripheral blood mononuclear cells xenografted into immunocompromised NOD/SCID/γc^{null} (NSG) mice can proliferate in the murine spleen, and reports suggest that transplanted CLL cell proliferation is dependent on co-engrafted human T-cells *in vivo* [104]. Other studies have also confirmed that NSG mice support proliferation of CLL cell xenografts and these observations directly correlate with disease characteristics in donor patients [105]. These models have been used to study *in vivo* effects of small molecule inhibitors in clinical development. Because of the differences between murine and human microenvironment this model is not suitable to screen anti-cancer activities of immune therapeutic agents [106].

1.1.10 Treatment of CLL

Over the past few decades, treatment options in CLL have improved, aided by the understanding that the disease is heterogeneous with a varied clinical course [107]. Whilst some patients may remain asymptomatic and not require intervention for many years, others require immediate treatment with traditionally employed alkylating agents such as Chlorambucil or Cyclophosphamide [108]. Advancements include the use of combinations of chemotherapy, monoclonal antibodies, the use of novel compounds such as PI3K and BTK inhibitors, immunomodulatory agents, and epigenetic modifiers. However, despite these advances, CLL remains incurable.

1.1.10.1 Chemotherapy or Single agent therapies

The DNA alkylating agent Chlorambucil has been used in the treatment of CLL for over 40 years and is the most widely used single agent compound, with an overall response rate of 51% [109]. Chlorambucil has been shown to intercalate with DNA leading to apoptosis [110].

Cyclophosphamide is a pro-drug that once metabolised *in vivo* causes alterations in the cytoskeleton and induces expression of pro-apoptotic proteins. It is converted by CYP450 in the liver to an active metabolite (4-hydroxy cyclophosphamide) [111]. In addition, several studies suggest that it can act as an immunomodulatory compound [112]. Despite several cellular effects of these drugs, their exact mechanism of action in CLL is still unknown [108]. The overall response and survival rates following single agent use is quite low [112]. The FDA approved Bendamustine, another alkylating agent, in 2008 for the treatment of CLL and the drug shows higher efficacy when compared to Chlorambucil and Cyclophosphamide [113, 114].

1.1.10.2 Purine analogues

Purine analogues such as Fludarabine, Cladribine, and Pentostatin are frequently used in the treatment of mature B-cell malignancies such as CLL and Hairy cell leukaemia. Fludarabine is the most commonly used purine analogue in CLL, either alone or in combination with Cyclophosphamide [115]. The mode of action of these drugs is to block several enzymes involved in DNA synthesis and repair, leading to apoptosis. Fludarabine is a pro-drug that undergoes de-phosphorylation prior to entry into CLL cells

where it is converted into the active metabolite F-ara-ATP [116]. It is often used with various DNA damaging agents, and combinations include Fludarabine and Chlorambucil; Fludarabine and Mitoxantrone; Fludarabine and Epirubicin; and Fludarabine, Cyclophosphamide and Mitoxantrone. The use of Fludarabine can lead to prolonged hypogammaglobulinemia and neutropenia, and lymphomas [117]. Allogenic transplantation was the only option for Fludarabine refractory patients until the arrival of monoclonal antibodies in CLL therapy [118].

1.1.10.3 Monoclonal antibody therapy

Monoclonal antibodies are now established agents in the treatment of CLL. CD20 and CD52 are phosphoproteins that are highly expressed in CLL [119]. Rituximab is a chimeric anti-CD20 antibody used to treat CLL [120]. Overall, the effectiveness of Rituximab in CLL is low when used as single agent. However, when used in combination with Fludarabine and Cyclophosphamide, it shows higher efficacy and can result in the clearance of circulating malignant cells [121]. Ofatumumab is a humanised anti-CD20 antibody that shows higher efficacy and better recognition of CD20 than Rituximab [122]. Alemtuzumab, an anti-CD52 antibody, has been shown to be more effective in the treatment of CLL patients compared to Rituximab [123]. The results of using antibody therapies in the treatment of CLL have been encouraging but little is known about the effect of microenvironment on these therapies [124]. Other monoclonal antibodies such as Lumiliximab (anti-CD23 monoclonal antibody) are less effective as single agents [125]

and should likely be combined with other chemotherapeutic agents for better responses.

1.1.10.4 Chemo-immunotherapy

Several studies suggest that combining chemotherapy with monoclonal antibodies (**Table 6**) results in cell death through synergy. Rituximab sensitises resistance tumour cell lines and can enhance susceptibility to Fludarabine and other agents [126]. Other groups have shown that Fludarabine decreases CD55 and CD59 expression and sensitises CLL cells to Rituximab [127]. Based on the above observations, addition of Rituximab to Fludarabine and Cyclophosphamide (FCR) has resulted in highly effective CLL front line therapy. Six median cycles of FCR can achieve good overall and complete responses (90% and 75%). The regimen is less effective in relapsed/refractory CLL patients [128, 129]. In order to improve the efficacy of FCR, several investigators have studied dose escalation of Rituximab. However, results were not considerably different from that of FCR [130, 131]. In relapsed settings, Pentostatin, Cyclophosphamide and Rituximab (PCR) based regimens have been evaluated but results were not significantly different to the FCR regimen [132].

Table 6: Summary of FCR alone and in combination with other agents in refractory or relapsed CLL.

Study	Treatment	No of patients	No of previous treatments	OR	CR	Ref
Wierda (2005)	FCR	179	2	73%	25%	[129]
REACH (2009)	FCR	276	1	70%	24%	[133]
Byrd (2009)	FCR+L	31	2	65%	52%	[134]
Wierda (2006)	CFAR	74	3	65%	24%	[135]
Hillmen (2007)	FCM+R	23	2	70%	43%	[136]

1.1.10.5 Novel Pharmaceutical agents

Increased understanding of the biology of CLL, brought about by modern genomic, proteomic and analytical techniques, has allowed significant advances in the clinical management and personalisation of CLL therapy. The era of B Cell Receptor siganlling inhibitors (BCRi) for example, has altered the landscape of CLL management. Currently licensed BCRis include: Ibrutinib, a Bruton's Tyrosine Kinase inhibitor (BTKi); Idelalisib, a PI3kinase inhibitor, and Venetoclax (ABT-199) which is a BCL2 receptor antagonist. BTK plays a vital role in the downstream signalling from the BCR in B-cells. Several studies have observed that BTK expression is high in CLL and other B-cell malignancies [75, 137]. Ibrutinib is a specific BTK inhibitor that targets its kinase activity through irreversibly binding to the cysteine at

position 481 and induces apoptosis in CLL [137-139]. Ibrutinib has now been approved by the National Institute for Clinical Excellence (NICE) for first line therapy in TP53 mutated CLL. Adverse effects include platelet dysfunction leading to haemorrhage, cardiotoxicity, diarrhoea and fatigue. Second generation BTKis such as Acalabrutinib are currently in trial. Several clinical trials examining the combination of Ibrutinib with Ofatumumab or Rituximab have shown promise in refractory CLL patients yielding response rates of 50-75% [140].

Pl3K is an indispensable molecule for transmitting signals from the BCR. There are three classes of PI3Ks, with class I containing four different isoforms. PI3Kδ has been shown to be critical for B-cell survival and function [141]. Idelalisib, a PI3Kδ specific inhibitor, inhibits phosphorylation and activation of AKT and ERK, leading to inhibition of cell migration and a reduction in cell viability [142]. Recently, Idelalisib in combination with Rituximab has shown positive results in CLL [141, 143]. Idelalisib is currently licensed in the UK as second line therapy in patients who have previously received a BTKi. Like Ibrutinib, whilst initiation of therapy improves lymphadenopathy, inhibition of migration of CLL cells from the blood to lymphoid tissues causes an initial lymphocytosis. This improves with ongoing treatment. Unfortunately the significant toxicity profile of Idelalisib has mitigated its use as a first line agent, being associated with pneumonitis, transaminitis and colitis [57].

1.1.10.6 Treatment Paradigm

Whilst the clinical course of CLL remains heterogeneous, with some patients displaying a very indolent natural history and others exhibiting aggressive and multiply relapsing disease, cytogenetic and molecular testing has allowed for more accurate prognostication. This has led to significant changes in treatment decision making in regard to systemic anti-cancer therapies and optimal drug combination. TP53 mutation or 17p- in CLL confers a significantly poorer prognosis, and current treatment paradigms are based around the presence or absence of mutated TP53 or 17p-, alongside clinical considerations such as patient age, co-morbidities and performance status. As earlier stated, UK standard of practice for first line treatment of TP53 mutated/17p- CLL is Ibrutinib. In patients able to tolerate optimal therapy, non-mutated TP53 is offered Fludarabine based therapy, in combination with Cyclophosphamide and Rituximab (FCR) [144].

The ability to screen for the presence of the IGHV mutation has also significantly altered prognostication in CLL, although at present in the UK this has not directly altered treatment paradigms. Patient with CLL and mutated IGHV, or the M-CLL cohort, exhibit a significantly improved prognosis, such that FCR therapy achieves a 12.8year progression free survival (PFS) of 53.9% (compared with 8.7% in unmutated IGHV, or U-CLL) [145].

Table 7 summarises recent efforts in the CLL field with BCR inhibitors and other novel agents**.** Despite the emergence of novel therapies and strategies,

disease recurrence remains the norm and hence further basic and clinical research to improve patient outcomes is essential.

Table 7: Drugs in Clinical trials – CLL.

Class	Clinical Trail	Sponsors
BTK inhibitors		
Ibrutinib	Refractory/ relapsed CLL	Pharmacyclic, Inc
CC-292	Refractory/ relapsed CLL Combinations CC-292 with Lenalidomide	Celgene Corporation
ONO-4059	Refractory/ relapsed CLL	Ono Pharma Co Ltd
ACP-196	Phase 1	Acerta Pharma
Pl3K gamma/delta inhibitors		
Idelalisib	Combination regimens with either Rituximab Ofatumumab, or Bendamustine or CC-122	Gilead Sciences
GS-980	Phase I	Gilead sciences
IPI-145	Phase III for progressive CLL	Infinity Pharma
AMG-319	Relapsed/ refractory CLL	Amgen
SYK Inhibitors		
GS-9973	Phase I	Gilead Sciences
Cerdulatinib	Phase I	Portola Pharma
Bcl-2 inhibitors		

ABT-199	Various combination studies with Rituximab, Obinutuzumab, Bendamustine	Roche
AT-101	Phase I/II trial	Mayo Clinic

1.1.10.7 Drugs that target BH3 proteins

Since most CLL cells express Bcl-2 protein, inhibition of this pathway by small molecules has emerged an an attractive therapeutic option in CLL [146]. Normally, Bcl-2 is an anti-apoptotic protein that regulates the intrinsic pathway of apoptosis. It has been well documented that overexpression of Bcl-2 counteracts pro-apoptotic proteins in CLL [147]. Its overexpression may be explained by epigenetic mechanisms such as hypomethylation of the Bcl-2 gene, or deletion of miR-15a and miR-16-1 [148, 149]. Early efforts at targeting Bcl-2 with a BH3 mimetic Oblimersen failed to improve efficacy of Fludarabine and Cyclophosphamide in a phase III trial. The use of Obatoclax, a pan-Bcl-2 inhibitor, was abandoned due to low response rates and toxicity. ABT-737 was the first BH3 mimetic with more potency and high affinity for all anti-apoptotic proteins. ABT-293 (Navitoclax) substituted ABT-737, as the latter was not suitable for oral administration [150]. In a phase I trial, Navitoclax achieved 35% ORR in refractory/relapsed CLL patients but there was no effect on 17p- or Fludarabine refractory patients. Due its superior pharmacokinetic profile, there has been a recent switch to the BH3 mimetic Venetoclax (ABT-199) [151]. The next generation highly selective Bcl-2 inhibitor, Venetoclax, has a greater degree of similarity to the BH3 domains

of Bcl-2 and Bcl-xL. ABT-199 induces caspase dependent apoptosis by disrupting the Bcl-2/BIM complex in variety of haematological malignancies including CLL, Diffuse large B-cell lymphoma (DLBCL), Follicular lymphoma (FL) and Mantle cell lymphoma (MCL) [152]. In a phase I trial, ABT-199 showed 84% overall response rate in patients with relapsed or refractory CLL [153] and is very effective in 17p- and TP53 mutated disease. Interestingly, clinical responses were observed in more than 87.5% of patients in combination with Rituximab [154]. Also combination of ABT-199 with Ibrutinib induces more apoptosis in primary MCL and CLL cells compared to each single agent alone [155]. ABT-199 is effective as a single agent or in combination with Bendamustine, Rituximab or Obinutuzumab for relapsed or refractory CLL. FDA has recently approved ABT-199 for relapsed or refractory and 17p- CLL.

1.1.10.8 Immunomodulatory drugs (IMiDs)

In the1950s, thalidomide was developed to aid sleep and as an anti-emetic during pregnancy. In 1961, many studies identified that thalidomide resulted in abnormalities such as phocomelia, underdeveloped bones, cardiac defects and aplasia and hence the drug was subsequently withdrawn [156]. In 1975, the FDA accepted thalidomide for use in the treatment of lepromatous leprosy [156]. Subsequently, refractory myeloma patients treated with thalidomide achieved 50% reduction in disease. Thalidomide in combination with Dexamethasone and Cyclophosphamide has improved outcomes in refractory or relapsed myeloma [157].

The predominant action of thalidomide in myeloma was thought to be immunomodulation and hence based on structure-activity studies of thalidomide several analogues termed Immunomodulatory agents (IMiDs) have been developed. Structurally, thalidomide and newer IMiDs (such as Lenalidomide and Pomalidomide) have common phthalimide and glutarimide moieties with small structural differences in the phthalimide ring [158]. IMiDs have a wide range of biological activities including anti-angiogenic properties [159], inhibition of nuclear factor-κB (NF-κB), inhibition of anti-apoptotic factors and disruption of the PI3K/AKT pathway [160, 161].

In 2010, the primary target of thalidomide was identified as Cereblon (CRBN) [162]. The gene for CRBN is located on chromosome 3 and encodes a 442 amino acid protein [163, 164]. CRBN functions as an adaptor protein that interacts with CUL4, DDB1, and Roc1 to form an E3 ubiquitin ligase complex [165]. Many studies have demonstrated that following binding of IMiDs, CRBN predominantly interacts with Ikaros (IKZF1) and Aiolos (IKZF3) leading to their degradation [162, 166-168].

The Ikaros family of transcription factors consist of five members: Ikaros (IKZF1), Helios (IKZF2), Aiolos (IKZF3), Eos (IKZF4) and Pegasus (IKZF5) [169]. They all share two common zinc finger domains. Their N-terminal domains aid DNA binding domain whilst their C-termini mediate dimerization [170]. Each member can homo-dimerise or form hetero-dimers with any of the other Ikaros family members [169]. The Ikaros family of proteins are

critical for multiple aspects of haematopoiesis including B-cell maturation and development [171].

Lenalidomide (LEN), a stereo-chemical derivative of thalidomide, has an amino and oxo group in its phthaloyl ring (**Figure 1.3**) [172]. LEN is an immunomodulatory agent that shows potent drug activity in non-Hodgkin lymphoma, multiple myeloma, and DLBCL [173]. It is FDA approved for use in the treatment of multiple myeloma (MM) and myelodysplastic syndromes (MDS) [172]. Whilst it is known that LEN (and other IMiDs) bind to and alter the specificity of Cereblon, the exact mechanism of their action in different diseases remains to be elucidated. Several studies have observed that LEN leads to inhibition of tumour growth by causing growth arrest in the G0/G1 stage of the cell cycle and induction of apoptosis [174]. LEN also has effects on the tumour microenvironment, by reducing the expression of several cytokines, and enhances Th1 type cellular immunity [175].

Recent studies in CLL have shown that LEN has single agent activity. LEN is shown to activate non-malignant immune cells, especially NK and CD4 T-cells and induce anti-leukemic effects in CLL [176]. CC-4047 and CC-5013, both derivatives of LEN, also possess anti-tumour activity by activating/modulating NK cells (upregulation of IL-2), whilst not having any direct cytotoxic effects on tumour cell lines or primary tumour cells [177]. Pomalidomide is also approved for use in the treatment of refractory or relapse myeloma in combination with Cyclophosphamide and Bortezomib [178, 179].

CC-122, is a novel immunomodulatory drug that hosts a glutarimide moiety but lacks the phthalimide ring common to other IMiDs. Like other IMiDs, CC-122 binds to CRBN, leading to degradation of target molecules that include Ikaros and Aiolos [180]. When used to treat DLBCL, CC-122 enhances the expression of IRF7 [181]. It has been also observed that c-Myc expression levels are decreased following CC-122 treatment of high-risk DLBCL patients previously treated with chemo-immunotherapy. Such reduced c-Myc levels have been observed to affect the activity of BCL6 in murine models [182]. Other effects of CC-122 include perturbation of the major histocompatibility complex class II (MHC II) in lymphocytes, and modulation of interferon gene transcription [180]. CC-122 also leads to activation of T-cells and de-repression of interferon (IFN) stimulated genes (ISG) that may contribute to the tumoricidal activity observed in DLBCL cells [183, 184]. Despite these observations, the precise mechanism of how the $CRL4^{CBRN}$ complex functions in the presence of CC-122 is not fully understood. These promising effects of CC-122 have helped in development of newer IMiDs compounds such as CC-220 and CC-885 that are currently in clinical trials. CC-885 has shown anti-proliferative properties in acute myeloid leukaemia and reduces the activity of the translation termination factor GSPT1 [185]. The role or activity of CC-122 on CLL cells is currently under investigation but not yet reported.

1.1.10.9 Future treatments

In recent years there has been a move towards harnessing T-cell driven immune surveillance in the management of cancer. One modality of T-cell directed immunotherapy is the engineering of autologous T lymphocytes *ex vivo* to target specific cell surface antigens expressed by malignant cells, known as chimeric antigen receptor T-cells (CAR-Ts) [186]. This involves harvesting autologous T lymphocytes, prior to genetic modification to allow expression of a cell surface receptor directed against the desired antigen, usually utilising viral or lentiviral techniques. These CAR-Ts are generated *ex vivo*, prior to infusion in to the patient. The CAR consists of a cell surface antigen binding domain, anchored by a transmembrane region bound to an intracellular co-stimulatory signalling domain. Upon antigen binding, activation of the intracellular region allows propagation and expansion of cancer cell directed T-cells *in vivo.* CAR-Ts expressing anti-CD19 have shown significant success in paediatric relapsed B cell Acute Lymphoblastic Leukaemia (B-ALL), the leading cause of cancer related death in children, with clinical trials reporting remission rates of 70-90%. However, the use of CAR-T therapy is tempered to some extent by off target effects. CD19 is expressed both in normal and malignant B cells, and as a result, CAR-T therapy can cause complete B cell depletion. Moreover, large tumour burden has been associated with 'cytokine release syndrome' (CRS), due to substantial release of cytokines including IL6 following massive T-cell activation. As well as leading to fluid shifts due to capillary leakage, CRS is associated with neurotoxicity, frequently requiring intensive care support.

These effects can in part be ameliorated by the IL6 directed monoclonal antibody, Tocilizumab, however CAR-T therapy may be more suited as a means of minimal residual disease (MRD) clearance [187].

Unfortunately, anti-CD19 CAR-T therapy in CLL has not demonstrated the same level of success as in B-ALL. This may be a result of CLL related B cell anergy leading to immune dysfunction, as well as T cell defects owing to the increased age in the CLL patient cohort. Trials are currently in development using CARs directed against alternative antigens such as ROR1 [57, 188].

1.1.11 Summary

Despite significant advances in the therapy of CLL the disease remains incurable. The advent of novel drugs does, however, provide a unique opportunity to explore relevant interactions and synergies. Such understanding may lead to the development of rational, effective, and personalised combinations for better outcomes. Our laboratory group has been studying the differential proliferation response of immunomodulatory drugs. This is described in chapter 3 **(Figure 3.1).**

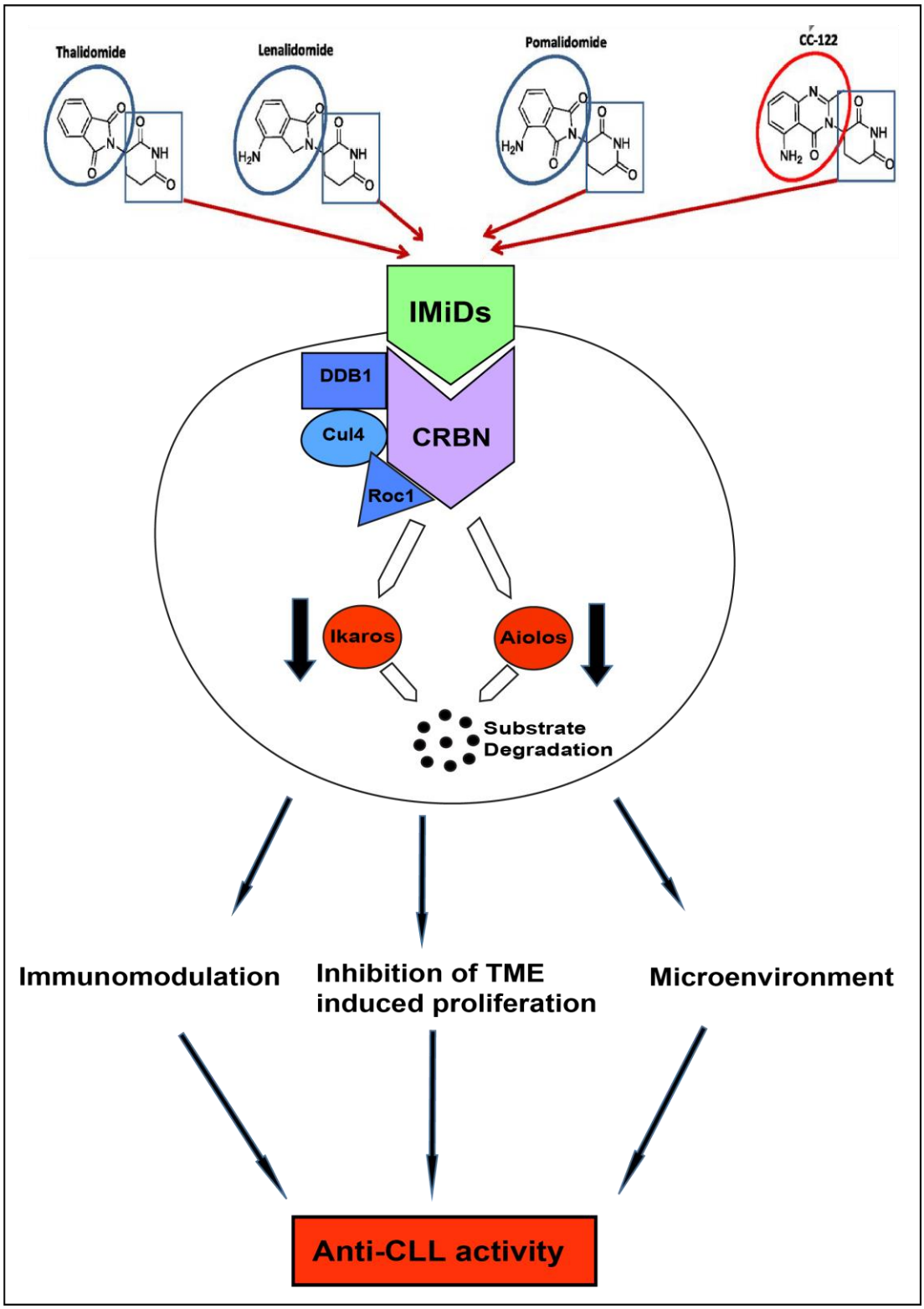


Figure 1.3: Structure of IMiDs and their mechanisms of action.

1.2 Hypothesis

Preliminary observations in our lab have demonstrated that CC-122 has activity in CLL and may be more potent than Lenalidomide as a single agent. We hypothesised that a better functional and mechanistic understanding of the activity of CC-122 on specific pathways that are critical for CLL cell survival can lead to information that is relevant to develop rational drug combinations in future strategies and help personalised approaches for therapy.

1.3 Aims

The **aims** of this thesis, therefore, are:

1. Compare the effects of LEN and CC-122 on B-CLL cell lines and primary CLL cells with regards to their growth, survival, and apoptosis.
2. Examine the effects of LEN and CC-122 treatment on BH3 containing apoptotic family proteins in B-CLL cell lines and primary CLL cells.
3. Understand the modulation of BCR signalling proteins upon anti-IgM stimulation in CLL cells in the context of treatment with LEN or CC-122.
4. To study the effects and relevance of epigenetic priming for LEN or CC-122 responses in B-CLL cell lines and primary CLL cells.

The following chapter (**Chapter 2**) describes the general materials and methods used in this study. Four experimental chapters are followed by overall conclusions and suggestions for future work.

Chapter 2 : Materials and Methods

This chapter describes general materials and laboratory methodologies employed to test the hypothesis and to answer research questions. Also described are the cell culture and molecular biology techniques employed throughout this study. Details of techniques that are specific to each chapter, or where general techniques were modified slightly, are addressed in the relevant chapters. Buffer recipes, drugs, reagents, relevant software is detailed in **Appendix D** and disease characteristics of primary CLL cases provided in **Appendix E**.

2.1. Cell Culture

2.1.1. Culturing of cell lines

Cell lines referred to in this thesis, their origin, growth media, and doubling times, are summarised in **Table 8**. They were purchased from Leibniz Institute (DSMZ) German Collection of Microorganism and cell cultures (Germany) except for CD154 expressing fibroblasts that were purchased from the American type culture collection (ATCC), USA.

All cell lines were cultured and maintained in vented culture flasks under aseptic conditions within a class II tissue culture cabinet. Complete media was prepared by adding L-Glutamine (final concentration 2mM), foetal bovine serum (10% v/v), penicillin (1000 units/mL) and Streptomycin (1 mg/mL) to the relevant base media when required. Cell densities and viability were

determined by trypan blue dye exclusion unless otherwise stated. All cell lines were tested frequently for mycoplasma.

2.1.2. Maintenance of suspension cell lines

Suspension cultures were maintained in Roswell Park Memorial Institute (RPMI) medium for EHEB and HG3 cells or Iscove's modified Dulbecco's medium (IMDM) media for MEC1 and MEC2 cells. Cells were passaged every three days at a 1:3 ratio of cells:new complete media. Alternatively, if required, they were seeded at a specific cell density in fresh complete media. Cells were maintained in a humidified incubator at 37˚C with 5% carbon dioxide (CO_2).

2.1.3. Maintenance and culture of CD154 expressing fibroblasts

CD154 expressing fibroblast cells were maintained in supplemented Dulbecco's Modified Eagle's Medium (DMEM), as described previously [189]. For passage, media was removed and discarded and the monolayer washed with phosphate buffer saline (PBS). Trypsin-Ethylene-diamine-tetra-acetic acid (EDTA) (1-2 mL) was added to the cells and incubated at 37˚C for 5 minutes. The flask was gently tapped to dislodge the cells for collection and centrifugation at 550×*g* for 5 minutes. The pellet was re-suspended with 5 mL of fresh complete DMEM media. To ensure confluence in a culture flask with 9.5 mL complete media, 0.5 mL of cells was added and the cells maintained in a humidified incubator at 37˚C with 5% CO_2.

Table 8: Characteristics of cell lines used in this study.

Cell line	Origin	Doubling time (h)	Karyotype	Reference
EHEB	69/Female/B-CLL	48-72	Normal or +11	[190]
MEC2	62/Male/B-CLL	31	12-, 17p-	[191]
MEC1	61/Male/B-CLL	40	12-, 17p-	[191]
HG3	70/Male/B-CLL	50-70	13q- ×2	[192]
MAVER-1	77/Male/MCL	23	t(11;14)	[193]
JeKo-1	78/Female/MCL	33	t(10;11;14)	[194]

2.1.4. Measuring cell viability and counting

Cell viability and counts were determined by using trypan blue exclusion assay to identify and enumerate live (unstained) and dead (stained) cells. In this assay, the dye readily penetrates dead or non-viable cells through ruptured cell membranes, and stains them dark blue, whereas live or viable cells are impermeable due to the presence of an intact cell membrane. The latter cells tend to exhibit a shiny bright halo. 20 µL of 1:1 diluted cell suspension with trypan blue were loaded onto a counting slide. A dedicated Nexcelom apparatus (Nexcelom Biosciences, UK) was used to measure cell count and viability.

2.1.5. Irradiation of CD154 fibroblasts

After harvesting, as described above, cells were irradiated (72 Gray by Gammacell® 3000 Elan) in two 10-minute pulses. Fibroblasts were counted as described and plated out at $4x10^5$ cells/mL per well in a 24 well plate and 5×10^4 cells per well in a 96 well plate in 100 µl. The plates were then incubated overnight at 37˚C, 5% CO_2 to allow the cells to adhere.

2.1.6. Cryopreservation of cell lines

Cell banks of each cell line at a low passage were prepared for use throughout the study. Highly viable cells were centrifuged at 500×*g,* 20˚C for 5 minutes. Supernatant was discarded and the pellet was re-suspended with ice-cold complete growth media such that the concentration of viable cells was $4x10^7$ per mL. An equal volume of ice-cold growth media with 20% Dimethyl sulfoxide (DMSO) was gradually added to the cell suspension in a drop-wise fashion over the course of 30 minutes. 1 mL of cell suspension was transferred to cryovials such that each contained $2x10^7$ cells. The vials were stored at -80ºC overnight and transferred to -150˚C the following day until further use.

2.1.7. Revival of cryopreserved cells

Frozen cells were quickly thawed by gently swirling in a 37˚C water bath. Thawed cells were transferred into pre-chilled 20 ml universal tubes and then diluted by adding cold complete growth media drop-wise. Cells were pelleted at 4˚C, 500×*g* for 5 minutes. The cell pellet was re-suspended in 5 mL

complete media and incubated in a humidified incubator at 37˚C with 5% CO_2.

2.1.8 Thawing and culturing of primary CLL cells

Cryopreserved primary CLL samples, obtained from the Liverpool Leukaemia biobank, were thawed on ice then transferred into pre-chilled labelled 20 mL universal tubes placed on ice. Ice-cold RPMI-1640 supplemented with 1% bovine serum albumin (BSA) (thawing media) was added drop-wise to the cells for 30 minutes with gentle agitation. The tubes were centrifuged at 550×*g* at 4˚C for 5 minutes and the pellet was washed with 5 mL of complete RPMI1640 media. The cells were re-suspended with 5 mL of complete media and allowed to recover for one hour in a humidified incubator at 37˚C with 5% CO_2. Cells were counted by trypan blue dye exclusion and the concentration was adjusted according to the specific experimental requirements. Ethical approval for the use of anonymised patient samples and data to perform this research was obtained by the Liverpool Leukaemia biobank.

2.2 Cell proliferation by BrdU assay

Cells were seeded at a density of 0.3-1×10^4 (cell lines) or 5×10^5 (primary CLL cells) cells in each well of a 96 well tissue culture plate. Cells were labelled by the addition of 3 µg/mL of BrdU reagent (Roche, Germany) and incubated according to the manufacturer's protocol. Following the incubation, plates were centrifuged at 300×*g* for 10 minutes, the supernatant was discarded and the plates were dried at 60˚C for 60 minutes. Then, 200 µL of FixDenat solution was added and the plates incubated for 30 minutes at

room temperature. The solution was removed and 100 µL of anti-BrdU mouse monoclonal antibody (diluted 1:100 with antibody dilution solution) was added and incubated for 90 minutes at room temperature. The plate was then washed three times with wash buffer (1xPBS) after which 100 µL of HRP substrate was added and incubated at room temperature for approximately 15–30 minutes to allow the colour to develop. 20 µL of 1 M Sulphuric acid (H_2SO_4) was then added to each well to stop the reaction. Finally, the absorbance was measured at 450 nm by using an enzyme-linked immunosorbent assay (ELISA) microplate reader (Bio-Tek, UK).

2.3 Protein electrophoresis

2.3.1 Cell lysate preparation

Cells were harvested and centrifuged at 550×*g* for 5 minutes, and the pellet then washed with 0.5 mL ice-cold PBS. The cell pellet was lysed with clear sample buffer (CSB) (1% SDS, 125 mM Tris pH 6.8, 5 mM EDTA, 10% glycerol, Protease and Phosphatase inhibitor). The lysed cells were sonicated on ice and then centrifuged for 10 minutes at 14000x*g,* 4˚C. The protein concentration of samples was measured and the clear lysates stored at 20˚C.

2.3.2. Protein quantification by Bradford assay

Total protein concentration was determined using a Bio-Rad DC protein assay kit (Bio-Rad laboratories Ltd, UK) according to the manufacturers' recommendations. In brief, a series of pre-made BSA protein standards (0, 0.5, 1, 1.5, 2, 2.5 and 3 mg/mL) were prepared. First, 5 µL of sample or

standard (in triplicate) was placed in a 96 well plate. Then, reagent A and reagent S were prepared at 1:50 dilution respectively and 25 µL of this mixture was added to each well followed by the addition of 200 µL of reagent B. The plate was gently agitated to ensure the reagents were mixed and then incubated at room temperature for 15 minutes. The absorbance was measured at 650 nm using a plate reader (Bio-Tek, UK).

2.4 Western blotting

2.4.1 Sample preparation

The volume of cell lysate required to yield 10 µg of protein was calculated and the volume made up to 20 µL with CSB buffer and 5x loading dye (5% SDS, 625 mM Tris pH 6.8, 50% glycerol, 5% β-mercaptoethanol and 0.04% bromophenol blue). Samples were heated at 95˚C for 5 minutes and separated on an appropriate percentage polyacrylamide gel.

2.4.2 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The resolving and stacking gel compositions are described in **Table 9 & 10**. When set, the gels where assembled in a Bio-Rad electrophoresis chamber and the central reservoir filled with 1×SDS running buffer (0.1% SDS, 25 mM Tris and 192 mM glycine). Then, equal volumes of protein (calculated as described previously) were added to the wells along with the Precision Plus Protein™ Kaleidoscope™ marker (Bio-Rad laboratories Ltd, UK) in the first lane. The unused wells were filled with an equal volume of 1X loading dye. Gels where run at 30 mA for approximately one hour.

Table 9: Resolving gel compositions used for protein gel electrophoresis.

% gel	**8%**	**10%**	**12%**
ddH_2O (mL)	9.7	8.3	7
30% acrylamide (mL)	5.3	6.7	8
Resolving buffer (mL)	5	5	5
10% APS (µL)	200	200	200
TEMED (µL)	20	20	20

Table 10: Stacking gel (5%) composition used for protein gel electrophoresis.

Components	**Volume**
ddH_2O (mL)	2.9
30% acrylamide (mL)	0.85
Resolving buffer (mL)	1.25
10% APS (µL)	25
TEMED (µL)	5

The separated proteins were transferred from the gels to PVDF (Roche, Germany) membranes pre-soaked in methanol. Before transfer, sponges and Whatman filter papers were soaked in ice-cold transfer buffer (25 mM Tris and 192 mM glycine) (Geneflow, UK). A sandwich of sponge: filter paper: gel:

PVDF membrane: filter paper: sponge was prepared and clamped together. The prepared cassette was then placed into the transfer tank (the gel being closest to cathode) along with ice-cold transfer buffer and an ice block. Proteins where transferred at 400 mA for one hour.

Following transfer, the membrane was transferred into blocking buffer (5% non-fat milk or BSA in Tris-buffered saline with 0.1% Tween 20 (TBS-T)) for 60 minutes with gentle agitation. Membranes were then incubated overnight with primary antibody diluted in blocking buffer at 4˚C with agitation. The final antibody concentrations are listed in **Table 11**. The following day, the membrane was washed three times (for 5 minutes each) with TBS-T and incubated with the relevant horseradish peroxidase (HRP) conjugated secondary antibody diluted in blocking buffer at room temperature for 60 minutes with agitation. The membrane was then washed again with TBS-T (3 times for 5 minutes each).

To visualise the target protein bands, the membrane was incubated with WESTAR® Supernova Enhanced Chemiluminescence (ECL) substrate reagent (Geneflow, UK). The image was developed using an LAS-1000 (Fujifilm, Japan). Densitometry was performed using ImageJ software.

2.5 Statistical analysis

The data in this thesis were analysed by using GraphPad Prism 6 and ImageJ (western) software. Details of analyses are indicated in the relevant results section.

This section highlights the materials and methods that generally apply to most chapters. Where applicable, other specific and relevant methods and materials are described in appropriate chapters.

Table 11: List of primary and secondary antibodies used in this study.

Primary Abs	Type	Host	Dilution	MW (kDa)	Supplier
Aiolos (O-21)	Polyclonal	Rabbit	1:3000	58	Santa Cruz Biotechnology
Ikaros (E-2)	Monoclonal	Mouse	1:2000	50	Santa Cruz Biotechnology
Cyclin A	Monoclonal	Mouse	1:1000	55	Cell signaling technology®
Cyclin B1	Monoclonal	Mouse	1:1000	55	Cell signaling technology®
Cyclin E	Monoclonal	Mouse	1:1000	48	Cell signaling technology®
Anti-$p21^{WAF1}$	Monoclonal	Mouse	1:1000	21	Merck Millipore
Mcl-1	Monoclonal	Rabbit	1:2000	35 & 40	Cell signaling technology®
Bcl-2	Monoclonal	Rabbit	1:2000	26	Cell signaling technology®
Bcl-xL	Monoclonal	Rabbit	1:2000	30	Cell signaling technology®
Bax	Monoclonal	Rabbit	1:2000	20	Cell signaling technology®

Primary Abs	Type	Host	Dilution	MW (kDa)	Supplier
Bak	Monoclonal	Rabbit	1:2000	25	Cell signaling technology®
Bim	Monoclonal	Rabbit	1:2000	12,15 & 23	Cell signaling technology®
p-PLCϒ2	Polyclonal	Rabbit	1:2000	150	Cell signaling technology®
PLCϒ2 (B-10)	Monoclonal	Mouse	1:2000	150	Santa Cruz Biotechnology
p-Akt	Monoclonal	Rabbit	1:2000	60	Cell signaling technology®
Akt (Pan)	Monoclonal	Mouse	1:2000	60	Cell signaling technology®
p-Erk (E-4)	Monoclonal	Mouse	1:2000	42 & 44	Santa Cruz Biotechnology
p44/42 MAPK (Erk1/2)	Polyclonal	Rabbit	1:2000	42 & 44	Cell signaling technology®
c-Myc	Monoclonal	Rabbit	1:5000	57	Abcam
p27	Monoclonal	Mouse	1:1000	27	Santa Cruz Biotechnology
Anti-β-actin	Monoclonal	Mouse	1:10,000	42	Sigma
Secondary Abs					
Anti-mouse IgG	Horseradish peroxidase conjugate	Goat	1:5000		Santa Cruz Biotechnology
Anti-rabbit IgG	Horseradish peroxidase conjugate	Goat	1:5000		Santa Cruz Biotechnology

Chapter 3 : Cell cycle related activities of IMiDs in CLL

3.1 Introduction

In recent years, IMiDs such as LEN have emerged as important therapeutic agents in various lymphoid malignancies [160, 195]. Several studies have illustrated that Lenalidomide inhibits cell proliferation in several B-cell lines, (e.g. Namalwa, LP-1 and U266), primary myeloma cells and cell lines (e.g. H929) [196, 197]. It is reported that LEN and Pomalidomide induce growth arrest by increasing expression of the cyclin dependent Kinase (CDK) inhibitor p21 in Namalwa cells [196] and U266 [197]. Other studies suggest that upregulation of p21 by LEN and subsequent binding to CDK2 inhibits cyclins resulting in cell cycle arrest [198]. More recent studies have shown that LEN inhibits the growth of primary CLL cells [199], and that the drug has activity in high-risk disease (11q- or 17p-) and in relapsed/refractory CLL patients [200-202]. More recently, CC-122 a novel IMiD compound with pleiotropic effects, has been demonstrated to have anti-tumour and immunomodulatory activities in a number of DLBCL cell lines [180] and is showing promising activity in various haematological malignancies. Hence understanding its effects on CLL cells and their critical biological pathways is of relevance.

Our lab has been interested in the activity of novel IMiD analogues such as CC-122 in CLL. In recent unpublished studies conducted by Dr. Jemma

Blocksidge & Dr. Mark Glenn, CC-122 is consistently superior to LEN in inhibiting proliferation of CLL cells *in vitro*. In these experiments 'responsiveness' to CC-122 or LEN was defined as >25% inhibition of proliferation compared to untreated or DMSO control. Such activity allows categorisation into 4 distinct response cohorts based on differential anti-proliferative responses of primary CLL cells to LEN or CC-122 (**Figure 3.1**). In this chapter, the effects of the two drugs on CLL cells with respect to cell cycle and relevant proteins are compared, characterised, and reported.

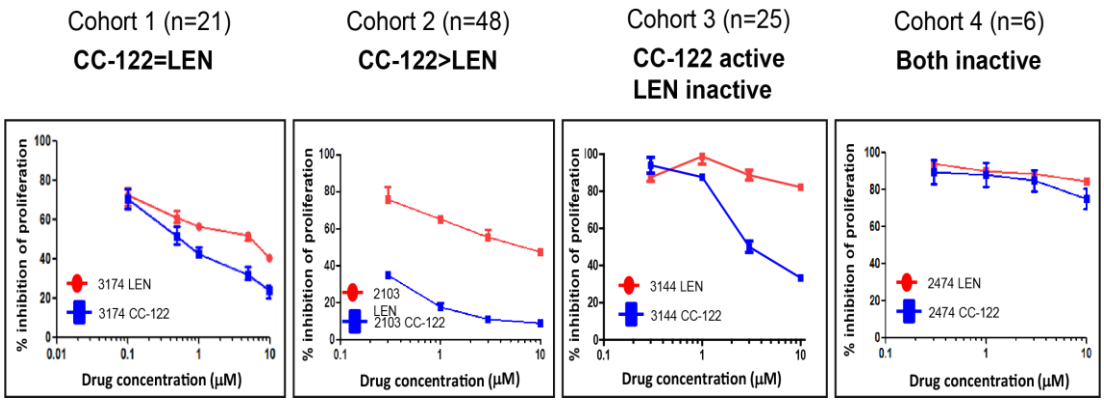


Figure 3.1: Activity based definition of CLL cohorts in response to LEN and CC-122.

As well as confirming the previously demonstrated anti-proliferative activities of LEN and CC-122 in primary CLL cells representative of the 4 cohorts, we additionally studied 4 B-CLL cell lines (EHEB, MEC2, MEC1 and HG3) to understand the effects of these drugs in the disease. We acknowledge that the cell lines indicated may not be truly representative of primary CLL cells and are derived from patients likely in the pro-lymphocytic transformation phase. Nevertheless, in the absence of other representative cell line models

that are a true reflection of CLL behaviour, they are the closest models for *in vitro* studies and are widely used by researchers in the field.

The **aims** of this chapter, therefore, were:

To compare the effects of the novel IMiD CC-122 to LEN with respect to proliferation and effects on cell cycle regulators.

a) In B-CLL cell lines and
b) In primary CLL cases representative of the 4 identified response cohorts

3.2 Methods

3.2.1. Studies of the effect of IMiDs on cell lines

Cell lines were seeded at a density of 0.5×10^5/mL in 96 well plates to determine the effects of CC-122 or LEN on cell proliferation. Cells were treated with the IMiDs using a range of concentrations (0.3-10 μM) and proliferation assessed in a BrdU incorporation assay (see below). To determine the effects of the IMiDs on relevant proteins in our panel of cell lines, cells where seeded at 0.5×10^6/mL and treated with IMiDs (1 and 10 μM) for 24 hours. The expression of target proteins in untreated and treated samples was examined by immunoblotting (see below).

3.2.2. Treatment of primary CLL cells with Lenalidomide and CC-122

Freshly thawed primary CLL cells were counted and plated out on previously irradiated CD40 expressing fibroblast monolayers at a density of 5×10^6 cells/mL for proliferation studies and 7.5×10^6 cells/mL for western blot experiments respectively. Cells were plated in complete RPMI medium supplemented with recombinant human interleukin-21 (rhIL-21) at a final concentration of 25 ng/mL, in accordance with a previously established protocol in our lab, to promote CLL cell proliferation and survival *in vitro* [203]. The cells were initially treated with increasing concentrations (0.3 μM–10 μM) of LEN or CC-122 for 3 days. Following the incubation, 50% of spent medium was removed and replaced with fresh media containing drug, rhIL-21, and BrdU and incubated for a further 3 days and cell proliferation measured using a BrdU assay (**Figure 3.2**).

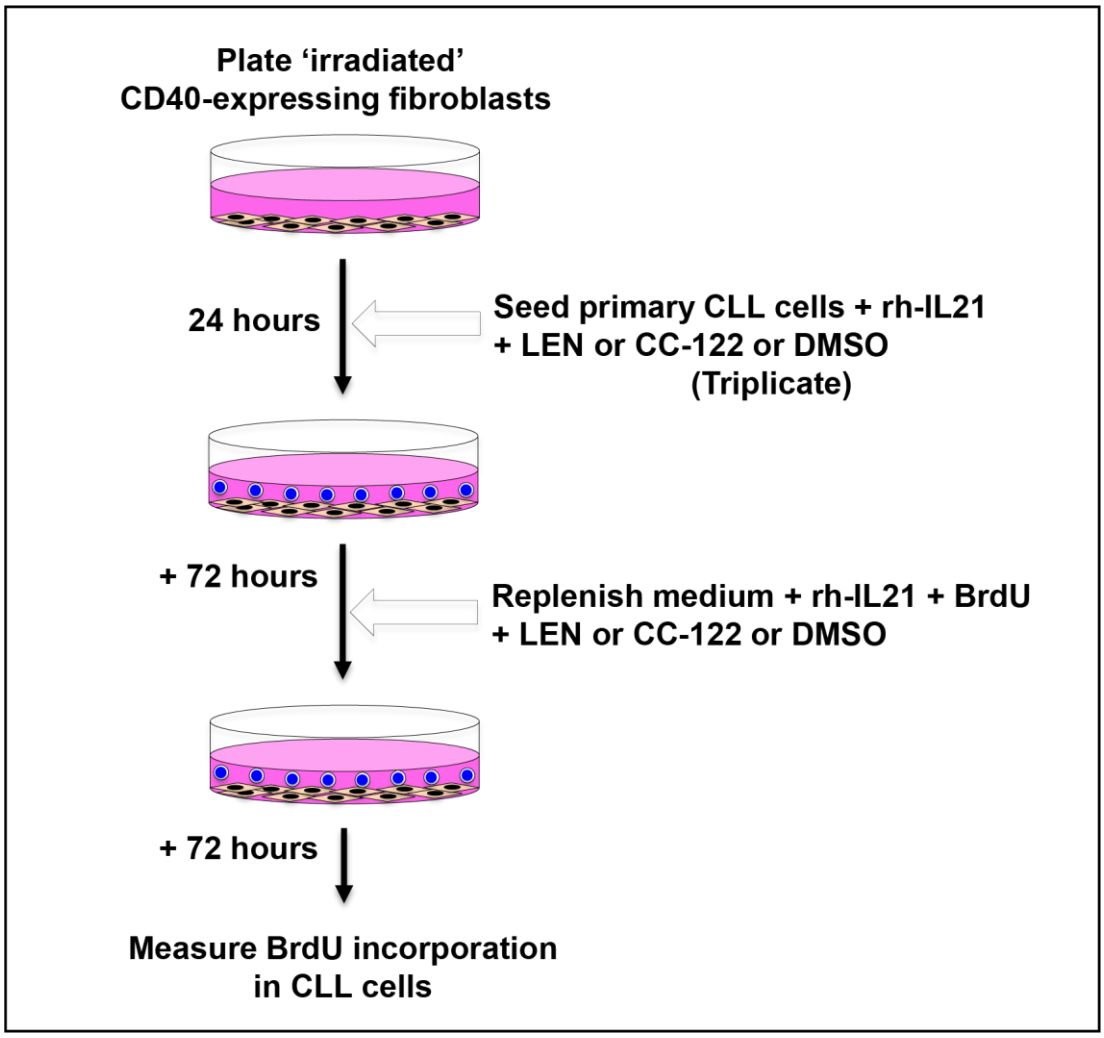


Figure 3.2: Schematic representation to illustrate the protocol for maintenance of primary CLL cells in culture.

3.3. Results

To explore the anti-proliferative activities of LEN and CC-122 (IMiDs), the above-mentioned CLL derived cell lines were initially tested.

3.3.1. Effects of Lenalidomide and CC-122 on proliferation in B-CLL cell lines

It is well established that LEN inhibits the proliferation of various B-cell and myeloma cell lines [196]. Firstly, we sought to examine proliferation at serial time points in the B-CLL cell lines EHEB, MEC2, MEC1 and HG3 to assess drug response. Proliferation responses were examined up to 72 hours since the cell lines had a doubling time of approximately 30-72 hours. Cells were treated with a range of concentrations of LEN or CC-122 (0.3-10 μM) for up to 72 hours and proliferation was then measured by a BrdU incorporation assay. The percentage of proliferating cells was determined by comparison to untreated cells at each time-point.

Figure 3.3 shows that both LEN and CC-122 begin to inhibit proliferation in EHEB, MEC2, MEC1 and HG3 cells after 24 hours treatment in a dose dependent manner. However, maximum inhibition was achieved after a 72-hour incubation with both drugs. LEN and CC-122 showed three distinct proliferation patterns across the studied B-CLL cell lines. Upon treatment, the percentage of actively proliferating cells decreased in a dose dependent manner for all cell lines except HG3 cells. EHEB cells **(Figure 3.3 A)** were highly sensitive to low doses of both LEN and CC-122 with comparable sensitivity. MEC1 and MEC2 cells exhibited similar proliferation patterns

upon exposure to LEN or CC-122 and likely reflect their origins from the same patient. While both cell lines were sensitive to both drugs, CC-122 was clearly more effective than LEN **(Figure 3.3 B & C)**. In contrast, HG3 cells were relatively resistant to both drugs **(Figure 3.3 D)**. At higher concentrations both drugs showed some inhibition of proliferation (data not shown). We present data generated with concentrations up to 10 µM that reflects physiologically achievable doses in the clinic [204]. The data for earlier (24 and 48 hour) time-points is shown elsewhere (see appendix **A-1**).

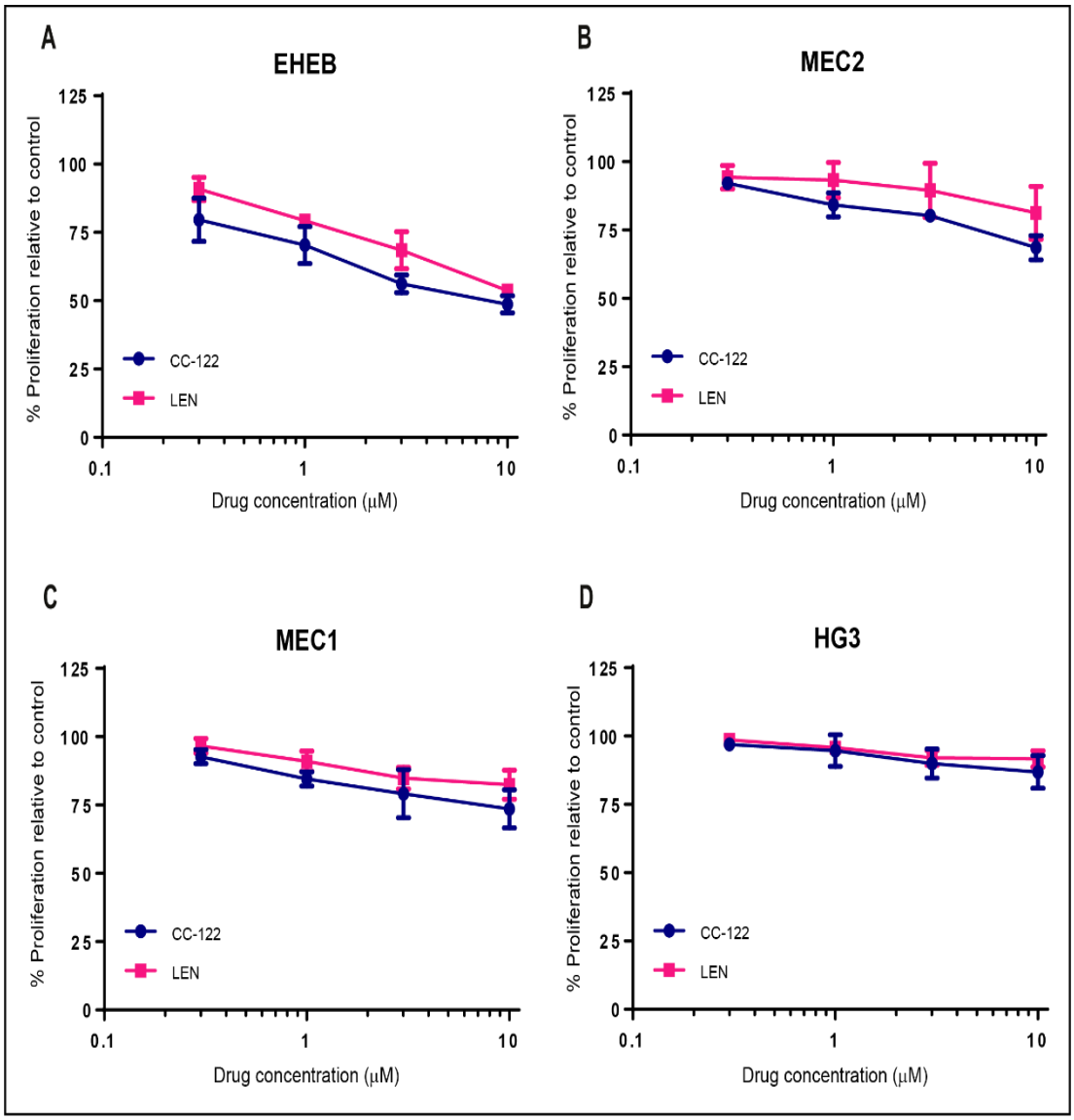


Figure 3.3: Effects of Lenalidomide and CC-122 on proliferation in B-CLL cell lines. B-CLL cell lines were treated with various concentrations from 0.3, 1, 3 and 10 μM of LEN or CC-122 or DMSO (negative control) and incubated at 37˚C for up to 72 hours. Cell proliferation was measured at 24, 48 and 72 hours after treatment by using BrdU incorporation assay. Data is represented as percentage of cell proliferation relative to the negative control. Cell lines treated were: **A.** EHEB; **B.** MEC2; **C.** MEC1; **D.** HG3; Pearson correlation coefficient analysis was used to determine statistically significant differences between LEN and CC-122 (n=3).

3.3.2. Levels of cyclin proteins expression in Lenalidomide and CC-122 treated B-cell lines

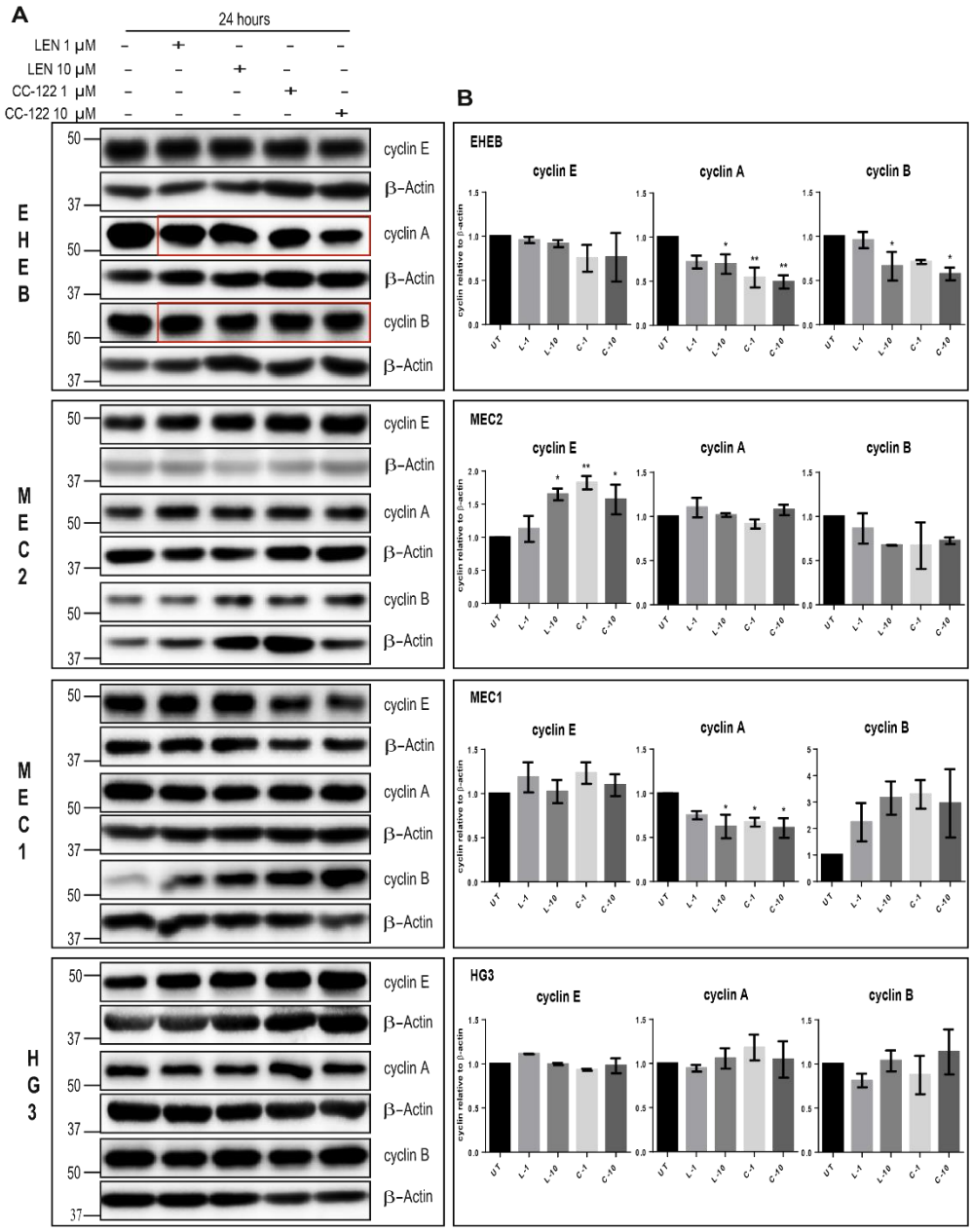
Because of the observed variable inhibition of proliferation by LEN and CC-122 we sought to examine levels of cyclin proteins that dictate cell cycle transitions. We examined the effects of these drugs on the expression of cyclin proteins to see if there was any correlation with the observed proliferation profiles in EHEB, MEC2, MEC1 and HG3 cells.

Previous studies have demonstrated that both drugs consistently downregulate Ikaros and Aiolos transcription factors within 24 hours. The levels of Aiolos and Ikaros were studied in parallel to ensure the drugs were active. We examined the effects of both drugs at 24 and 72 hour time points on cyclin levels in the 4 cell lines. For western blot analysis the cell lines were treated with 1 µM and 10 µM of LEN, CC-122 or DMSO control for 24 and 72 hours and cyclin A, B and E levels were determined respectively. For densitometry purposes, the expression of cyclins was compared to β-Actin levels.

Figure 3.4 shows that both LEN and CC-122 had similar effects on levels of cyclin expression, but each cell line showed variable effects at different time points. At 24 hours, levels of cyclins A and B were significantly decreased in EHEB cells. In MEC1 cells, only cyclin A expression was significantly reduced. In contrast, cyclin E levels increased with the treatment (**Figure 3.4 A**). At 72 hours, both drugs significantly inhibited cyclin A and B levels in

EHEB cells. But cyclin A levels alone were considerably reduced in MEC1 cells (**Figure 3.4 C**).

The densitometry plots show the results of 2 independent experiments. Close examination of the levels of cyclins by densitometry suggests a relative decrease in cyclin B levels at 24 hrs. HG3 cells in contrast did not show any significant changes in cyclin levels and likely reflects the non-responsiveness of this cell line to IMiDs.


A
24 hours
LEN 1 µM
LEN 10 µM
CC-122 1 µM
CC-122 10 µM
EHEB
MEC2
MEC1
HG3
cyclin E
β-Actin
cyclin A
cyclin B
50
37
B
EHEB
MEC2
MEC1
HG3
cyclin E
cyclin A
cyclin B
cyclin relative to β-actin
UT
L-1
L-10
C-1
C-10

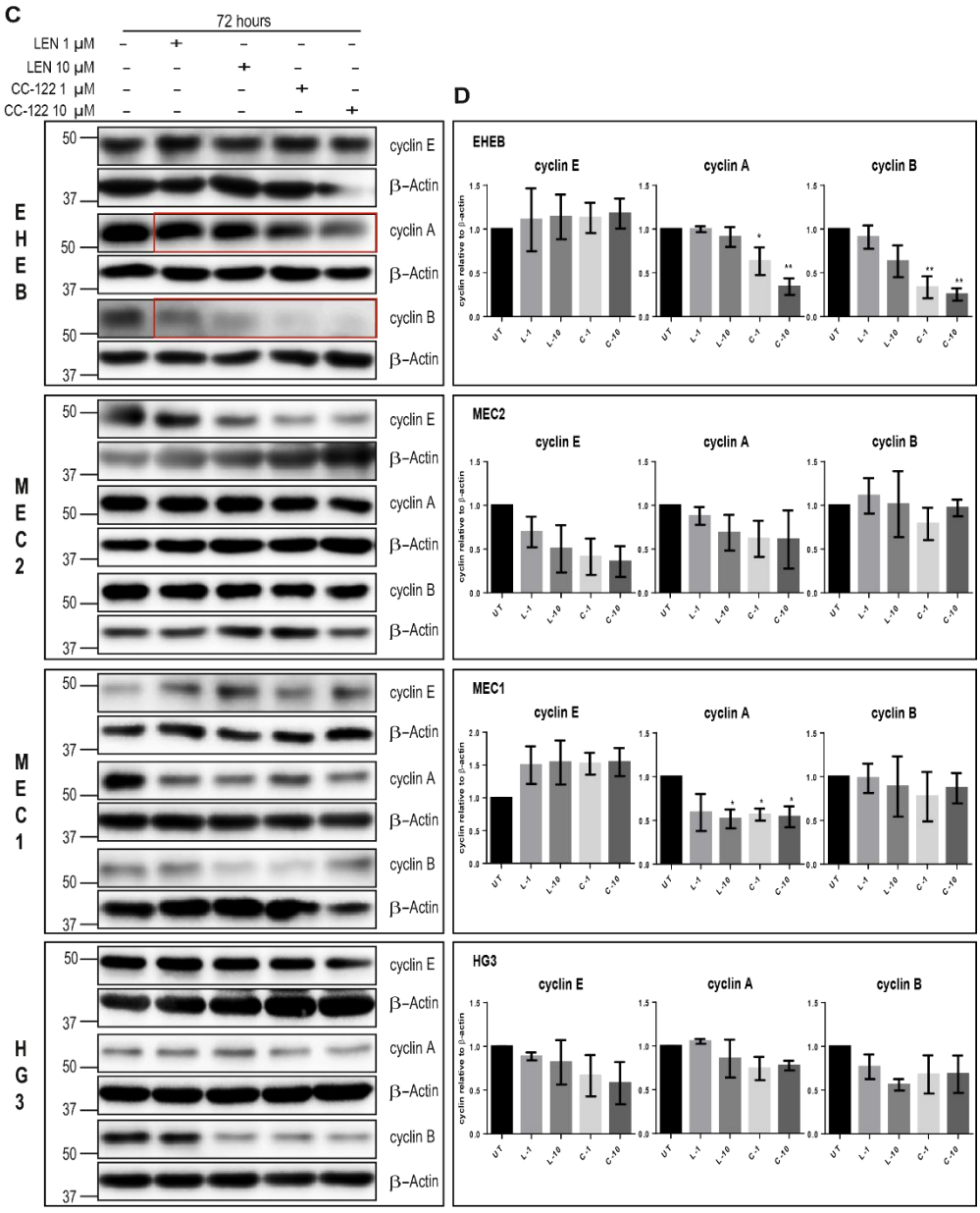


Figure 3.4: Expression levels of cyclin proteins in Lenalidomide or CC-122 treated B-cell lines. EHEB, MEC2, MEC1 and HG3 cells were left untreated (UT) or treated with LEN or CC-122 (1 µM and 10 µM) and incubated for 24 and 72 hours. Cells were collected at indicated time points, lysates separated by SDS-PAGE and immunoblotted for cyclins E, A, B and β-actin (loading control). One-way analysis of variance (ANOVA) and Dunnett's multiple comparison test were used to determine statistically significant differences from UT. **p*<0.05; ***p*<0.01; ****p*<0.001; (mean ± standard error of the mean [SEM]; n=2). **A & C**. Western blots; **B & D** Densitometry; **A & B** are **24 hours** and **C & D** are **72 hours.**

3.3.3. Lenalidomide and CC-122 induce p21 expression in B-CLL cell lines

The findings above suggest that LEN and CC-122 may inhibit the cell cycle transitions to S and G_2/M phases of the cell cycle in EHEB cells. Hence we additionally monitored the expression of the CDK inhibitors $p21^{Waf1}$ and $p27^{Kip1}$. $p21^{Waf1}$, in particular, is a universal cyclin inhibitor which functions both in the G_1 and G_2 phases and restricts the entry of cells into M phase [205]. Recent studies have reported that p21 expression is increased in Namalwa [196] and U266 [197] cells after 24 hours of treatment with LEN. Therefore, we next analysed p21 expression in B-CLL cell lines following treatment with either LEN or CC-122 (1 µM and 10 µM) to investigate if there was any correlation between p21 expression and the observed proliferation patterns and changes in cyclin expression.

Figure 3.5 shows that at 24 and 72 hours, LEN did not induce p21 expression in the cell lines examined. CC-122, in contrast, upregulated p21 expression in all the B-cell lines at 24 hours. At 72 hours, the upregulated p21 expression was only maintained in EHEB cells, but not in MEC2, MEC1 or HG3 cells. This observation is in keeping with the better anti-proliferative response observed in EHEB cells that showed the greatest degree of inhibition of proliferation at 72 hours, which likely reflects the higher sustained levels of p21. The lack of sustained induction of p21 expression in MEC1, MEC2 and HG3 cells likely explains the inferior responses of these lines to CC-122 and LEN. Although there does seem to be a relative induction of p21

in all cells using CC-122 (1 and 10 µM) at 24 hours this does not seem to clearly correlate with proliferation responses.

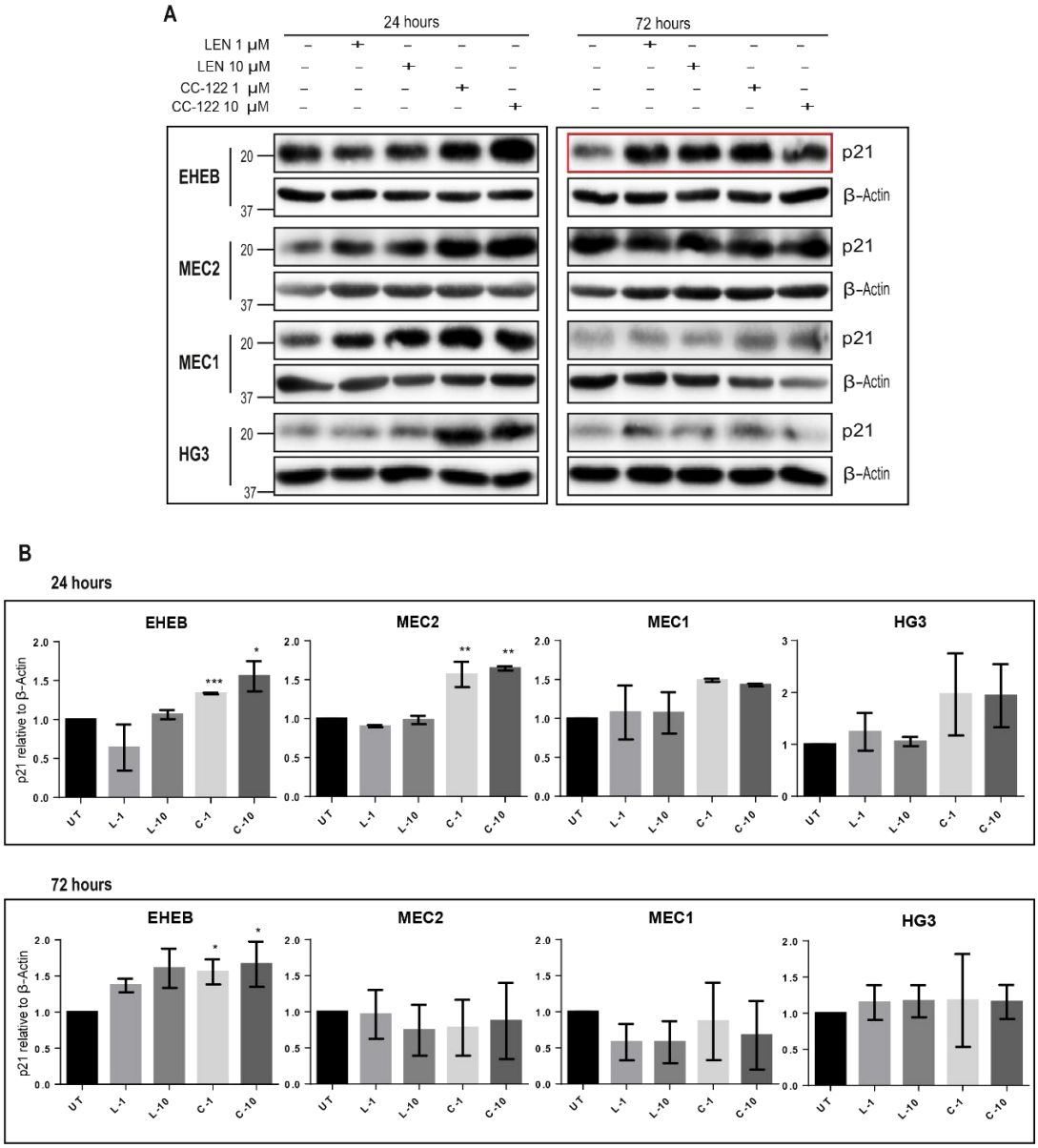


Figure 3.5: Lenalidomide and CC-122 induce p21 expression in B-CLL cell lines. EHEB, MEC2, MEC1 and HG3 cells were left untreated (UT) or treated with either LEN or CC-122 (1 and 10 µM) and incubated for 24 and 72 hours. Levels of p21 and β-actin were then examined by western blot. One-way ANOVA and Dunnett's multiple comparison test were used to determine statistically significant differences from UT. **p*<0.05; (mean ± SEM; n=2). **A.** Western blots; **B.** Densitometry;

3.3.4. Primary CLL cells show variable inhibition of proliferation upon treatment with Lenalidomide and CC-122.

Despite the fact that primary CLL cell are resistant to apoptosis and have long survival time *in vivo* they undergo spontaneous apoptosis *in vitro* [206]. It is well established that a combination of CD40 expressing fibroblasts with IL-4 as a co-stimulant restricts spontaneous apoptosis and promotes longer CLL cell survival *in vitro* [207-209]. Recently Maria et al., have reported that a combination of CD40L and rhIL-21 induces proliferation in CLL cells [210]. Studies in our lab using CD40/rh-IL21 corroborate such findings. We hence, utilised the latter combination to study the effects of LEN and CC-122 on proliferation of primary CLL cells. Patient derived CLL cells were plated on CD40 expressing fibroblast monolayers and media supplemented with rhIL-21, and LEN or CC-122 and incubated for 72 hours. Subsequently, 50% of spent media was removed and replaced with fresh media containing rhIL-21 and BrdU and incubated for a further 3 days. Cell proliferation was subsequently measured in a BrdU incorporation assay.

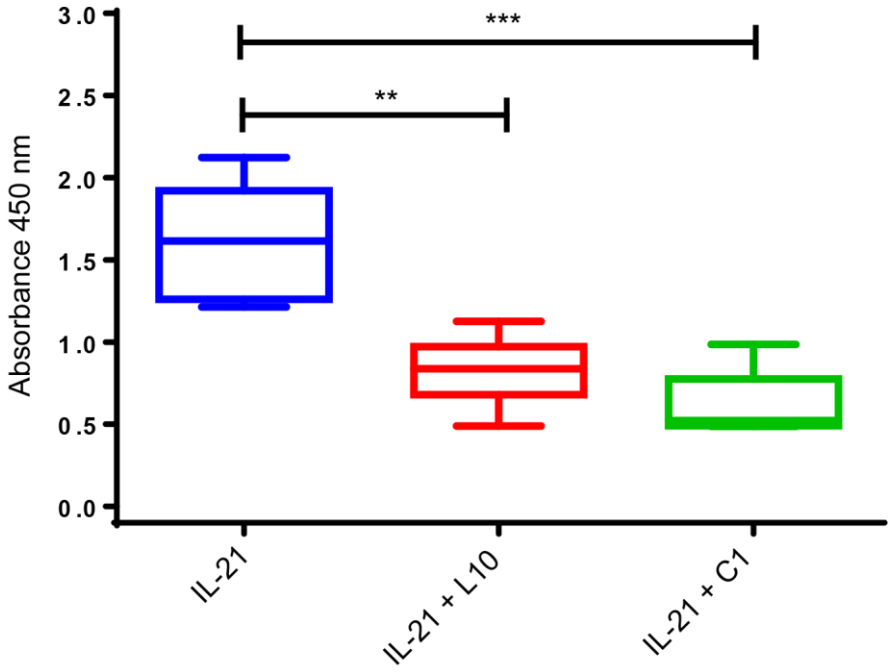


Figure 3.6: Proliferation of primary CLL cells on CD40 expressing fibroblasts and rhIL-21 system. Freshly thawed primary CLL cells were co-cultured with CD40 expressing fibroblasts supplemented with rhIL-21, LEN or CC-122 (10 µM) or DMSO as control for 3 days. Then, 50% of the media was replaced with fresh media that contained LEN or CC-122 plus rhIL-21 and BrdU reagent, and incubated for a further 3 days. The amount of BrdU incorporated into cellular DNA, which is directly proportional to the number of proliferating cells, was measured at 450 nM. Data is presented as percentage of cell proliferation relative to the control and plotted on a line graph. One-way ANOVA and Dunnett's multiple comparison test were used to determine statistically significant differences from rhIL-21. ** p<0.01 and *** p<0.001 (mean ± SEM; n=8).

In our studies LEN and CC-122 exhibit variable inhibition of proliferation in primary CLL cells. Thus, whilst in the majority of the primary CLL samples the percentage of proliferating cells is decreased in a concentration dependent manner with either drug, the degree of inhibition varied between cases and treatment. Based on these findings, 4 cohorts of patients were confirmed (representative examples are shown in **Figure 3.7 A-H**). In the first cohort, cells were found to be sensitive to both LEN and CC-

122 (**Figure 3.7 A & B**). **Figure 3.7 C & D** shows a representative case from the second cohort in which although both LEN and CC-122 have activity, CC-122 shows a greater effect than LEN. In the third cohort CC-122 alone, but not LEN, showed inhibition of proliferation (**Figure 3.7 E & F).** In the final cohort**,** neither LEN nor CC-122 had any effect on proliferation (**Figure 3.7 G & H**). Evaluation of the proliferation data allowed us to determine that the inhibitory concentration 50 (IC_{50}) for LEN is 10 µM, whilst that for CC-122 is 1 µM. These concentrations were hence chosen for all further experiments.

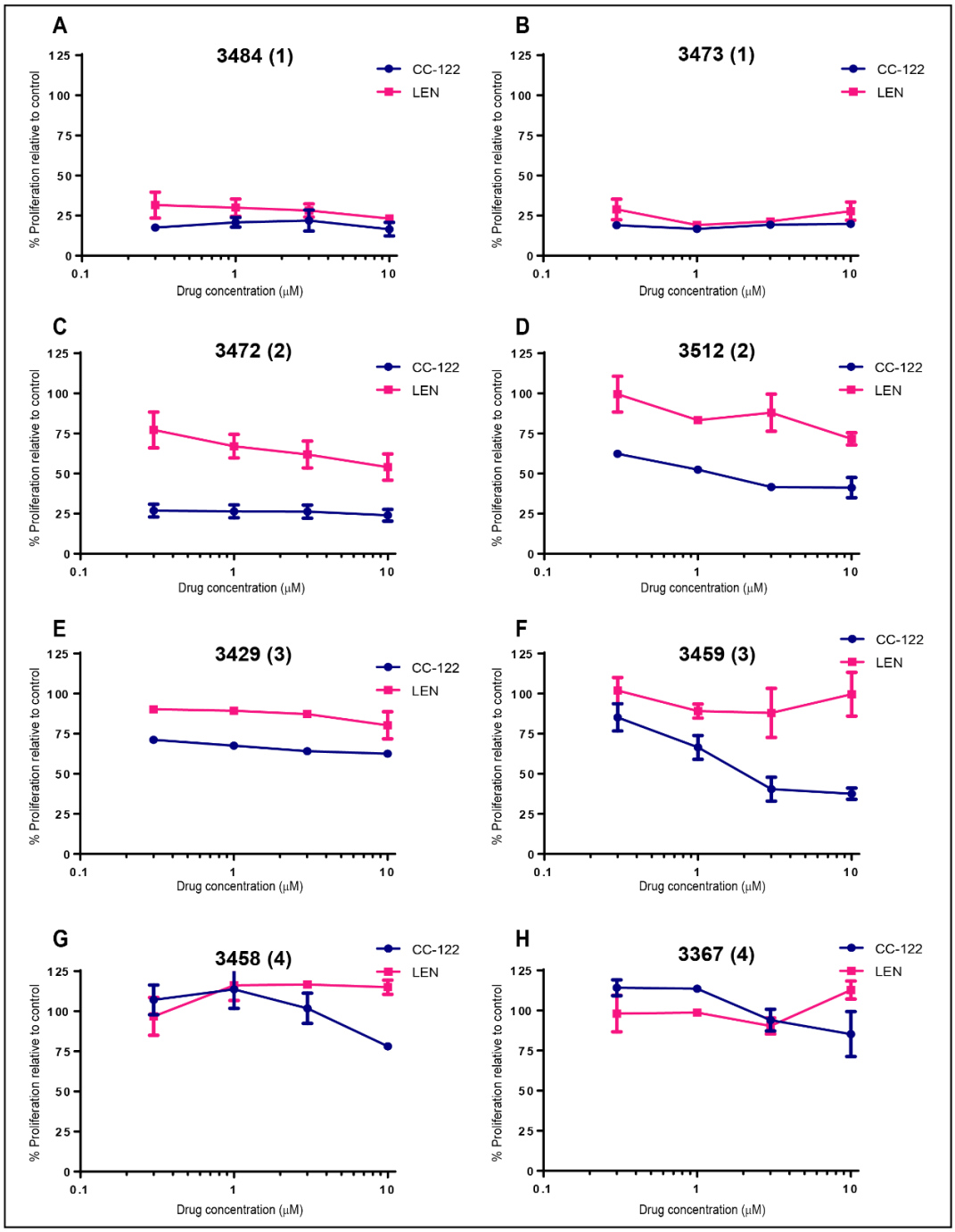


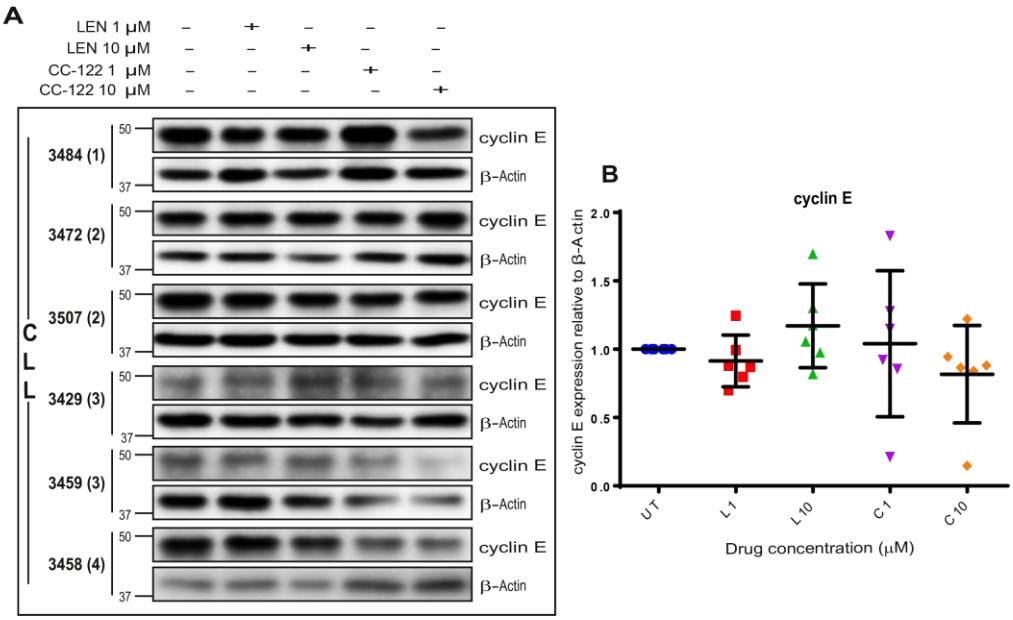
Figure 3.7: Primary CLL cells show variable proliferation responses upon treatment with Lenalidomide and CC-122. Freshly thawed primary CLL cells were co-cultured with CD40 expressing fibroblasts supplemented with rhIL-21, and exposed to varying concentrations of LEN or CC-122 (0.3 μM and 10 μM) or DMSO as a control for 72 hours. After 3 days, 50% of the media was replaced with fresh media containing LEN or CC-122 plus rhIL-21 and BrdU reagent, and cells incubated for another 3 days. The amount of

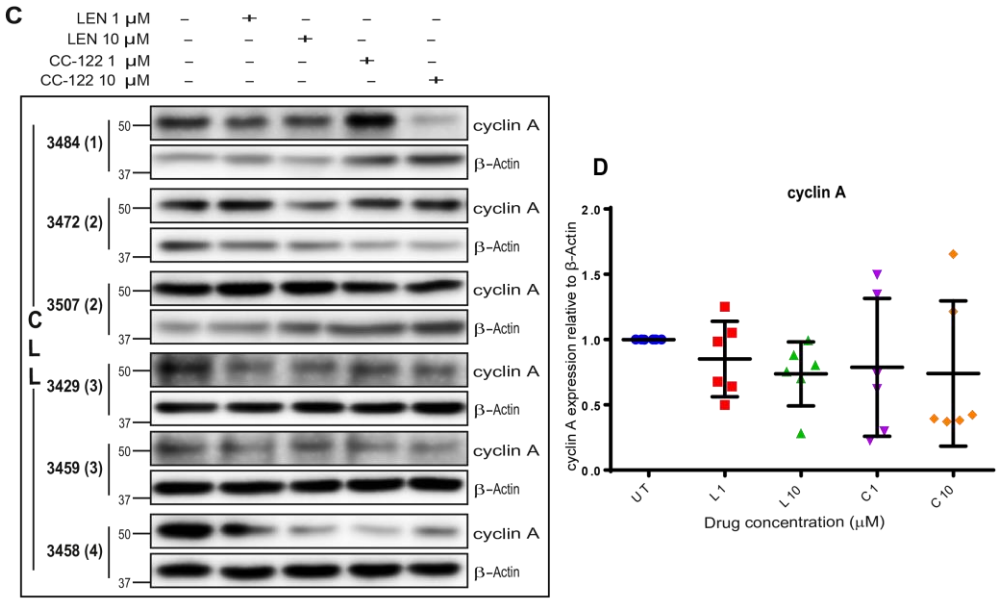
BrdU incorporated in the DNA was measured at 450 nM, which is directly proportional to the number of proliferating cells. Data is represented as percentage of cell proliferation relative to the control and plotted on a line graph. Pearson correlation coefficient analysis was used to determine statistically significant differences between LEN and CC-122 (n=8).

3.3.5. Expression of cyclins in Lenalidomide and CC-122 treated primary CLL cells

As with the data generated from cell lines, we hypothesised that inhibition of proliferation in primary CLL cells may reflect changes in expression of proteins that affect cell cycle progression. Therefore, we examined the effects of LEN and CC-122 on the expression of cyclin proteins to see if there was any correlation with the observed proliferation profiles in primary CLL cells and cohorts described above. Cells were treated with 1 µM and 10 µM of LEN or CC-122 or DMSO for 24 hours and expression of cyclins E, A and B levels determined by immunoblotting.

Figure 3.8 shows that LEN and CC-122 had variable effects on cyclin protein expression in primary CLL cells after 24 hours. The levels of cyclin A decreased in all four CLL cohorts upon LEN and CC-122 treatment. There was no consistent or significant pattern observed with cyclins E or B.


A
LEN 1 μM
LEN 10 μM
CC-122 1 μM
CC-122 10 μM
CLL
3484 (1)
3472 (2)
3507 (2)
3429 (3)
3459 (3)
3458 (4)
cyclin E
β-Actin
50
37
B
cyclin E
cyclin E expression relative to β-Actin
Drug concentration (μM)
UT
L 1
L 10
C 1
C 10


C
LEN 1 μM
LEN 10 μM
CC-122 1 μM
CC-122 10 μM
CLL
3484 (1)
3472 (2)
3507 (2)
3429 (3)
3459 (3)
3458 (4)
cyclin A
β-Actin
50
37
D
cyclin A
cyclin A expression relative to β-Actin
Drug concentration (μM)
UT
L 1
L 10
C 1
C 10

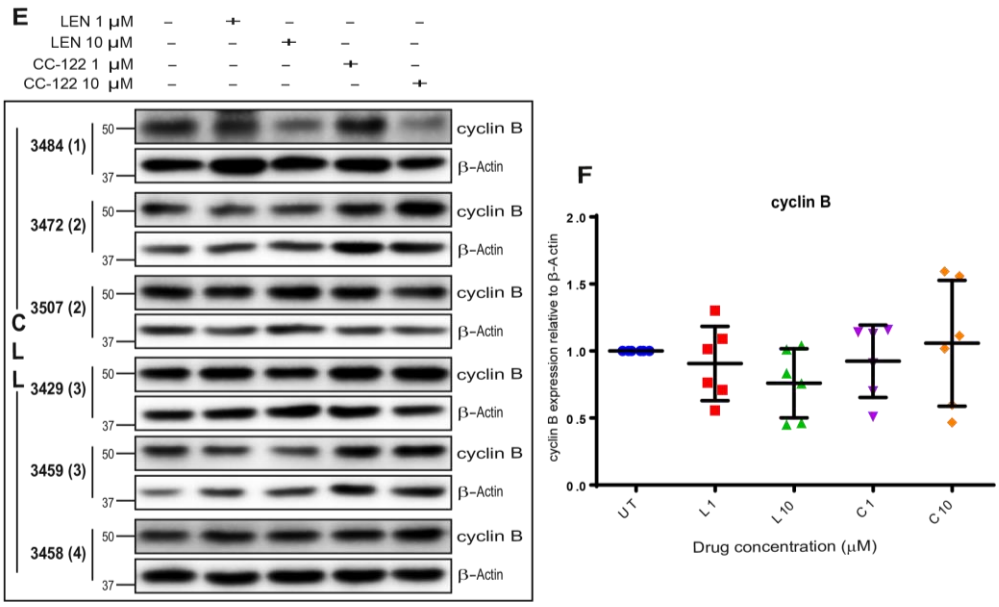


Figure 3.8: Expression of cyclins in Lenalidomide and CC-122 treated primary CLL cells. Primary CLL cells were left untreated (UT) or treated with either LEN or CC-122 (1 µM and 10 µM) and incubated for 24 hours. Cell lysates were separated by SDS-PAGE and immunoblotted for cyclin E, A, B and β-actin (loading control). Immunoblots and Densitometry (mean ± SEM; n=6): **A & B;** cyclin E; **C & D;** cyclin A; **E & F;** cyclin B;

3.3.6. Lenalidomide and CC-122 induce p21 expression in primary CLL cells

Recent reports suggest that LEN increases p21 expression in primary CLL cells [199]. Hence, we analysed p21 protein expression in primary CLL cells following treatment with either LEN or CC-122 to examine if there was any correlation between p21 expression and the observed changes in proliferation.

Figure 3.9 shows that both LEN and CC-122 upregulate p21 expression in primary CLL cells. At 24 hours, p21 expression was induced in a dose

dependent manner in primary CLL cells. Interestingly, CC-122 induced higher p21 expression than LEN in all cohorts except 3458 where both drugs induced similar levels of p21 protein. The greater inhibition of proliferation with CC-122 does correlate with the induction of p21 expression.

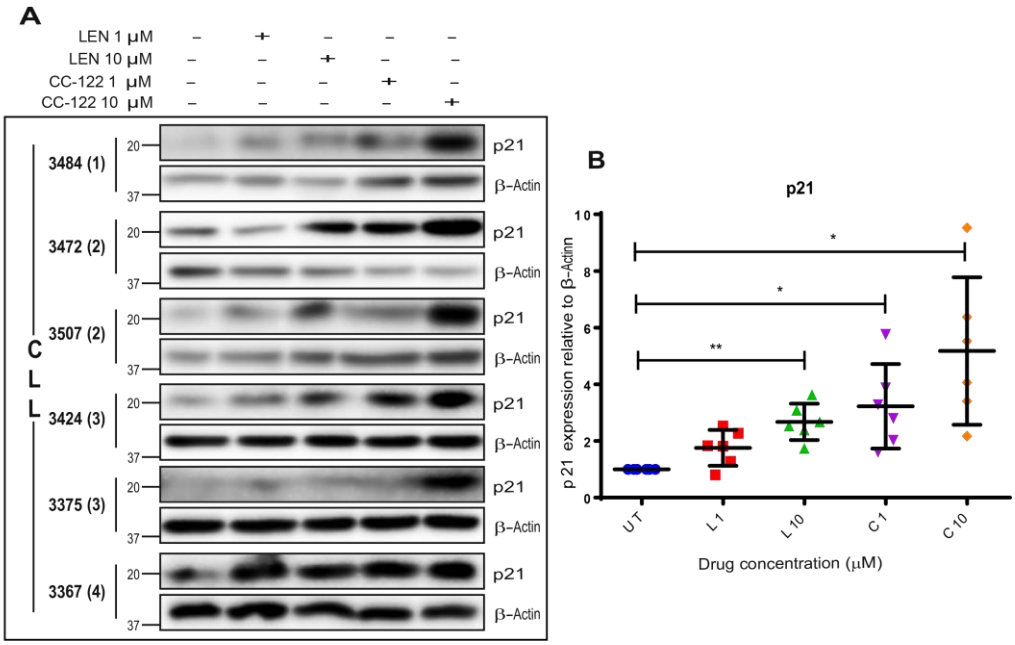


Figure 3.9: Lenalidomide and CC-122 induce p21 expression in primary CLL cells. Primary CLL cells were left untreated (UT) or treated with either LEN or CC-122 (1 µM and 10 µM) and incubated for 24 hours. Levels of p21 and β-actin were then determined by western blot. **A.** Western blot; **B.** Densitometry; One-way ANOVA and Dunnett's multiple comparison test were used to determine statistically significant differences from UT. *p<0.05 and **p<0.01 (mean ± SEM; n=6).

3.4 Discussion

The principle aim of this chapter was to investigate the anti-proliferative effects of LEN and CC-122 in both B-CLL cell lines and primary CLL cells. Initially, B-CLL cell lines (EHEB, MEC2, MEC1 and HG3) were chosen as a model to understand how IMiDs alter malignant cell proliferation.

In recent years, LEN has been shown to inhibit proliferation in B-lymphoma and multiple myeloma cell lines [195, 211] as well as in primary CLL cells [199]. A next generation IMiD analogue CC-122, has also been shown to have superior anti-tumour and immunomodulatory activity in DLBCL cell lines [180]. In our study, both LEN and CC-122 were observed to variably inhibit the proliferation of various B-CLL cell lines. One explanation for the variability might be that inherent genetic aberrations play a role in the cellular response to LEN and CC-122. For instance EHEB cells, which were found to be the most susceptible to the anti-proliferative effects of the drugs, are derived from B-CLL cells that have minimal karyotypic abnormalities [190]. MEC2 and MEC1 have similar cytogenetics (both harbour a deletion of 17p) [191] and show similar responses to drug treatment. In contrast, HG3 cells only harbour a deletion at 13q with loss of miR 15a/miR 16-1 [192] and yet are most resistant to both drugs. It is likely that other genetic changes such as mutations may impact such behaviour. CC-122 does consistently inhibit proliferation to a greater extent than LEN.

Expression levels of cyclins dictate cell cycle progression. In addition, p21 a universal cyclin-CDK inhibitor also impacts cell cycle status [212].

Overexpression of p21 inhibits proliferation in mammalian cells and is also found to inhibit a broad range of cyclin-CDK complexes [213]. It has previously been shown that LEN inhibits cell cycle arrest by upregulation of p21 levels [196, 199]. Other studies show that induction of p21 inhibits the binding of cyclin A or cyclin E to CDK2 [198]. Therefore, we studied levels of cell cycle proteins in the 4 B-CLL cell lines used in this study. Both the drugs significantly affect cyclin A and B levels in EHEB cells and likely also inhibit the CDK2/cyclin E complex at the G_1/S transition. There was a clear correlation between inhibition of proliferation and alterations in cyclin levels in EHEB cells. The other 3 cell lines, however, did not show any noticeable changes upon drug treatment. Both LEN and CC-122 did induce p21 expression in EHEB cells and this correlated with changes in proliferation. Although CC-122 did induce p21 levels in all 4 B-CLL cell lines at early time points, this was not sustained.

In addition to B-CLL cell lines, we have characterised the effects of LEN and CC-122 on the proliferation of primary CLL cells in our system that utilises CD40L and rh-IL21. The observed effects allowed us to define four distinct cohorts of response. Using our CLL cohorts, we confirm the findings of Fecteau et al [199] who showed that LEN inhibited the proliferation of CLL cells. Our studies are the first to compare the effects of CC-122 with those of LEN on primary CLL cells. We find that both LEN and CC-122 variably inhibit the proliferation of primary CLL cells. CC-122 was shown to be more efficacious than LEN. The IC_{50} for LEN and CC-122 was found to be 10 and

1 μM respectively. The effects of CC-122 seem to be more independent of karyotypic abnormalities compared to LEN.

The studies of changes in cyclin expression showed that cyclin A levels were consistently reduced in primary CLL cells on drug exposure whereas expression of cyclins E and B did not change. There were no consistent effects on expression of cyclins that clearly tracked with the observed proliferation profiles in primary CLL cells or identified cohorts. We have confirmed that LEN upregulates p21 expression in CLL cells [199]. The results obtained suggest that CC-122 induces higher p21 expression in primary CLL cells. Although CC-122 induced a greater effect than LEN, these effects are not correlated with the degree of proliferation inhibition as this was also seen in cohort 4 that was consistently resistant to the anti-proliferative effects of both drugs. The reason for this observation remains unclear and may reflect as yet unknown changes that impact cell cycle progression.

In conclusion, both LEN and CC-122 show differential anti-proliferative effects in B-CLL cell lines and primary CLL cells. CC-122 seems superior to LEN for its effects on CLL cells *in vitro* and may provide therapeutic opportunities. Cohort 3 clearly demonstrates the existence of a scenario where CC-122 may still be effective in cases that are resistant to LEN. Although cyclin levels and p21 expression correlated with the observed proliferation inhibition in EHEB cell lines this was not observed with primary CLL samples and other B-CLL cell lines.

Consistent changes in cell proliferation may eventually promote apoptosis *in vitro*. The changes in the expression of proteins that impact apoptosis upon treatment with LEN or CC-122 are investigated in more detail in **Chapter 4.**

Chapter 4 : Investigation of potential synergistic or antagonistic effects of IMiDs on BH3 containing proteins in CLL

4.1 Introduction

Several lines of evidence suggest that Thalidomide and its analogues cause growth inhibition. In addition IMiDs promote apoptosis in MM cell lines and primary cells from MM patients [160]. Both anti-proliferative and cytotoxic effects of IMiDs are predominantly due to proteasomal degradation of the critical B-cell transcriptional factors Ikaros and Aiolos [211]. In other studies, high concentrations of Lenalidomide are shown to induce apoptosis in UT-7 and MUTZ cells [195]. A recent study has also demonstrated that the novel IMiD compound CC-122 is cytotoxic to DLBCL cell lines at low concentrations [180].

Several studies have demonstrated that overexpression of Bcl-2, Mcl-1 and Bcl-xL are commonplace in most haematological malignancies including CLL [214, 215]. In CLL, elevated Bcl-2 levels are particularly linked to loss of the miR 15a and 16-1 locus in chromosome 13q [216]. Whereas the relevance of Mcl-1 expression in CLL is a matter of debate the protein does contribute to cell survival and drug resistance [217]. Similarly, elevated Bcl-2/Bax and Mcl-1/Bax ratios are associated with poor response to conventional therapy [218, 219].

We have demonstrated that LEN and CC-122 differentially inhibit proliferation in B-CLL cell lines and primary CLL cells (**Chapter 3**). Whether this robust inhibition of proliferation is linked to apoptosis is hence a relevant line for investigation. It is also possible that IMiD treatment may impact the expression of BH3 containing proteins and may provide clues to potential synergies or antagonistic interactions. In order to study this, we measured the levels of apoptotic proteins in 4 B-CLL cell lines and primary CLL cells following treatment with LEN or CC-122.

The **aims** of this chapter, therefore, were:

1. To determine the effects of LEN and CC-122 on apoptosis in both B-CLL cell lines and primary CLL cells using flow cytometry.
2. To examine the levels of anti- and pro-apoptotic proteins in B-CLL cell lines and primary CLL cells following LEN and CC-122 treatment.

4.2 Methods

4.2.1 Measurement of apoptosis by flow cytometric analysis

Fluorescence-activated cell sorting (FACS) using 3, 3'-dihexyloxacarbocyanine iodide (DiOC6) and propidium iodide (PI) was used to categorise cells into viable, early apoptotic, and late apoptotic populations. Cell lines were seeded at a density of 0.5×10^6/mL and incubated for up to 5 days. Primary CLL cells were used at a density of 7.5×10^6/mL on previously plated fibroblast monolayers and incubated for up to 3 days. Cells were treated with different concentrations (1-10 μM) of LEN or CC-122 or an equal volume of (DMSO) (vehicle control) and were incubated at 37°C, 5% CO_2 in a humidified incubator. Cells were harvested at specific time points and labelled with DiOC6 and PI. Mean florescence intensity (MFI) was measured at wavelengths of 530 and 630 nM using a FACS machine (Attune, Life technologies, UK) and percentage of live and dead cells were estimated. (PI^-DiOC6^+ = live, PI^-DiOC6^- = early apoptotic, PI^+DiOC6^- = late apoptotic).

4.2.2 Determination of the protein expression levels of anti and pro-apoptotic proteins

B-CLL cell lines were used at a density of 0.5×10^6/mL and incubated for 24 and 72 hours. Primary CLL cells were seeded at a density of 7.5×10^6/mL on fibroblast monolayers and incubated for 24 hours. These cells were treated with LEN or CC-122 (1 and 10 μM) or an equal volume of (DMSO) (vehicle control) and cells were incubated at 37°C, 5% CO_2 (humidified) and harvested at specific time points. Whole cell lysates were probed for the

expression of anti and pro-apoptotic proteins by immunoblotting. The intensity of each band was quantified by ImageJ software and normalised to a loading control.

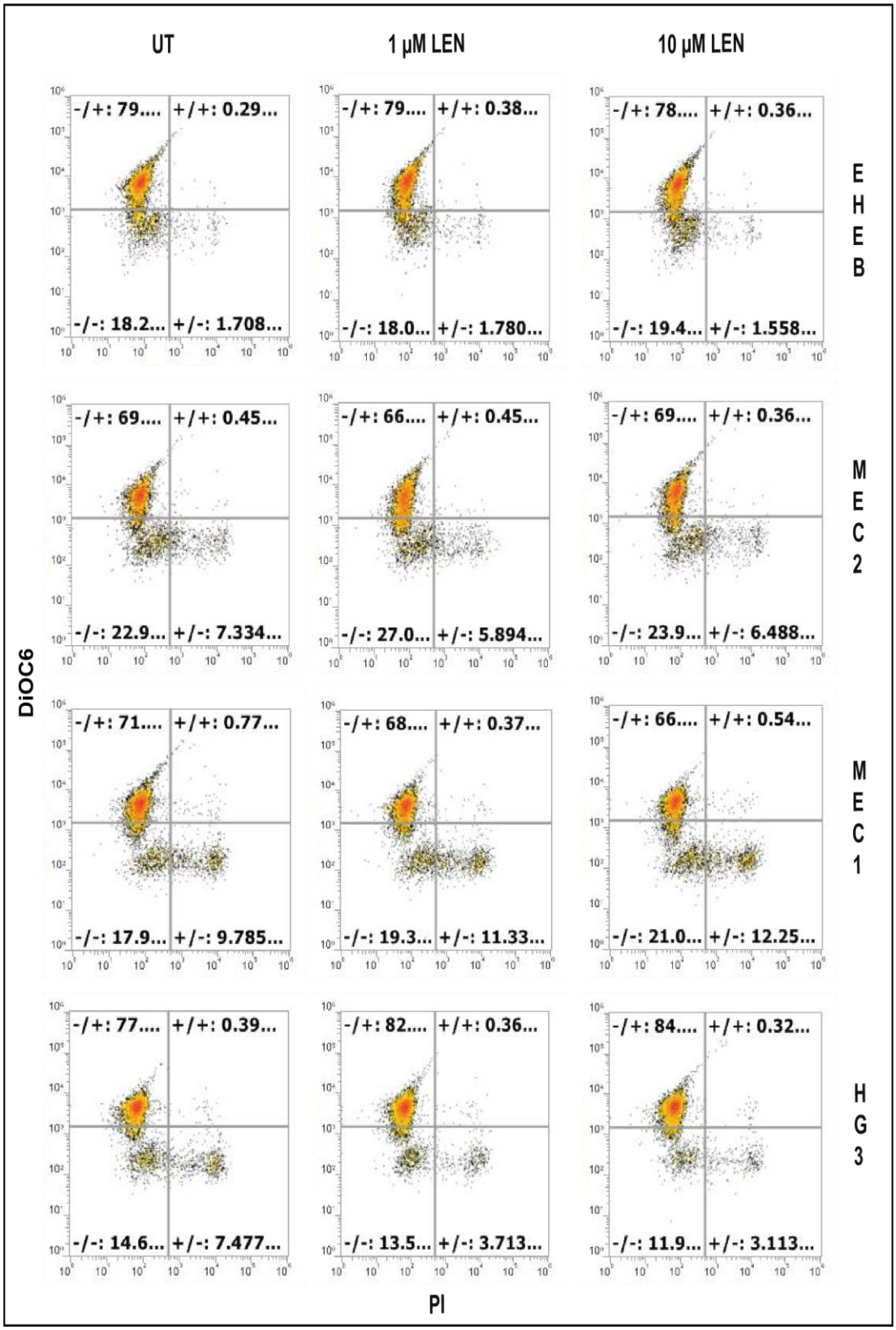
4.3 Results

4.3.1 Flow cytometric analysis of apoptosis in B-CLL cell lines after treatment with Lenalidomide and CC-122

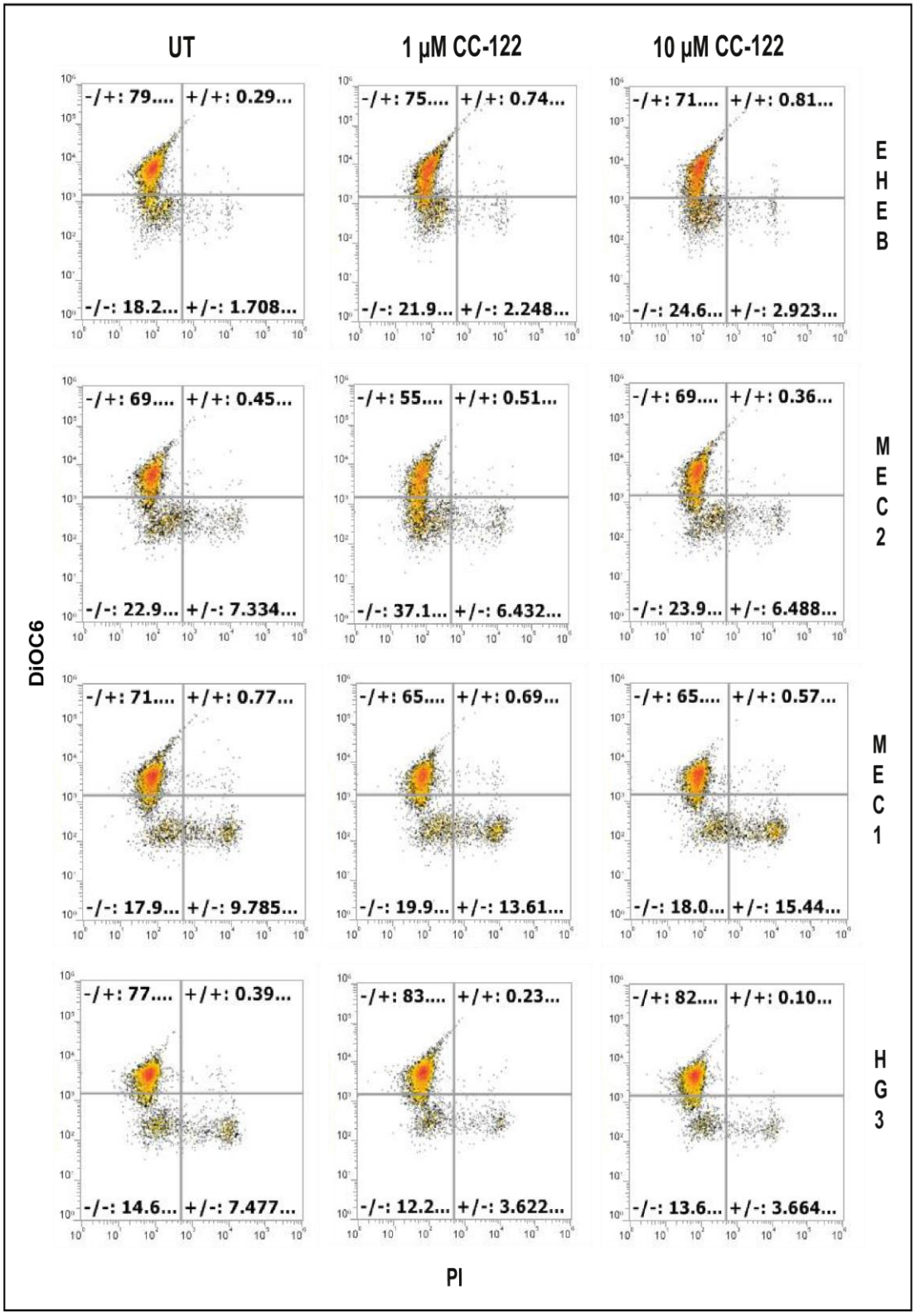
Previous studies have indicated that LEN and CC-122 induce apoptosis in multiple myeloma and DLBCL cell lines. Hence both drugs were tested for their ability to induce apoptosis in different B-CLL cell lines. Cells were treated with varying concentrations of LEN or CC-122 (1-10 µM) at multiple time points for up to 5 days. Programmed cell death was quantified by changes in the mitochondrial trans-membrane potential using DiOC6 and parallel measurement of PI stained nuclei by FACS assay. The percentage of viable cells were analysed for every treatment by comparing with untreated cells.

As shown in **Figures 4.1 A, B and C**, exposure of EHEB, MEC2, MEC1 and HG3 cells to neither LEN nor CC-122 induced apoptosis even up to 100 µM for 5 days. Cells consistently maintained their viability at all concentrations as with untreated cells. Taken together, these results suggest that LEN and CC-122 are not cytotoxic to B-CLL cell lines.

A) LEN apoptosis on Day 3



B) CC-122 apoptosis on Day 3



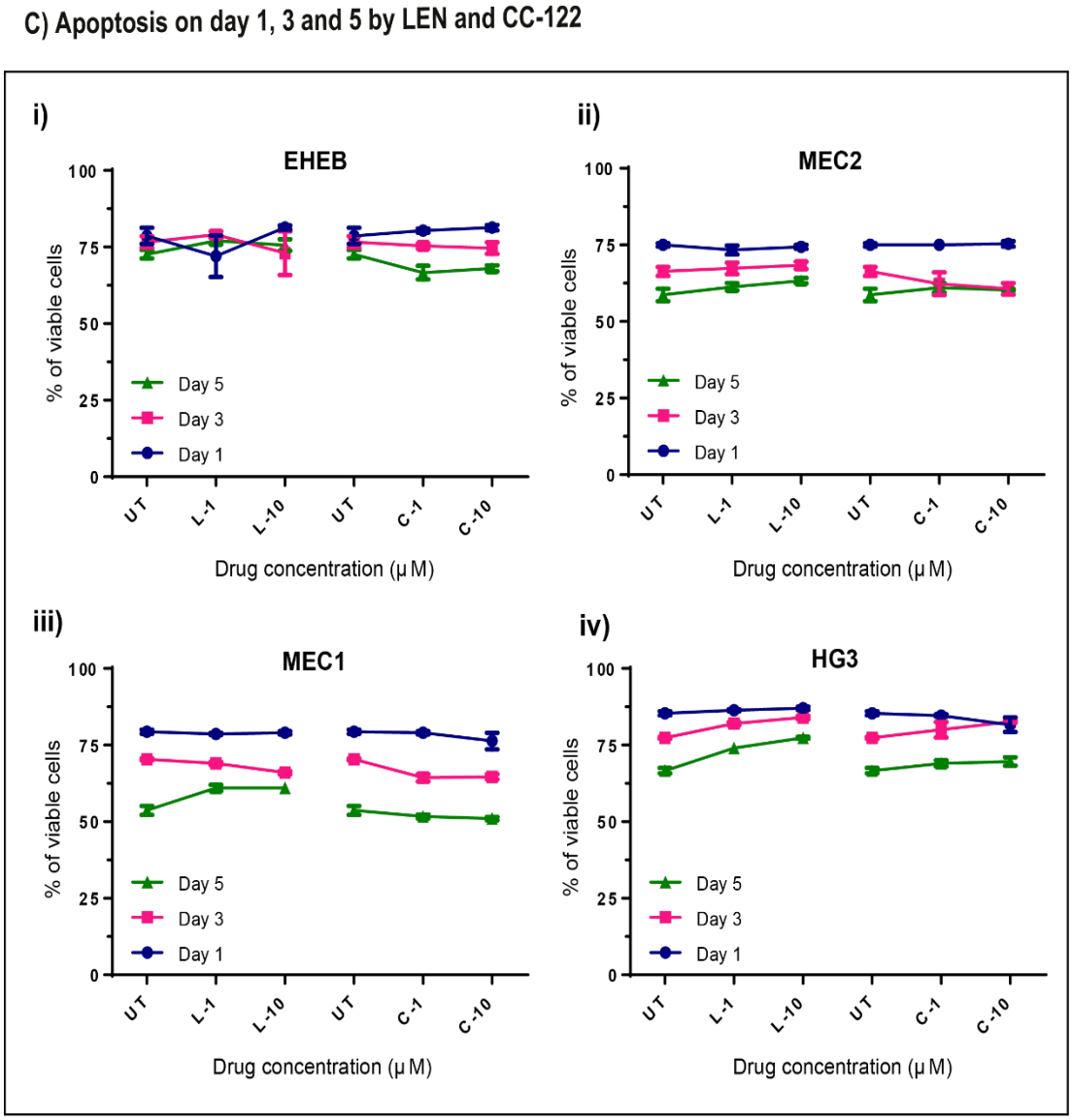


Figure 4.1: FACS based measurement of apoptosis in B-CLL cell lines following treatment with Lenalidomide and CC-122. B-CLL cell lines were incubated with increasing concentrations of LEN or CC-122 or DMSO (negative control). Cell viability was measured after 1, 3, and 5 days by FACS using combined DiOC6 and PI staining. FACS plots showing the viability of cells at day 3 treated with **LEN (A), CC-122 (B)** and Day 1 & Day 5 FACS plots are shown in **Appendix (A 4.1 - 4.4).** The percentages of viable cells following treatment relative to the untreated sample are plotted in **(C).**

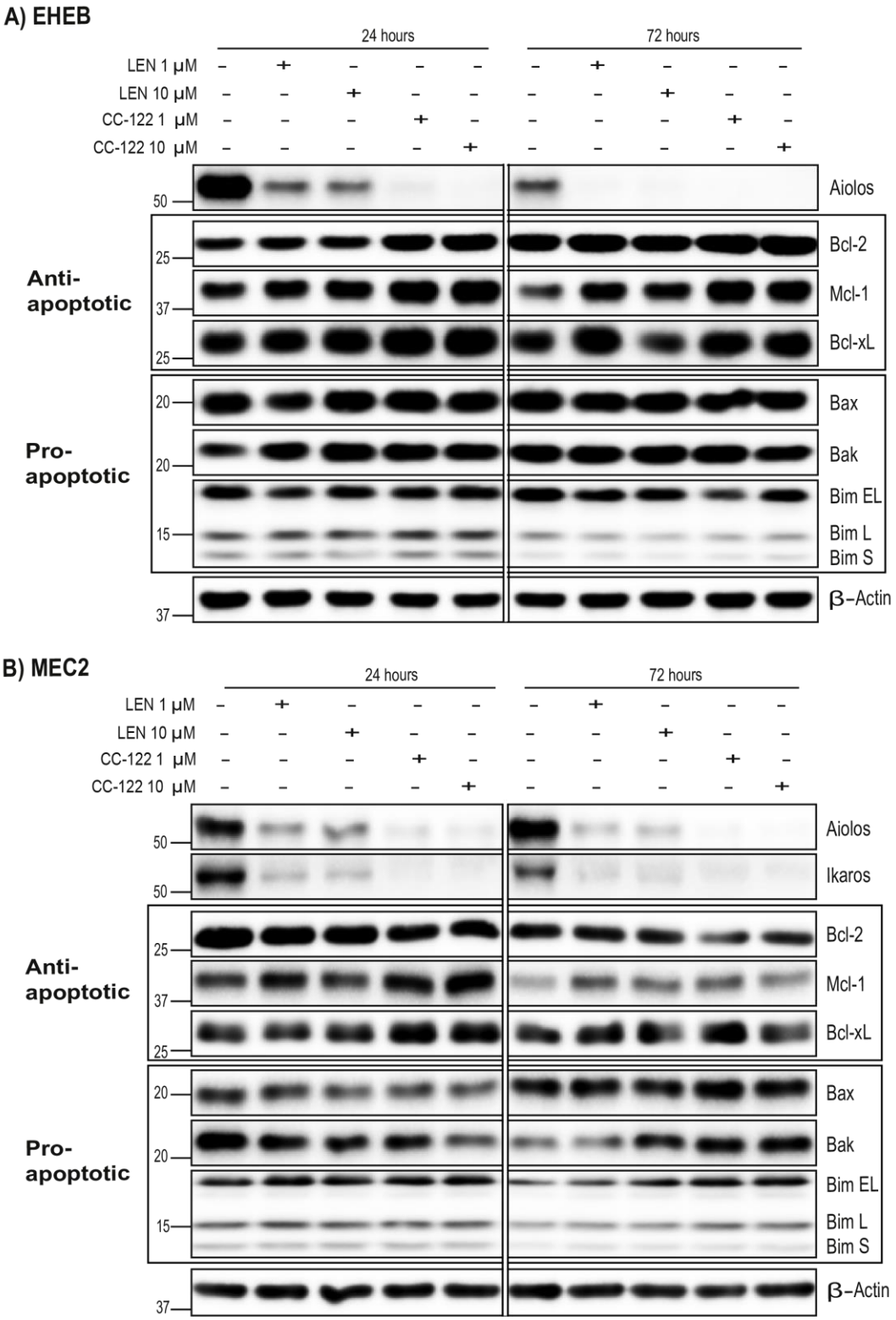
4.3.2 Effects of Lenalidomide and CC-122 on BH3-containing proteins in B-CLL cell lines

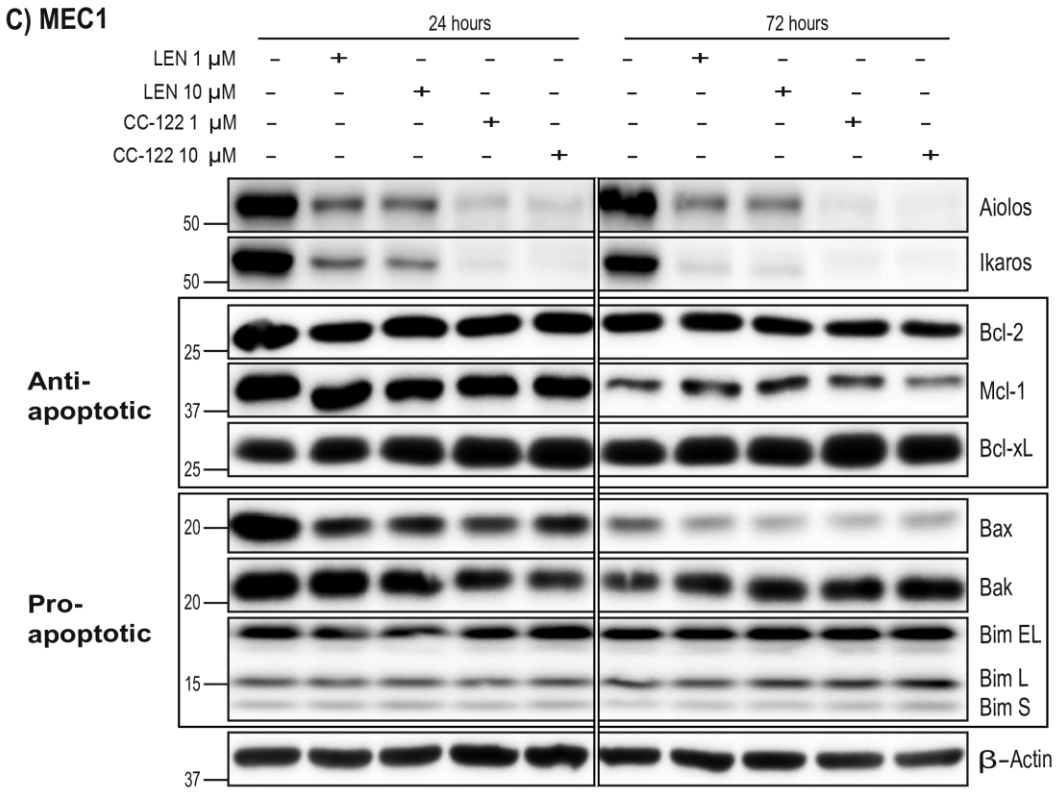
As the results show that LEN and CC-122 have no direct apoptotic affects in B-CLL cell lines, we next examined to see if the drugs had any effects on BH3-containing anti and pro-apoptotic proteins. B-CLL cell lines were exposed to 1 and 10 µM concentrations of LEN or CC-122 for 24 and 72 hours. Whole cell lysates were then analysed by western blot with antibodies against a pre-selected panel of anti- and pro-apoptotic proteins.

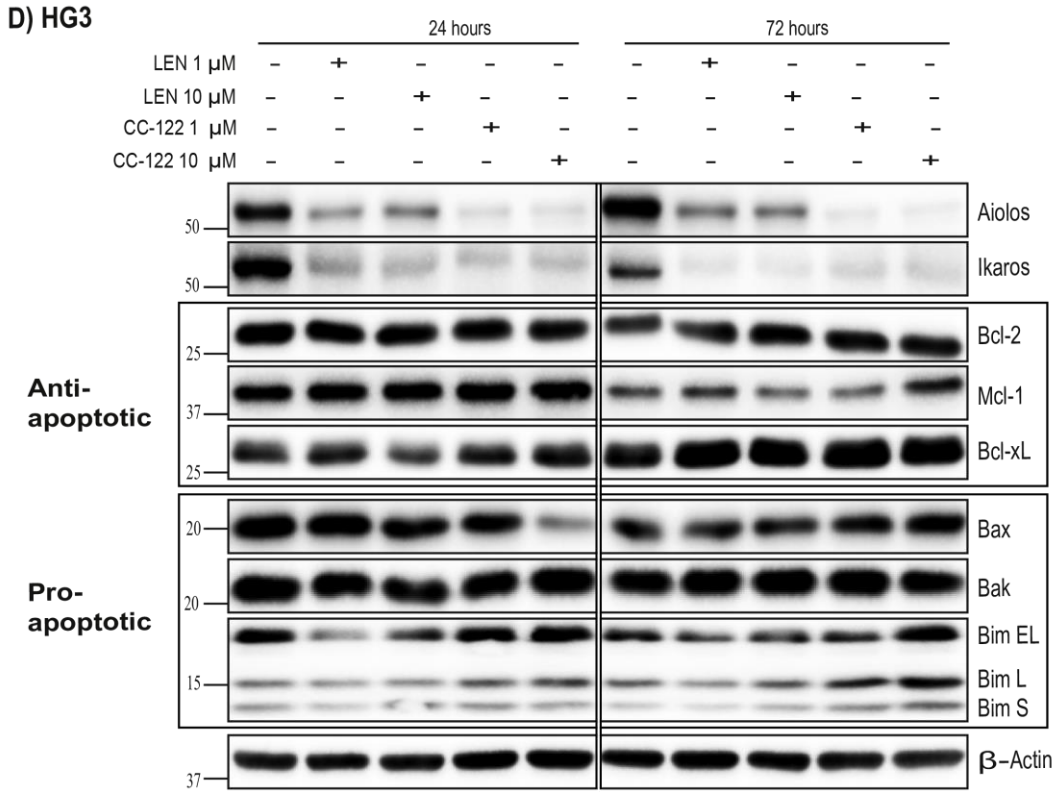
As shown in **Figure 4.2**, LEN and CC-122 induce variable alterations in the levels of anti- and pro-apoptotic proteins in EHEB, MEC2, MEC1 and HG3 cells. Both drugs promote degradation of transcription factors Aiolos and Ikaros at both time points and confirm drug activity **(A-D).** The degradation of Aiolos and Ikaros was more pronounced with CC-122 compared to LEN. The expression of anti-apoptotic proteins did alter in response to LEN and CC-122 after 24 hours with no further change in expression after 72 hours in most of cell lines. Upregulated Bcl-2 protein expression was noticed in EHEB and HG3 cells with both drugs. In contrast, Bcl-2 expression was down regulated in MEC2 and MEC1 cells. Mcl-1 expression gradually increased with treatment in all cell lines except MEC1 cells, while Bcl-xL was slightly increased only in MEC2 cells. In addition, pro-apoptotic proteins such as Bax were downregulated in all the cell lines at 24 and 72 hours except HG3 that showed upregulation after 72 hours treatment. In MEC2 and MEC1 cells, Bak expression was decreased at 24 hours but increased after 72 hours on drug

treatment. Only MEC2 cells showed consistent Bim-EL upregulation upon drug exposure at both time points.

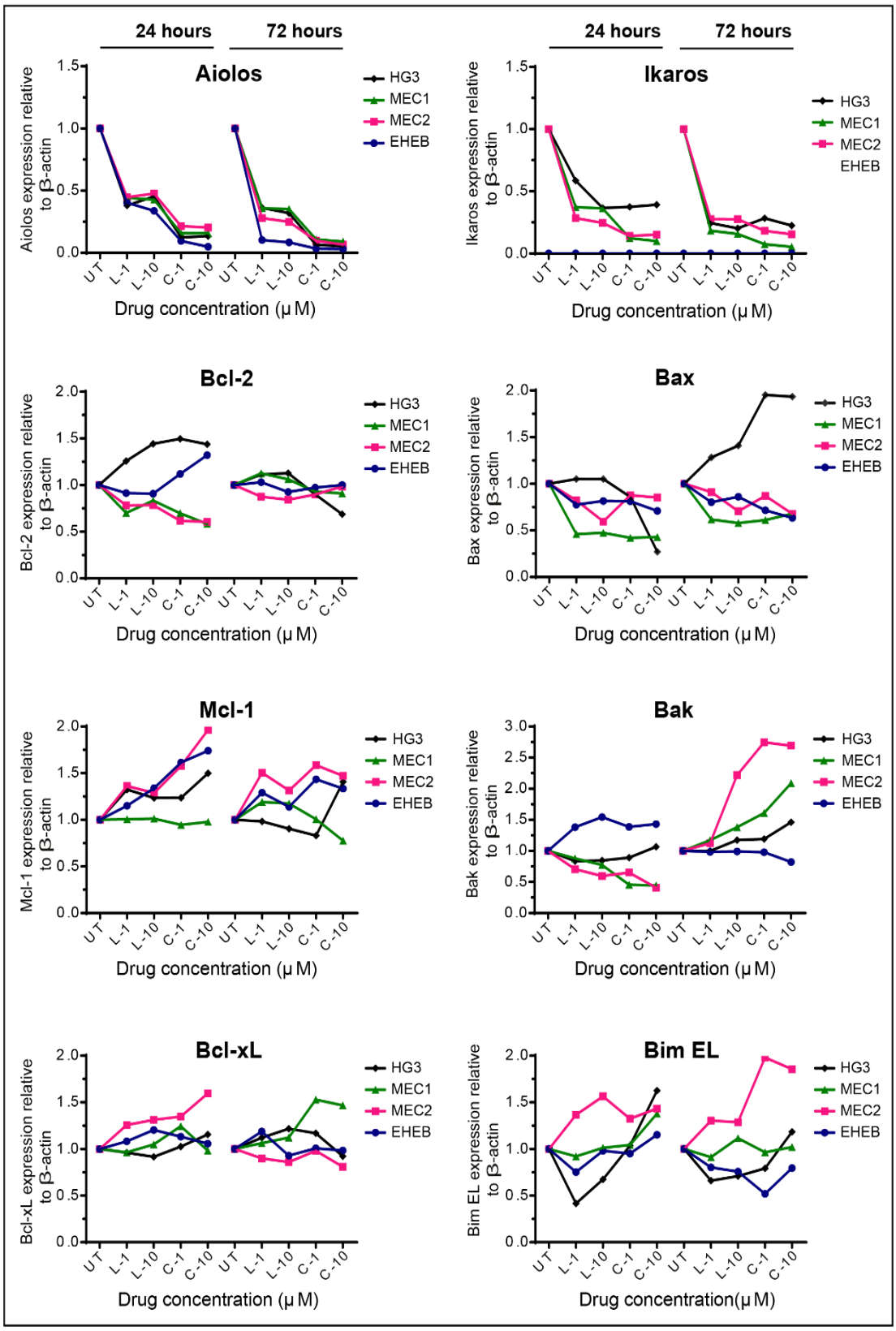
Interestingly, both drugs did increase Bcl-2/Bax ratios in EHEB & HG3 cells and additionally Mcl-1/Bax ratios in EHEB and MEC2 cells after 72 hours treatment. Other cell lines did not change either anti-apoptotic or pro-apoptotic proteins with IMiD treatment. Taken together, the patterns observed in the B-CLL cell lines upon drug treatment were not consistent to draw any significant conclusions.


A) EHEB
24 hours
72 hours
LEN 1 μM
LEN 10 μM
CC-122 1 μM
CC-122 10 μM
Aiolos
Bcl-2
Mcl-1
Bcl-xL
Anti-apoptotic
Bax
Bak
Bim EL
Bim L
Bim S
Pro-apoptotic
β-Actin
B) MEC2
24 hours
72 hours
LEN 1 μM
LEN 10 μM
CC-122 1 μM
CC-122 10 μM
Aiolos
Ikaros
Bcl-2
Mcl-1
Bcl-xL
Anti-apoptotic
Bax
Bak
Bim EL
Bim L
Bim S
Pro-apoptotic
β-Actin


C) MEC1
24 hours
72 hours
LEN 1 μM
LEN 10 μM
CC-122 1 μM
CC-122 10 μM
Aiolos
Ikaros
Bcl-2
Mcl-1
Bcl-xL
Bax
Bak
Bim EL
Bim L
Bim S
β-Actin
Anti-apoptotic
Pro-apoptotic
50
50
25
37
25
20
20
15
37


D) HG3
24 hours
72 hours
LEN 1 μM
LEN 10 μM
CC-122 1 μM
CC-122 10 μM
Aiolos
Ikaros
Bcl-2
Mcl-1
Bcl-xL
Bax
Bak
Bim EL
Bim L
Bim S
β-Actin
Anti-apoptotic
Pro-apoptotic
50
50
25
37
25
20
20
15
37

E) Densitometry analysis



F) Bcl-2 and Mcl-1: Bax ratio

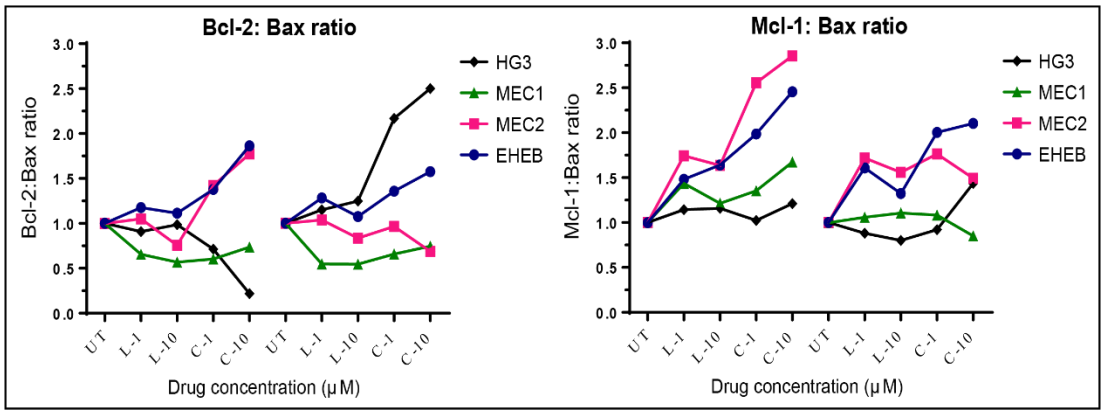


Figure 4.2 Effect of Lenalidomide and CC-122 on BH3-containing proteins in B-CLL cell lines. B-CLL cell lines were treated with LEN or CC-122 (1 and 10 µM) and incubated at 37˚C for 24 and 72 hours. Whole cell lysates were analysed for changes in expression of target proteins: **A.** EHEB; **B.** MEC2; **C.** MEC1; **D.** HG3; The changes in protein levels (regardless of the cell line) were compared to untreated control cells and plotted as shown in E (Densitometry analysis) and F (Bcl-2 and Mcl-1: Bax ratio).

4.3.3 Measurement of apoptosis by Flow cytometric analysis of primary CLL cells following treatment with LEN or CC-122

Primary CLL cells were treated with varying concentrations of LEN or CC-122 (1-100 µM) for up to 72 hours. Apoptosis rates were quantified using DiOC6 and PI by FACS assay as previously described. The percentage of viable cells for each treatment and at specific time points were compared to untreated cells **(Figure 4.3).**

The results show that exposure of primary CLL cells to either LEN or CC-122 for up to 72 hours did not induce significant apoptosis even at the highest concentration of either drug. As expected, Aiolos was degraded with both LEN and CC-122 treatment after 24 hours. These results suggest that

primary CLL cells, like the cell lines, exhibit cytostatic effects rather than apoptosis on exposure to LEN or CC-122.

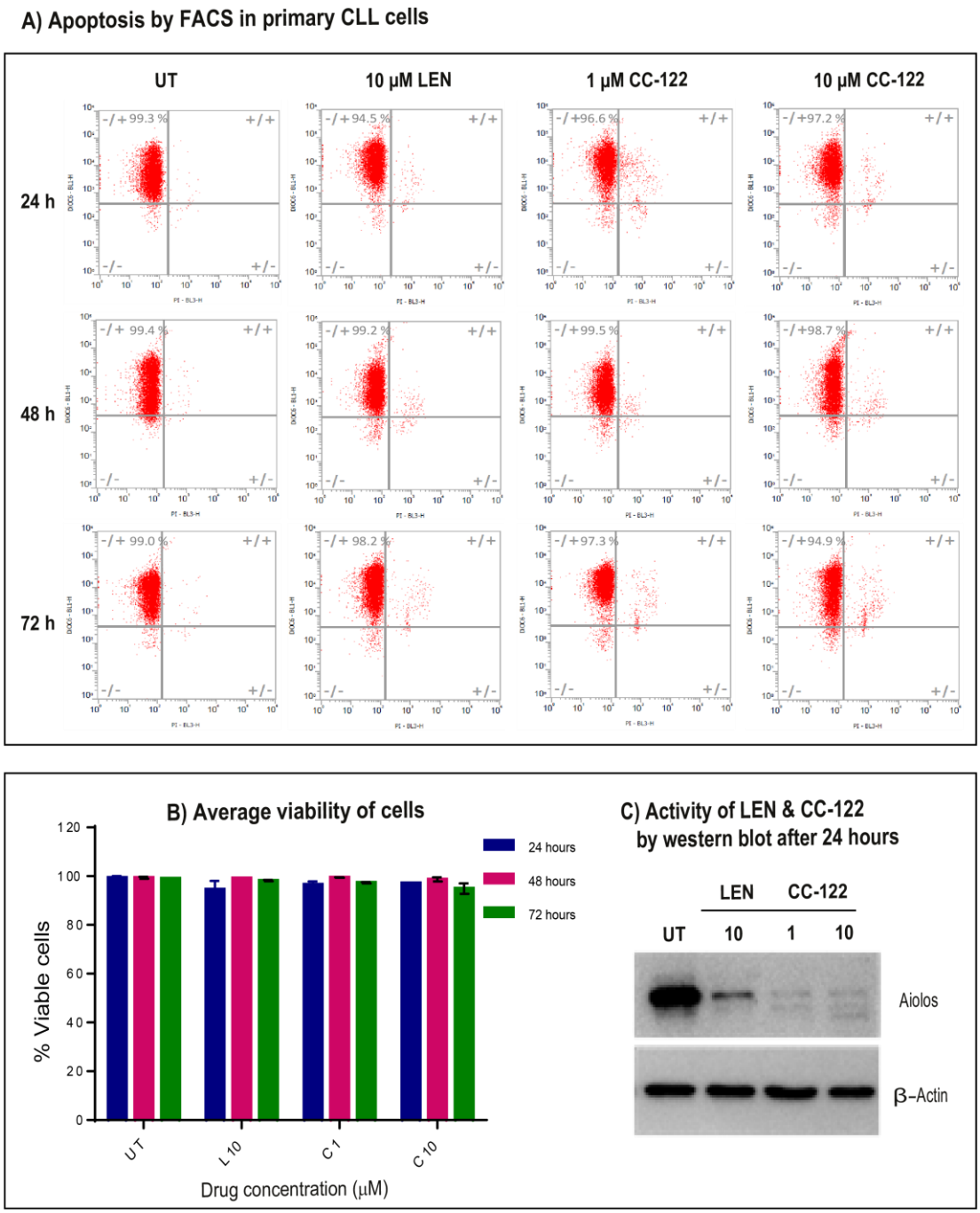


Figure 4.3: Flow cytometric analyses of apoptosis of primary CLL cells following treatment with Lenalidomide or CC-122. Primary CLL cells **(CLL# 3568)** were incubated with increasing concentrations of LEN, CC-122,

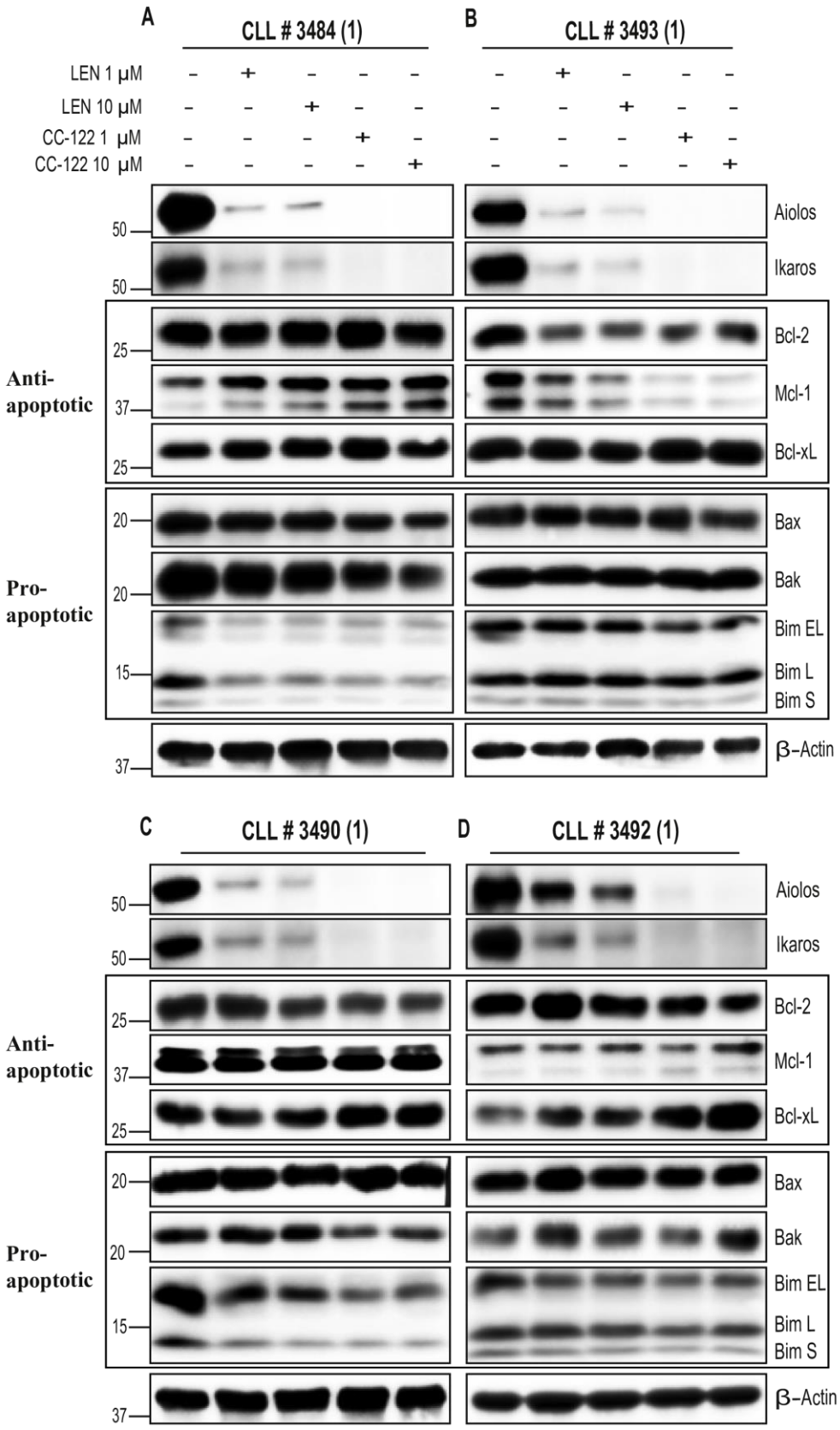
or DMSO (negative control) on fibroblast monolayers. Cell viability was measured after 24, 48 and 72 hours by DiOC6 and PI staining and FACS analysis. The representative cases of FACS plots show viability of cells up to 3 days following treatment with LEN or CC-122 **(A),** the percentage of viable cells relative to untreated control cells is plotted **(B)** and the Western blots were performed after 24 hours incubation **(C).** One-way ANOVA was used to determine statistically significant difference from UT (Mean ± SEM; n=6).

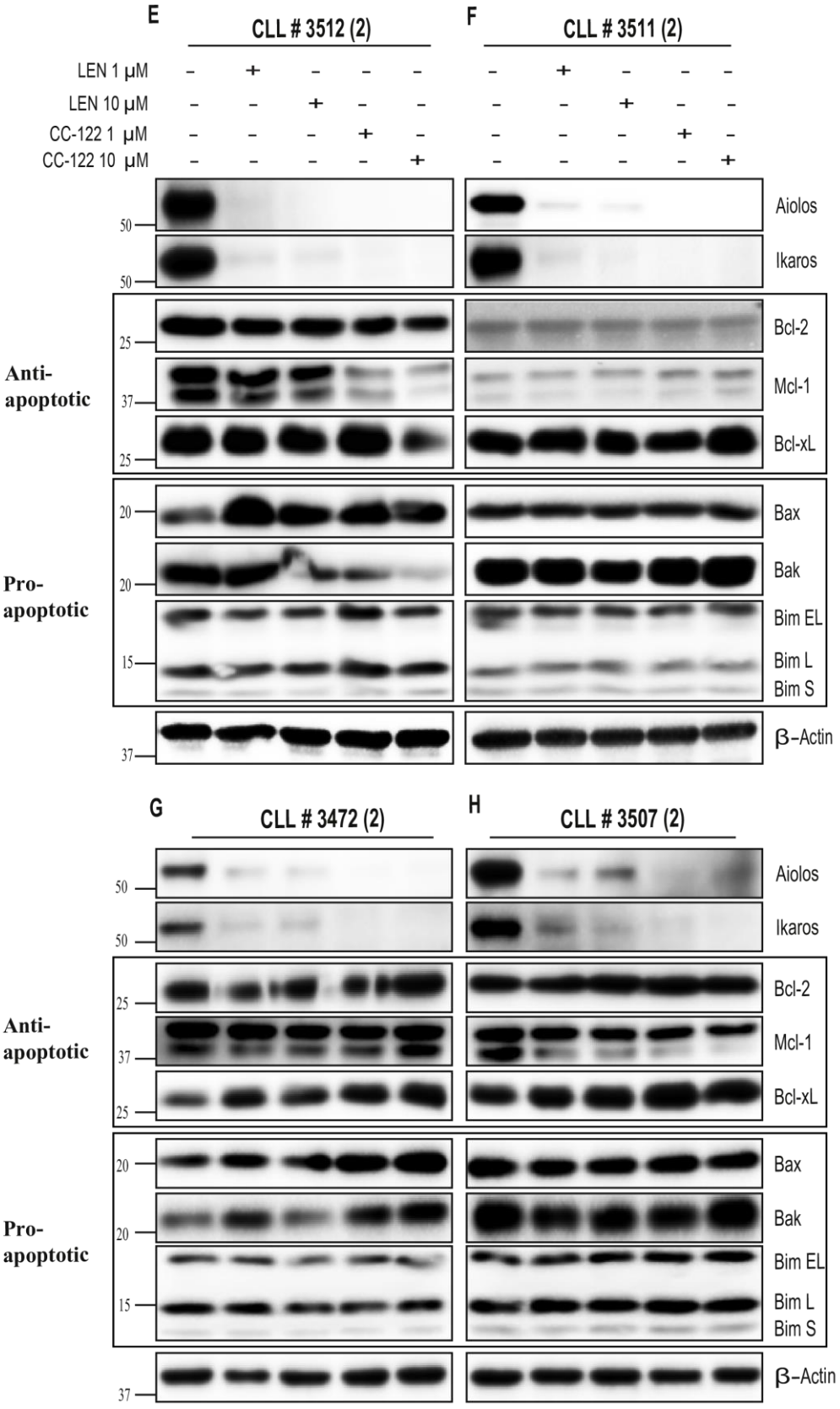
4.3.4 Effects of Lenalidomide and CC-122 on expression of BH3-containing proteins in primary CLL cells

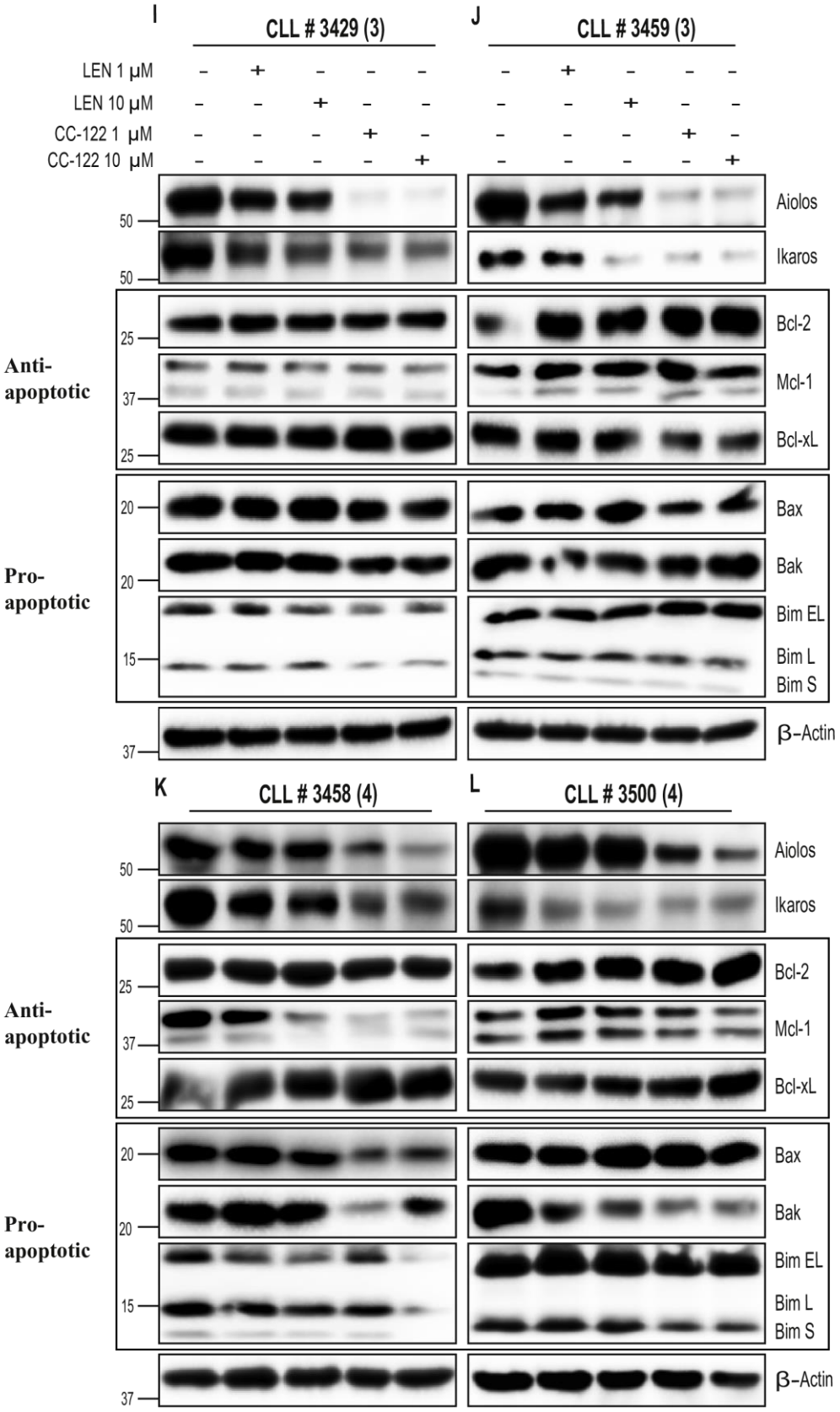
To assess whether LEN and CC-122 induce changes of relevant proteins in primary CLL cells, we treated CLL cells with LEN and CC-122 for 24 hours and analysed whole cell lysates by western blotting.

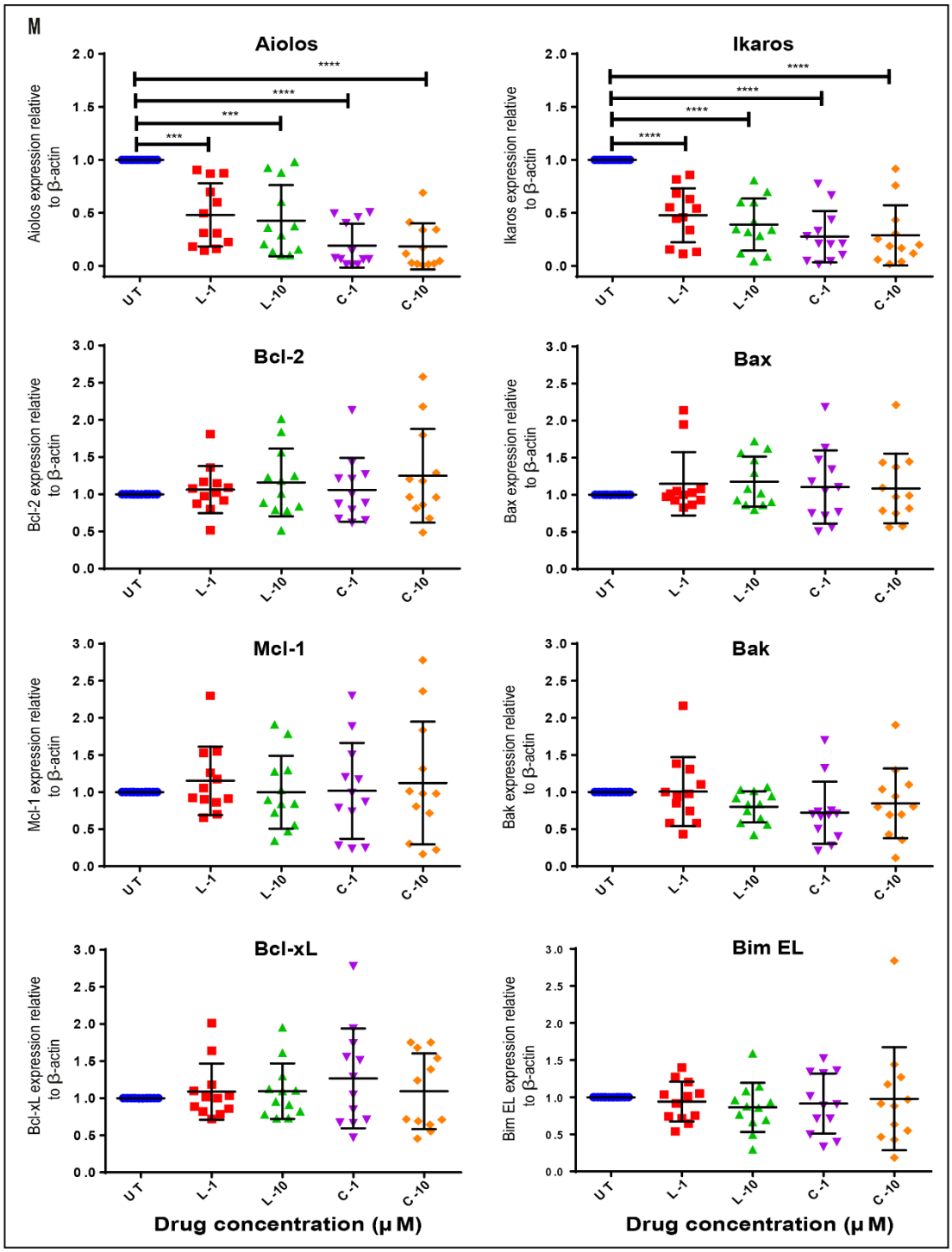
The results are summarized in **Figure 4.4** and **Tables 12 & 13**. All primary CLL samples expressed anti- and pro-apoptotic proteins. There was considerable inter-patient variability upon treatment with LEN or CC-122. Activity of both drugs was confirmed by demonstration of Ikaros and Aiolos degradation. There were no consistent changes across the samples in expression of specific members of the BH3 family. There were, however, instances of unique changes within the CLL cases. Samples 3507, 3548, 3493 and 3492 show changes in expression of at least one of the anti-apoptotic proteins whilst 3490, 3492 and 3500 show changes in pro-apoptotic members following treatment with the drugs. In some cohorts, treatment with LEN or CC-122 seemed to perturb the balance of the anti and pro-apoptotic proteins (**Figure 4.4 A, D & E**). Interestingly, the ratios of anti- and pro-apoptotic proteins are altered with LEN or CC-122 treatment. Although both drugs increase the ratio of Bcl-2: Bax (5/12), Mcl-1: Bax (6/12), BIM: Bcl-2

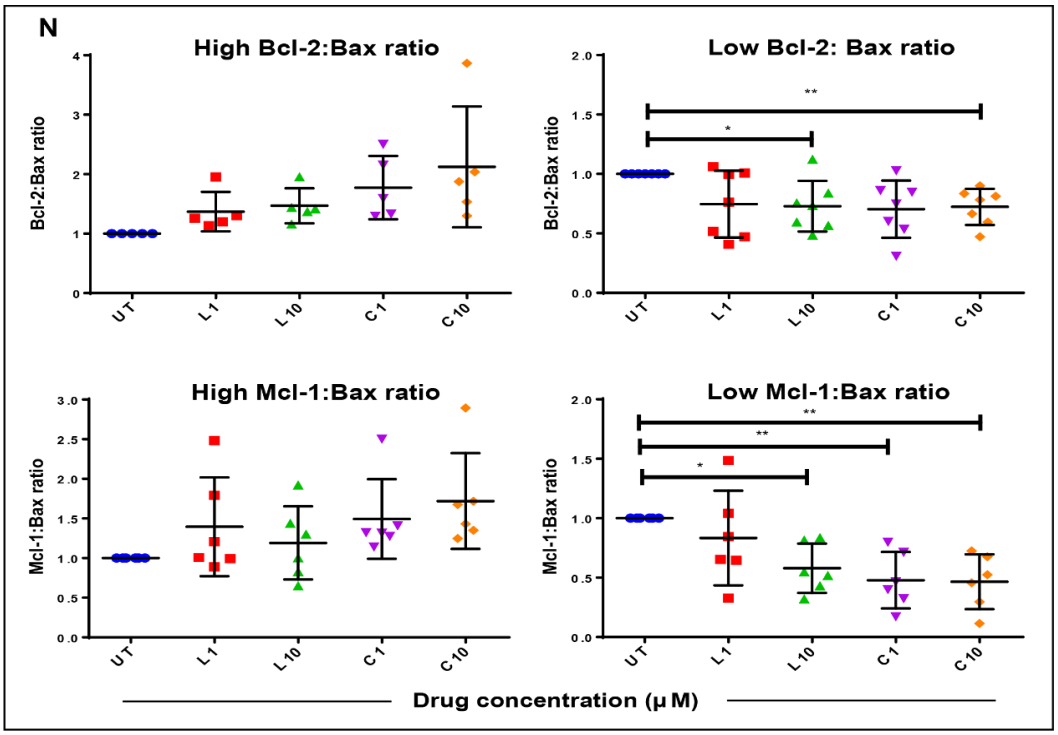
(3/12) and BIM: Mcl-1 (5/12) in specific cases they were not statistically different compared to untreated samples. Interestingly, all these ratios were significantly altered in more than 6 samples (see Table below). These results indicate that BH3 containing protein profiles and their ratios in primary CLL cells were differentially altered in the presence of LEN or CC-122 and may predict activity of IMiDs combined with ABT-199 in specific cases.


A
CLL # 3484 (1)
B
CLL # 3493 (1)
LEN 1 µM
LEN 10 µM
CC-122 1 µM
CC-122 10 µM
Aiolos
Ikaros
Bcl-2
Mcl-1
Bcl-xL
Bax
Bak
Bim EL
Bim L
Bim S
β-Actin
Anti-apoptotic
Pro-apoptotic
50
50
25
37
25
20
20
15
37
C
CLL # 3490 (1)
D
CLL # 3492 (1)


E
CLL # 3512 (2)
F
CLL # 3511 (2)
LEN 1 µM
LEN 10 µM
CC-122 1 µM
CC-122 10 µM
Aiolos
Ikaros
Bcl-2
Mcl-1
Bcl-xL
Anti-apoptotic
Bax
Bak
Bim EL
Bim L
Bim S
Pro-apoptotic
β-Actin
G
CLL # 3472 (2)
H
CLL # 3507 (2)


I
CLL # 3429 (3)
J
CLL # 3459 (3)
LEN 1 μM
LEN 10 μM
CC-122 1 μM
CC-122 10 μM
Aiolos
Ikaros
Bcl-2
Mcl-1
Bcl-xL
Bax
Bak
Bim EL
Bim L
Bim S
β-Actin
Anti-apoptotic
Pro-apoptotic
K
CLL # 3458 (4)
L
CLL # 3500 (4)
50
25
37
20
15


M
Aiolos
Aiolos expression relative to β-actin
Ikaros
Ikaros expression relative to β-actin
Bcl-2
Bcl-2 expression relative to β-actin
Bax
Bax expression relative to β-actin
Mcl-1
Mcl-1 expression relative to β-actin
Bak
Bak expression relative to β-actin
Bcl-xL
Bcl-xL expression relative to β-actin
Bim EL
Bim EL expression relative to β-actin
UT
L-1
L-10
C-1
C-10
Drug concentration (μM)



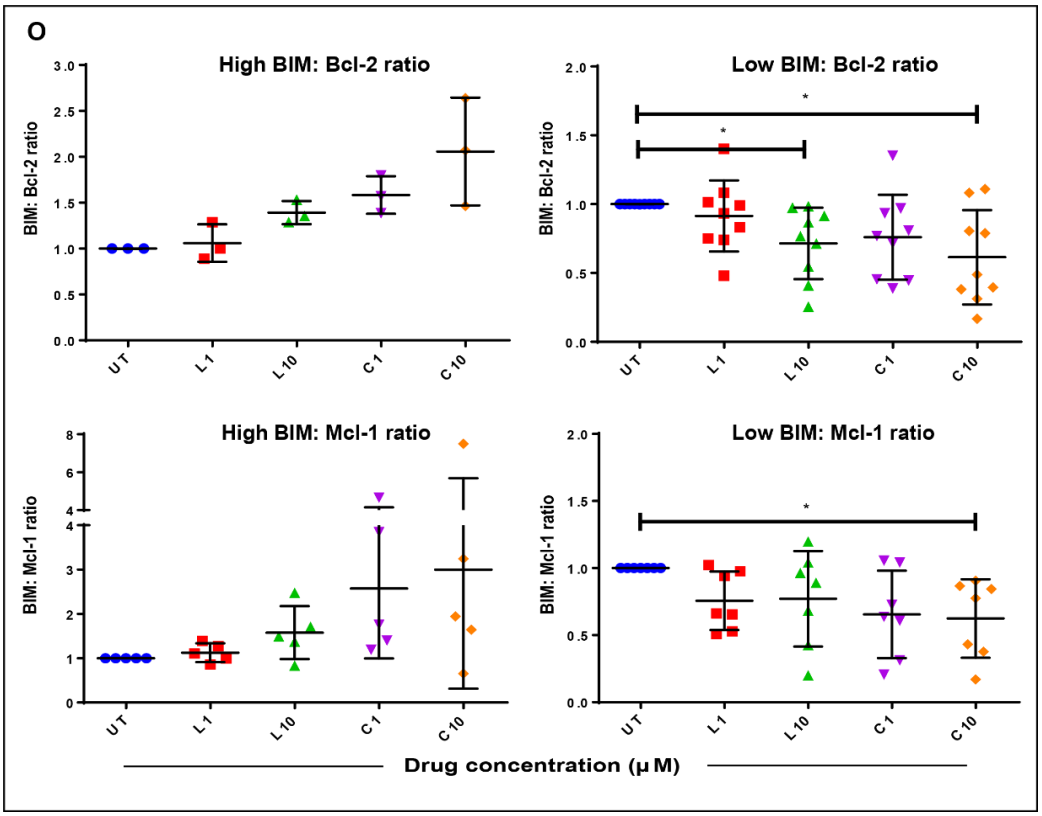


Figure 4.4: Effects of Lenalidomide or CC-122 on BH3-containing protein expression in primary CLL cells. Primary CLL cells were treated

with LEN or CC-122 for 24 hours. Whole cell lysates were analysed by western blotting for changes in the expression of target proteins **(A-L).** The expression of each protein in treated CLL cases, was normalised to untreated controls; densitometry **(M).** Ratios of Bcl-2: Bax, Mcl-1: Bax, BIM: Bcl-2 and BIM: Mcl-1 **(N & O);** ANOVA and Dunnett's multiple comparison test were used to determine statistically significant differences from UT. **p*<0.05; ***p*<0.01; ****p*<0.001; *****p*<0.0001; [SEM]; n=12).

4.4 Discussion

The aims of this chapter were to investigate if LEN and CC-122 induced apoptosis in B-CLL cell lines and primary CLL cell lines and also evaluate potential predictors of the sensitivity to LEN or CC-122 in combination with ABT-199 in primary CLL cells. The variable effects of these compounds on proliferation that triggered this study are described in **Chapter 3.** We hypothesised that such variability may be due to differential induction of apoptosis in the B-CLL cell lines and primary CLL cells.

Prior studies have shown that LEN and CC-122 induce apoptosis in multiple myeloma and DLBCL cell lines [180, 196]. The results obtained indicate that there was no demonstrable induction of apoptosis in either B-CLL cell lines or primary CLL cells upon exposure to LEN and CC-122 **(Figure 4.1)** and hence is not the cause for the variable inhibition of proliferation.

In addition, we measured the levels of BH3-domain containing anti- and pro-apoptotic proteins in B-CLL cell lines and primary CLL cells. In general, the members of the Bcl-2 family of proteins that are anti-apoptotic, protect cells from apoptosis. Whereas, pro-apoptotic proteins induce cell death [220]. Previous studies revealed that Bcl-2, Mcl-1, Bax and Bak proteins are overexpressed in CLL [214]. High levels of Mcl-1 are linked to chemo resistance and over expression of Bcl-2 (and a high Bcl-2: Bax ratio) is linked with an elevated white blood count in CLL [221]. B-CLL cell lines (MEC2 and MEC1) also show high levels of Bcl-2, Bcl-xL and Bax [191].

Despite down regulation of Ikaros and Aiolos, which served as the positive control for drug activity, the levels of individual proteins such as Bcl-2, Bax and Bak expression were only slightly decreased in MEC2 and MEC1 cells upon LEN or CC-122 treatment. Mcl-1, Bcl-xL and Bim levels, however, were slightly increased in MEC2 cells. Other cell lines showed little increase in the Mcl-1 and Bcl-xL expression level upon treatment without altering the pro-apoptotic protein levels (**Figure 4.2 E**).

Like B-CLL cell lines, LEN and CC-122 did not induce cytotoxic effects up to 3 days in primary CLL cells even at high concentrations. A variable alteration of anti- and pro-apoptotic proteins was observed in the primary CLL samples tested. Previous studies demonstrated that an elevated Bcl-2:Bax ratio is associated with poor response to conventional therapy [218]. Other studies report that the Mcl-1:Bax ratio is elevated patients resistant to Rituximab treatment [219]. Interestingly, in our study, a high Bcl-2: Bax ratio is associated with higher Mcl-1: Bax levels in 3 CLL cases. However, significantly lower ratios were found in 4 cases after IMiD treatment. Similarly, BIM displacement from Bcl-2 & Mcl-1 complex triggers the release of cytochrome C from the inner mitochondrial membrane spaces leading to apoptosis. In our study, high BIM: Bcl-2 (3/12) and BIM: Mcl-1 (5/12) ratios were observed in some CLL samples.

Table 12: Data summary of Bcl-2: Bax and Mcl-1: Bax ratios in primary CLL samples following LEN or CC-122 treatment

Primary CLL cohorts (n=12)			
Bcl-2: Bax ratio (ABT-199)		**Mcl-1: Bax ratio (S63845)**	
High (5/12) (42 %)	**Low (7/12) (58 %)**	**High (6/6) (50 %)**	**Low (6/6) (50 %)**
3490 (1), 3429 (3),	**3493 (1), 3512 (2),**	**3490 (1), 3429 (3),**	**3493 (1), 3512 (2)**
3459 (3)	**3472 (2), 3500 (4)**	**3459 (3)**	**3472 (2), 3500 (4)**
3507 (2)	**3484 (1), 3492 (1)**	**3484 (1), 3492 (1)**	**3507 (2)**
3458 (4)	**3511 (2)**	**3511 (2)**	**3458 (4)**
High Bcl-2: Bax & Mcl-1: Bax ratio		Low Bcl-2: Bax & Mcl-1: Bax ratio	
High Bcl-2: Bax ratio		Low Bcl-2: Bax ratio	
High Mcl-1: Bax ratio		Low Mcl-1: Bax ratio	

Table 13: Data summary of BIM: Bcl-2 and BIM: Mcl-1 ratios in primary CLL samples following LEN or CC-122 treatment

Primary CLL cohorts (n=12)			
BIM: Bcl-2 ratio		**BIM: Mcl-1 ratio**	
High (3/12) (25 %)	**Low (9/12) (75 %)**	**High (5/6) (42 %)**	**Low (7/6) (58 %)**
3512 (2)	**3484 (1), 3490 (1)**	**3512 (2), 3429 (3)**	**3484 (1), 3490 (1)**
3429 (3)	**3492 (1), 3472 (2)**	**3493 (1)**	**3492 (1), 3472 (2)**
3511 (2)	**3459 (3), 3500 (4)**	**3507 (2)**	**3459 (3),3500 (4)**
	3493 (1),3507 (2)	**3458 (4)**	**3511 (2)**
	3458 (4)		
High BIM: Bcl-2 & BIM: Mcl-1 ratio		Low BIM: Bcl-2 & BIM: Mcl-1 ratio	
High BIM: Bcl-2 ratio		Low BIM: Bcl-2 ratio	
High BIM: Mcl-1 ratio		Low BIM: Mcl-1 ratio	

Our results suggest that it may be possible to predict which CLL patients may benefit from a combination of IMiD and ABT-199.

To conclude, unlike MM and DLBCL cells, CLL cells do not undergo apoptosis on treatment with either LEN or CC-122. The predominant effect of both drugs seems to be cytostatic. Anti- and pro-apoptotic protein alterations could potentially help us to identify patients that may benefit from combining IMiDs with ABT-199.

Chapter 5 : Investigation of the effects of IMiDs on BCR signalling in CLL

5.1 Introduction

Lenalidomide has been shown to have therapeutic activity in haematological malignancies including CLL [222]. Its efficacy in relapsed or refractory CLL patients has been demonstrated [223].

More recently, the novel analogue, CC-122, has shown promise in DLBCL [180] but little is known about its effects on CLL cells. Low expression of BCR is a hallmark of CLL and aberrant signalling leads to abnormal growth and proliferation of malignant cells [224].

In general, BCR engagement triggers intracellular signalling events that result in downstream phosphorylation of critical kinases and other substrates [225]. A cascade of phosphorylation activates downstream substrates such as BTK, PLC-Ɣ2, PI3K, AKT, ERK and other signalling intermediates [226]. Following stimulation, there is upregulation of transcription factors including NF-κB which promote B-cell survival and proliferation [227]. Previous studies have reported that certain BCR pathway proteins (Lyn, PI3K, p38 and ERK 1/2) are constitutively phosphorylated which leads to survival and proliferation of CLL cells [73, 228, 229].

Therefore, we hypothesised that the inhibition of CLL cell proliferation by LEN and CC-122, may result in changes within the BCR signalling pathway. If true, it is necessary to understand the effects of the drugs on BCR signalling pathway proteins to uncover potential interactions and synergies.

To test this hypothesis, we first examined the effect of the IMiDs on MCL (MAVER-1 and JeKo-1) cell lines, as they are more sensitive to BCR stimulation than the previously mentioned B-CLL cell lines. We subsequently examined primary B-CLL cells for perturbations in BCR signalling on treatment with IMiDs.

The **aims** of this chapter, therefore, were:

1. To optimize BCR crosslinking methodology in Maver-1 cells.
2. To examine the effects of LEN and CC-122 upon Anti-IgM stimulation in a serum-containing and serum-free environment on proteins in the BCR signalling pathway in MAVER-1 and JeKo-1 cells.
3. To determine the effects of LEN and CC-122 on BCR signalling in primary CLL cells.
4. To additionally determine the effects of LEN and CC-122 on BCR signalling in primary MCL cells.
5. Correlate the effects of LEN and CC-122 on BCR signalling with Anti-IgM or CD40L stimulation in primary CLL cells.

5.2 Methods

5.2.1 B-cell receptor stimulation with human Anti-IgM

MAVER-1, JeKo-1 and primary CLL cells (on CD154 fibroblasts) were pre-incubated with LEN (10 µM) or CC-122 (1 and 10 µM) for 24 hours. Cells were pelleted and centrifuged at 550x*g*, 5 minutes, 20°C, then re-suspended in 100-200 µL of spent media and 10-20 µg/mL of AffiniPure F(ab')$_2$ Fragment Goat Anti-human IgM (Jackson ImmunoResearch, USA) antibody was added for 15 minutes at 37°C. To stop the reaction, 1 mL of ice-cold PBS was added and the cells pelleted and centrifuged at 550×*g*, 4°C for 5 minutes. The cell lysates were analysed for changes in expression of target proteins by immunoblotting.

5.2.2 B-cell receptor stimulation with human CD40 Ligand

Primary CLL cells were pre-incubated with LEN (10 µM) or CC-122 (1 µM) for 22 hours. Purified recombinant Human CD40 Ligand (500 µg/mL) and cross-linking antibody (Miltenyi Biotech, USA) were mixed in a 1:1 ratio then added to the cells for 30 minutes at room temperature. For CLL cells, 2 µL of this mixture was added and incubated at 37°C for a further 2 hours to give a total IMiD treatment time of 24 hours. Cells were collected by centrifugation at 550×*g* for 5 minutes and then washed with 1 mL of ice-cold PBS. Cell lysates were analysed as described above.

5.2.3 Preparation of growth-arrested MAVER-1 cells

MAVER-1 cells were plated at a density of 0.5×10^6/mL in serum free RPMI media for 24 hours. The cells were then treated for 24 hours with LEN (10 μM) or CC-122 (1 μM) before being stimulated with Human Anti-IgM for 15 minutes (see above). The cells were washed twice with 1 mL of ice-cold PBS and collected by centrifugation at 550×*g* for 5 minutes and lysates analysed as described previously.

5.3 Results

5.3.1 Optimization of concentration and duration of Anti-IgM stimulation in MAVER-1 cells

It is well established that MCL cell lines are highly sensitive to BCR stimulation upon Anti-IgM stimulation leading to the activation of downstream signalling proteins [230]. Hence, in the first instance the MAVER-1 cell line was selected as a model to understand the changes in BCR signalling pathway proteins in response to LEN or CC-122. Before performing the drug treatment experiments, it was essential to optimize the concentration of Anti-IgM and duration of stimulation at which MAVER-1 cells show maximal activation. MAVER-1 cells were treated with a range of concentrations of Anti-IgM 0-20 µg/mL for 15 minutes. Cells were stimulated for a range of time points up to 15 minutes for initial optimization. Cells were then harvested, lysed and analysed by western blotting. Protein transfer membranes were probed for p-PLCϒ2, p-ERK, p-AKT and corresponding total proteins as loading controls.

Figure 5.1 shows that phosphorylation of BCR intermediates in MAVER-1 cells was directly proportional to the Anti-IgM concentration as well as duration of incubation. BCR crosslinking with Anti-IgM resulted in phosphorylation of proximal BCR signalling proteins p-PLCϒ2, ERK and AKT in MAVER-1 cells when compared to unstimulated cells. The level of phosphorylation induction of p-PLCϒ2, ERK and AKT proteins was concentration dependent (**A**). Similarly, stimulation of MAVER-1 cells with 20

µg/mL showed higher phosphorylation induction with increasing time of incubation; whereas maximum induction was achieved within 5 minutes in PLCϒ2, maximal phosphorylation of ERK and AKT proteins (**C**) was seen after 15 minutes. Therefore, 20 µg/mL of Anti-IgM stimulation for 15 minutes was adopted as the optimal condition for further experiments in this chapter.

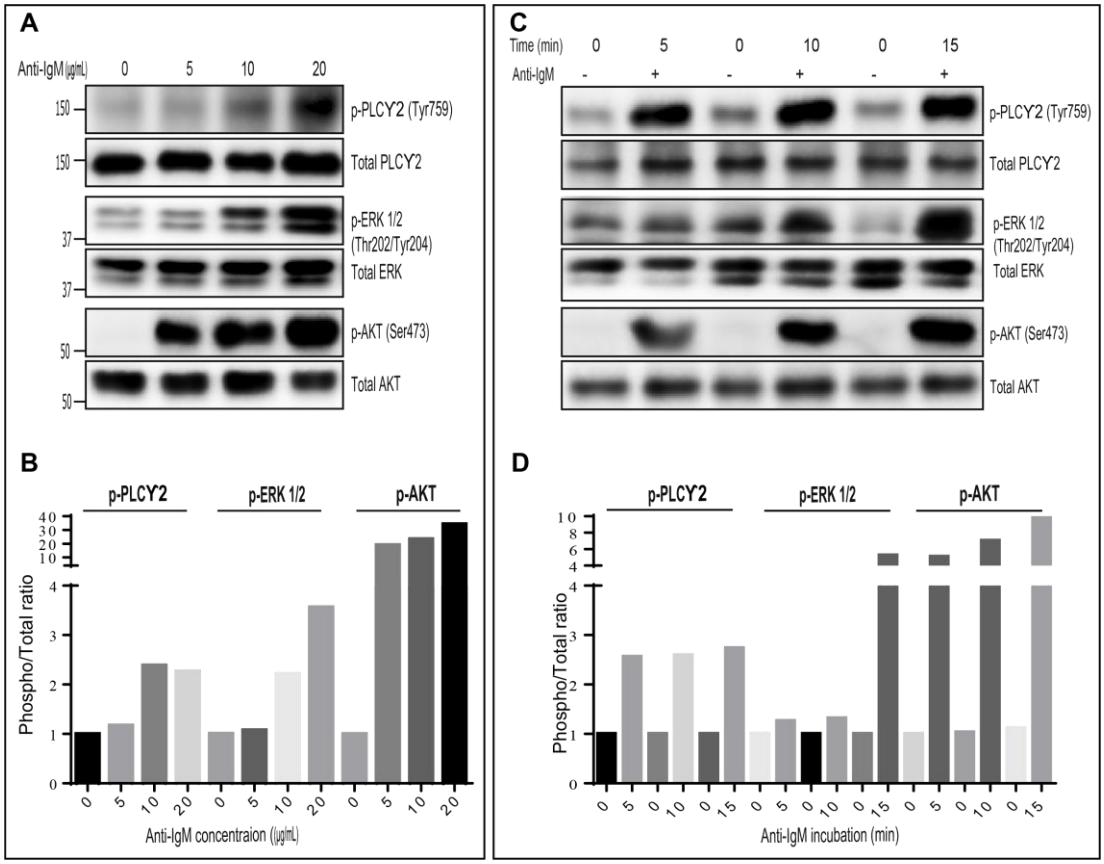


Figure 5.1: Optimization of concentration and duration of Anti-IgM stimulation in MAVER-1 cells. MAVER-1 cells were cultured and exposed to 0, 5, 10 and 20 µg/mL of Anti-IgM for 15 minutes **(A)** and 20 µg/mL of Anti-IgM for 0, 5, 10 and 15 minutes **(C)** and incubated at 37˚C. Lysates were prepared from harvested cells after each time point and then analysed by western blot. The membranes were probed for p-PLCϒ2, p-ERK, p-AKT and corresponding total protein antibodies. **B** and **D**; Densitometry;

5.3.2 Anti-IgM dependent activation of BCR signalling proteins in B-CLL and MCL cell lines

Having shown that MAVER-1 cells are sensitive to BCR stimulation (**Figure 5.1**), we next investigated the effect of stimulation with Anti-IgM, using this optimised method, on additional MCL (Jeko-1) and B-CLL (MEC1, MEC2, HG3 & EHEB) cell lines and compared them to MAVER-1 cells.

Upon IgM stimulation, MAVER-1 and JeKo-1 cells induced phosphorylation of PLCϒ2, ERK and AKT proteins. However, HG3 cells showed only a slight induction of p-PLCϒ2, whereas EHEB cells showed phosphorylation of AKT upon Anti-IgM treatment. In contrast, MEC1, MEC2, cells did not show any Anti-IgM induced activation of the above proteins when compared to unstimulated cells (**Figure 5.2**). Unlike MAVER-1 and Jeko-1 cells, B-CLL cell lines have constitutively phosphorylated BCR signalling proteins even in unstimulated cells. Total protein levels in both cell lines remained similar in both treated and untreated samples. This suggests that MAVER-1 and JeKo-1 cells were the most sensitive to Anti-IgM stimulation compared to the B-CLL cell lines and these were used for future studies.

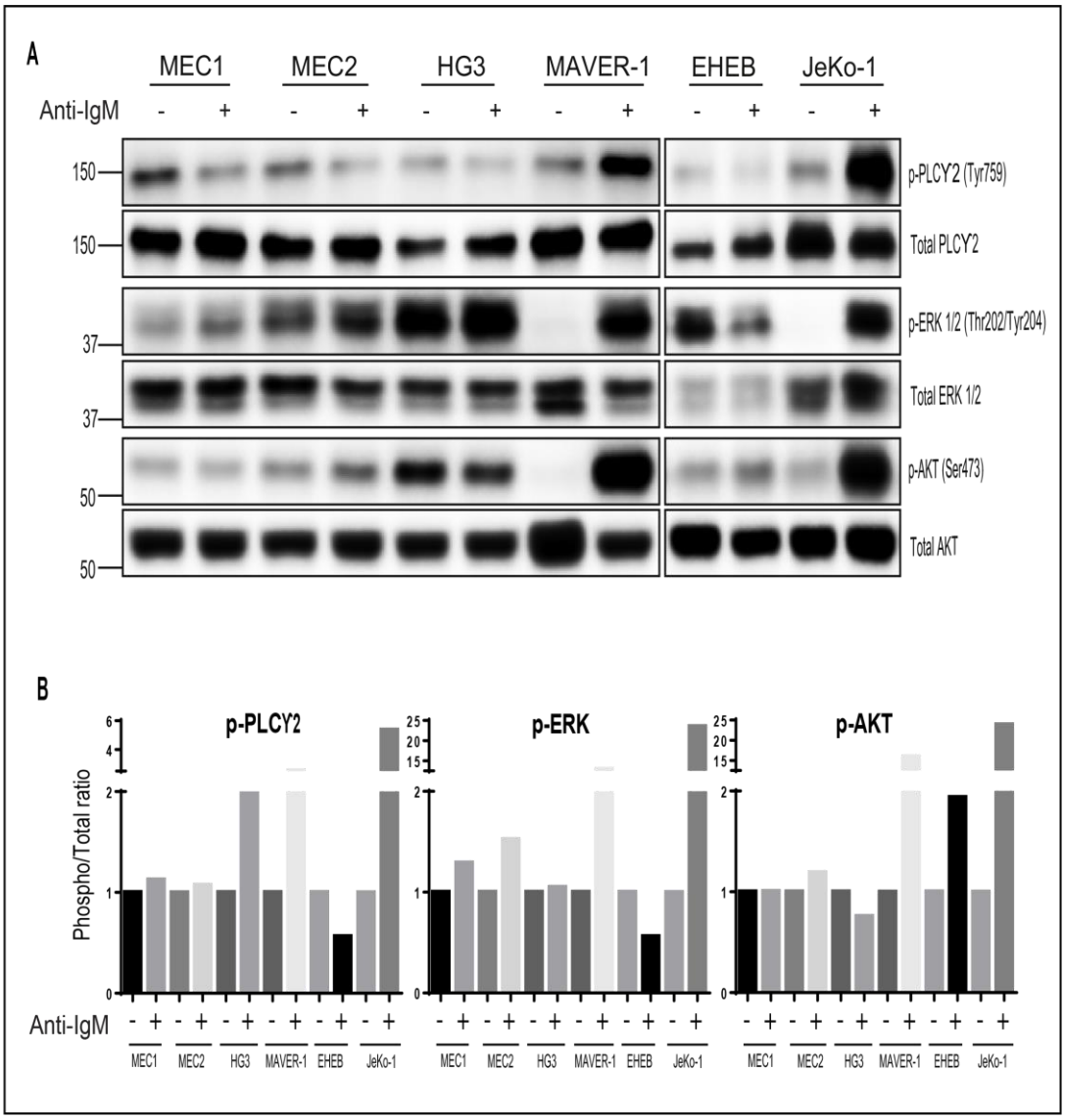
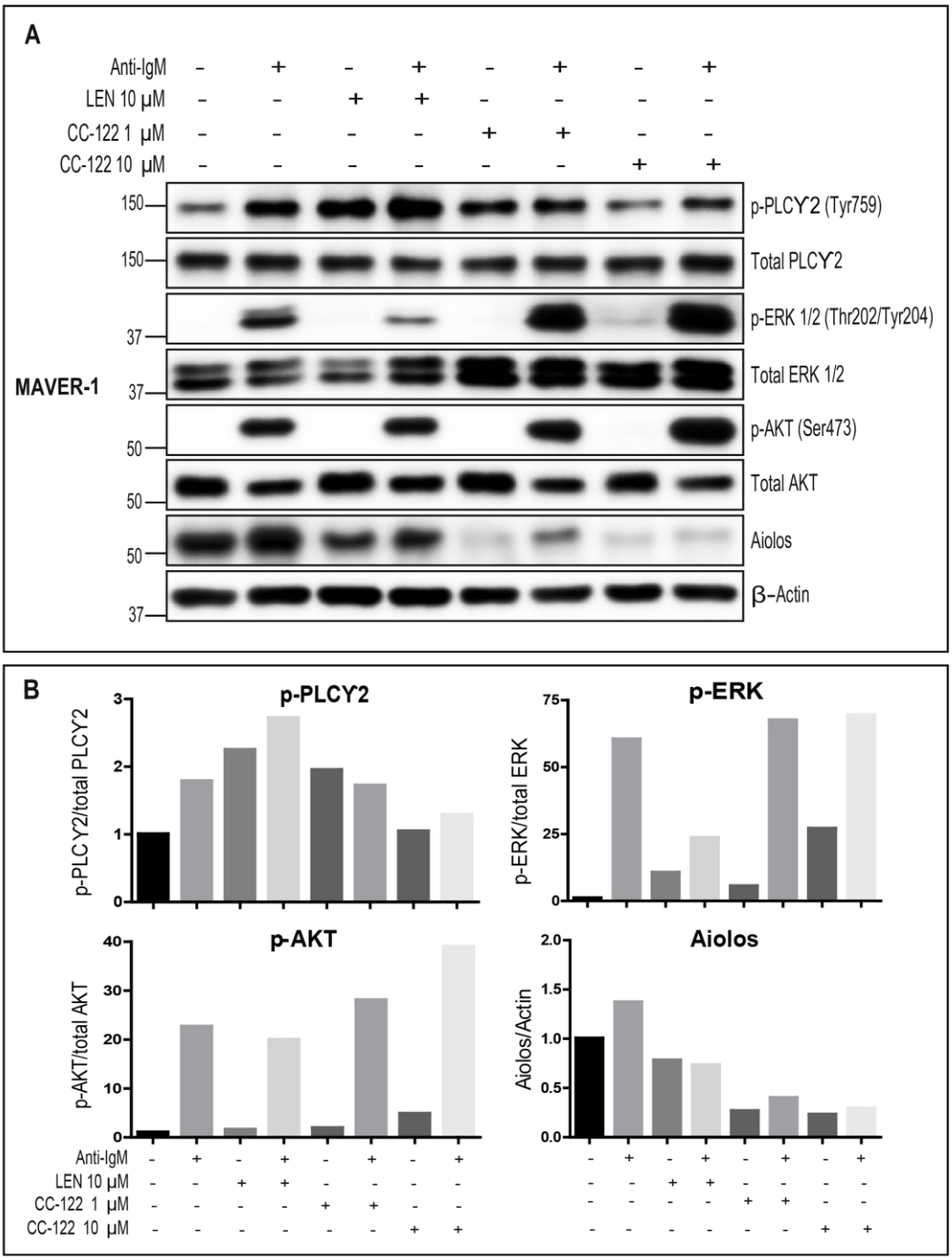


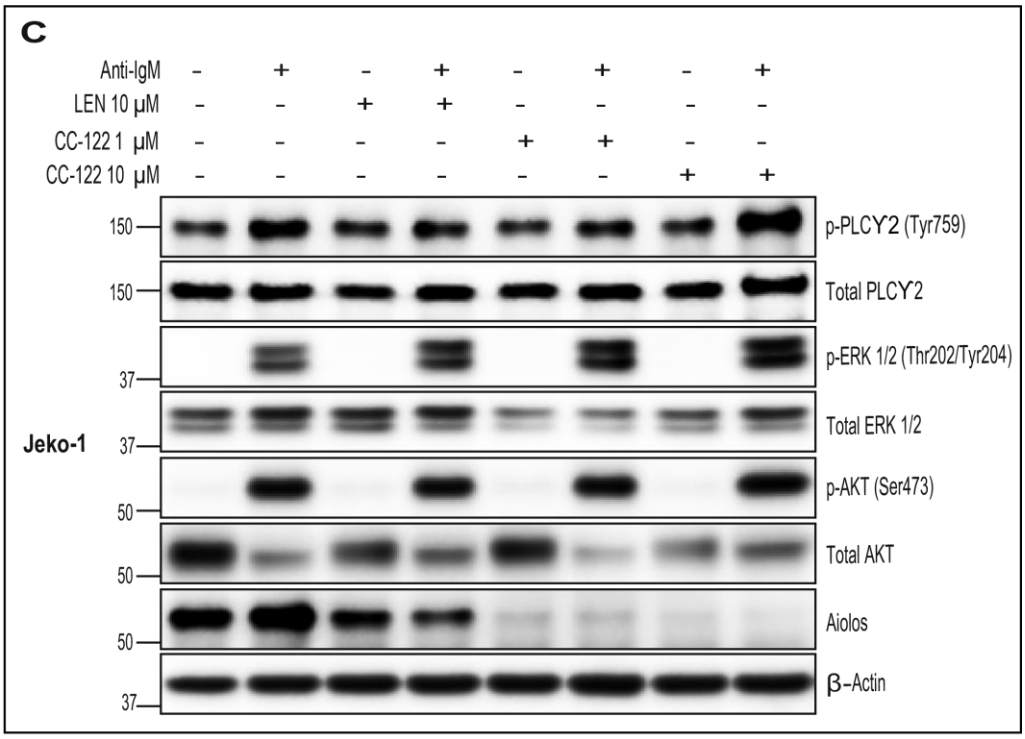
Figure 5.2: Activation of BCR signalling proteins in B-CLL and MCL cell lines by Anti-IgM. MCL (MAVER-1 and JeKo-1) and B-CLL (MEC1, MEC2, HG3 and EHEB) cells were cultured and exposed to 20 µg/mL of Anti-IgM for 15 minutes. Whole cell lysates were prepared and analysed by western blot. The membranes were probed for p-PLCY2, p-ERK, p-AKT and total proteins as loading controls. **A.** Western blot showing phospho and total protein expression in all the cell lines. **B.** Densitometry values of phospho-proteins relative to the respective total protein.

5.3.3 Effects of Lenalidomide and CC-122 on BCR signalling proteins in MAVER-1 and JeKo-1 cells stimulated by Anti-IgM

We next investigated the effects of LEN and CC-122 on proximal signalling events of BCR proteins upon stimulation with Anti-IgM. Both MAVER-1 and JeKo-1 cells were treated with LEN or CC-122 for 24 hours. Cells were then stimulated with 20 µg/mL of Anti-IgM for 15 minutes before examination by immunoblotting.

As shown in **Figure 5.3,** both LEN and CC-122 impact the phosphorylation status of downstream proteins in the BCR pathway upon Anti-IgM stimulation in both MAVER-1 (**A & B**) and JeKo-1 (**C & D**) cells. As expected, Anti-IgM alone induced phosphorylation of the studied BCR proteins. Following Anti-IgM stimulation and treatment with both drugs, constitutive phosphorylation was maintained in all examined BCR proteins (PLCϒ2, AKT and ERK) in MAVER-1 and JeKo-1 cells. Interestingly, irrespective of Anti-IgM stimulation, Aiolos was consistently degraded in both cell lines and was more evident with CC-122 than LEN. These results suggest that neither LEN nor CC-122 have a significant effect on BCR signalling in MAVER-1 and JeKo-1 cells.


A
Anti-IgM
LEN 10 µM
CC-122 1 µM
CC-122 10 µM
MAVER-1
150
150
37
37
50
50
50
37
p-PLCγ2 (Tyr759)
Total PLCγ2
p-ERK 1/2 (Thr202/Tyr204)
Total ERK 1/2
p-AKT (Ser473)
Total AKT
Aiolos
β-Actin
B
p-PLCγ2
p-PLCγ2/total PLCγ2
p-ERK
p-ERK/total ERK
p-AKT
p-AKT/total AKT
Aiolos
Aiolos/Actin
Anti-IgM
LEN 10 µM
CC-122 1 µM
CC-122 10 µM


C
Anti-IgM
LEN 10 µM
CC-122 1 µM
CC-122 10 µM
Jeko-1
150
150
37
37
50
50
50
37
p-PLCγ2 (Tyr759)
Total PLCγ2
p-ERK 1/2 (Thr202/Tyr204)
Total ERK 1/2
p-AKT (Ser473)
Total AKT
Aiolos
β-Actin

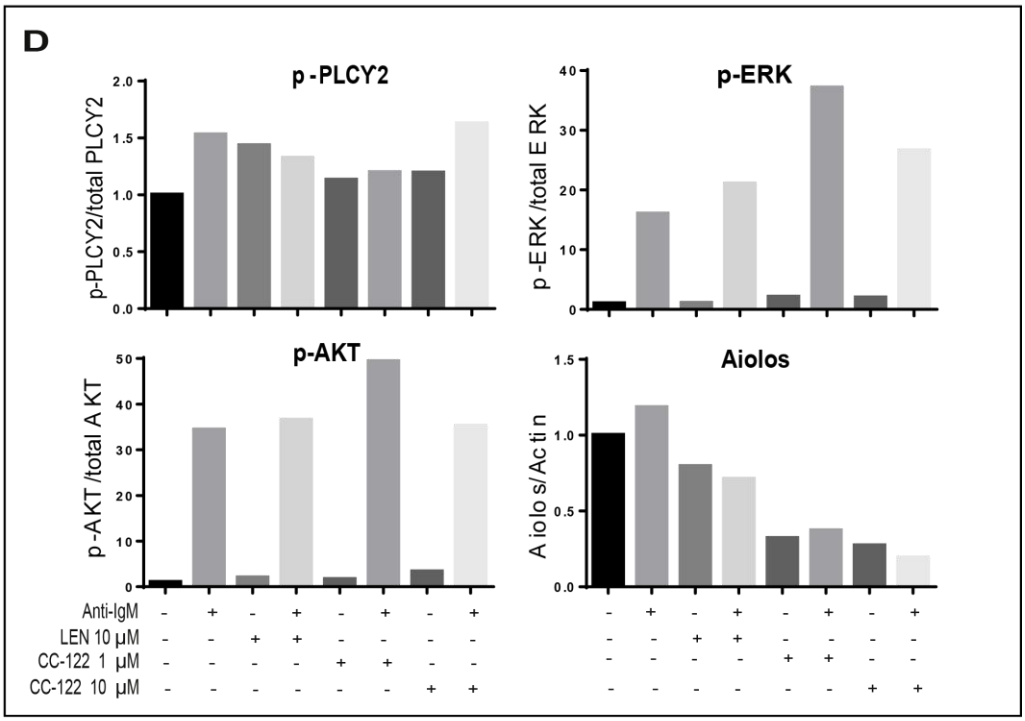

D
p-PLCγ2
p-PLCγ2/total PLCγ2
p-ERK
p-ERK /total ERK
p-AKT
p-AKT /total AKT
Aiolos
Aiolos/Actin
Anti-IgM
LEN 10 µM
CC-122 1 µM
CC-122 10 µM

Figure 5.3: Effects of Lenalidomide and CC-122 on BCR signalling proteins in MAVER-1 and JeKo-1 cells stimulated by Anti-IgM. MAVER-1 **(A)** and JeKo-1 **(C)** cells were cultured and treated with LEN (10 µM) or CC-122 (1 and 10 µM) or DMSO for 24 hours. Cells were collected and treated with 20 µg/mL of Anti-IgM for 15 minutes. The lysates were prepared and analysed by western blot for a panel of target proteins. Densitometry analysis was performed to compare levels of phospho- to total proteins and are shown for MAVER-1 **(B)** and Jeko-1 **(D).**

5.3.4 Effects of Lenalidomide and CC-122 on BCR signalling proteins in MAVER-1 cells stimulated by Anti-IgM under serum free conditions

Following withdrawal of serum, the ERK pathway is deactivated suggesting that continuous activation via Raf-MEK-ERK is necessary to maintain basal levels of ERK activity in proliferating cells [231]. We further investigated the effect of LEN and CC-122 on BCR signalling proteins upon stimulation in serum deprived cultures. MAVER-1 cells were cultured for 24 hours in serum free RPMI medium. Then, cells were treated with LEN or CC-122 for 24 hours before being stimulated with Anti-IgM as previously described.

Figure 5.4 shows that the phosphorylation levels of proteins in MAVER-1 cells were only slightly altered by LEN and CC-122 treatment even under serum free condition. However, upon Anti-IgM stimulation, all the proteins were consistently phosphorylated and showed high induction compared to unstimulated cells. Both LEN and CC-122 degraded Aiolos expression completely in MAVER-1 cells. Upon LEN and CC-122 treatment with IgM stimulation, ERK phosphorylation was slightly increased while p-PLCϒ2 and p-AKT levels were constantly maintained in both stimulated and unstimulated

(Anti-IgM) samples following treatment with LEN or CC-122. This suggests that both drugs were not effective at inhibiting the BCR signalling proteins in MAVER-1 cells even under serum free conditions.

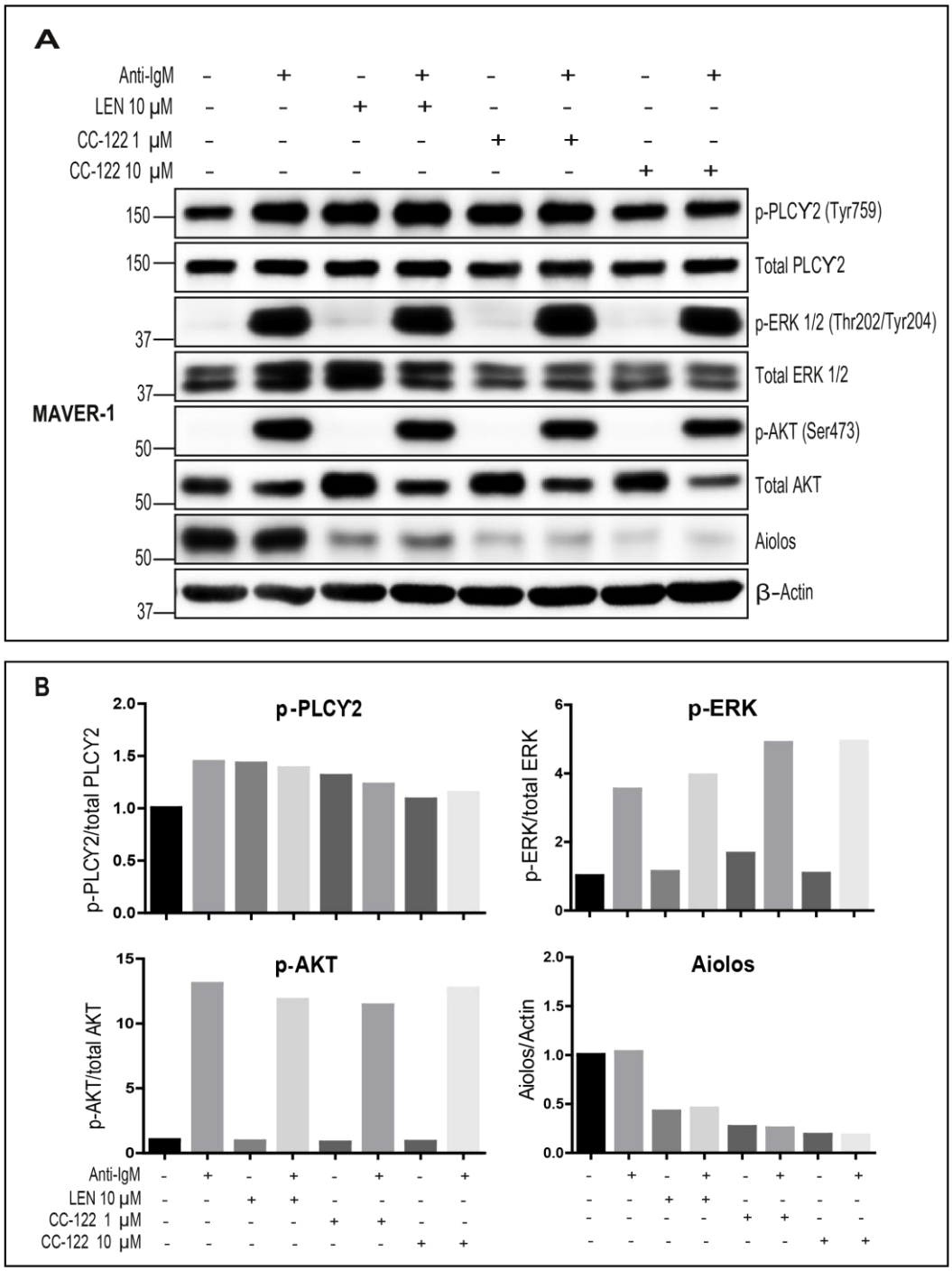


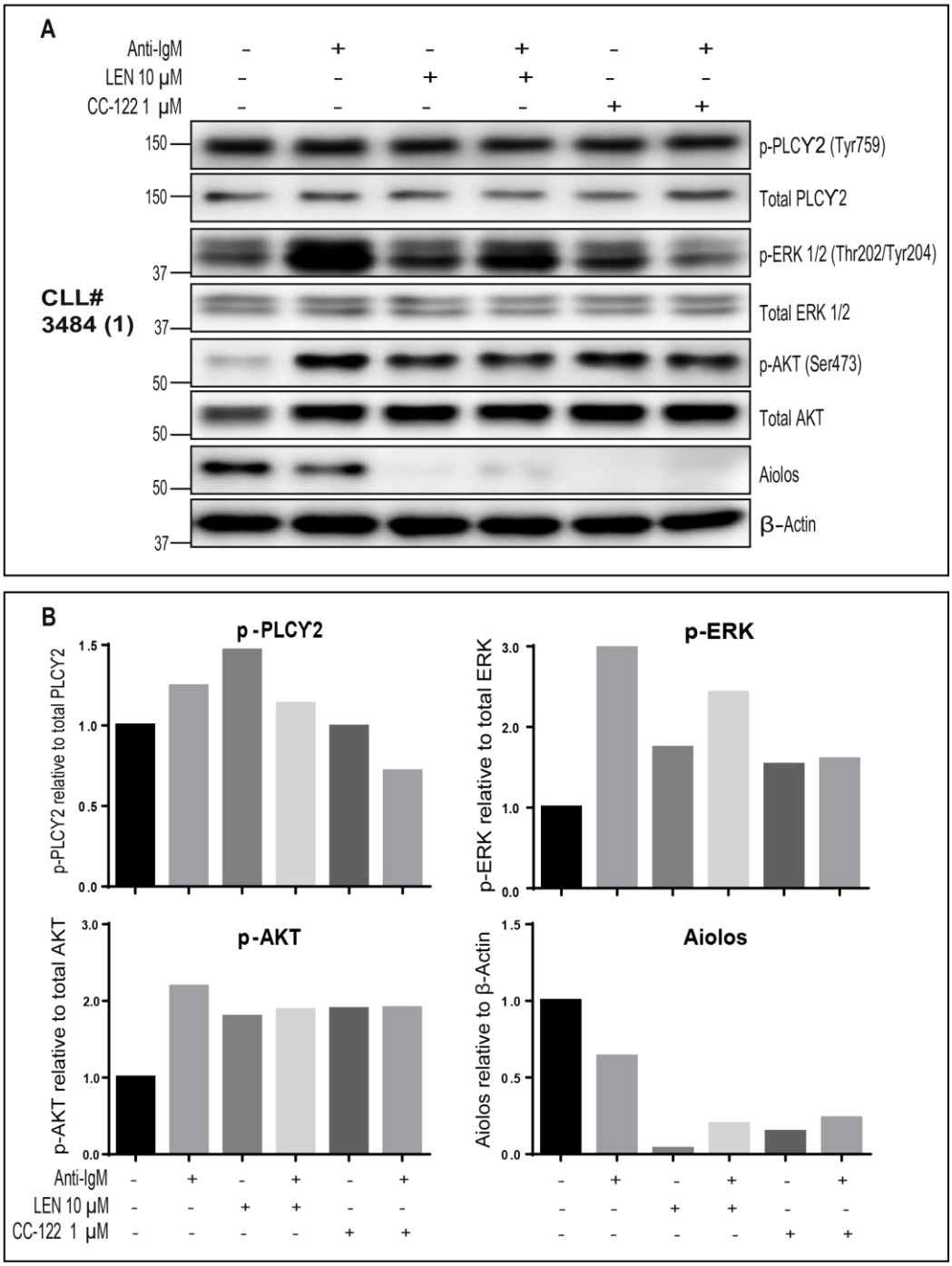
Figure 5.4: Effects of Lenalidomide and CC-122 on BCR signalling proteins in MAVER-1 cells stimulated by Anti-IgM in serum free conditions. MAVER-1 cells were initially cultured for 24 hours in serum free RPMI medium and treated with LEN (10 µM) or CC-122 (1 and 10 µM) or DMSO for additional 24 hours. Cells were collected and treated with 20 µg/mL of Anti-IgM for 15 minutes. Whole cell lysates were prepared and analysed by western blot. **A.** Western blots showing phospho- and total protein expression in MAVER-1 cells. **B.** Densitometry analysis was performed to compare the levels of phospho- to total proteins.

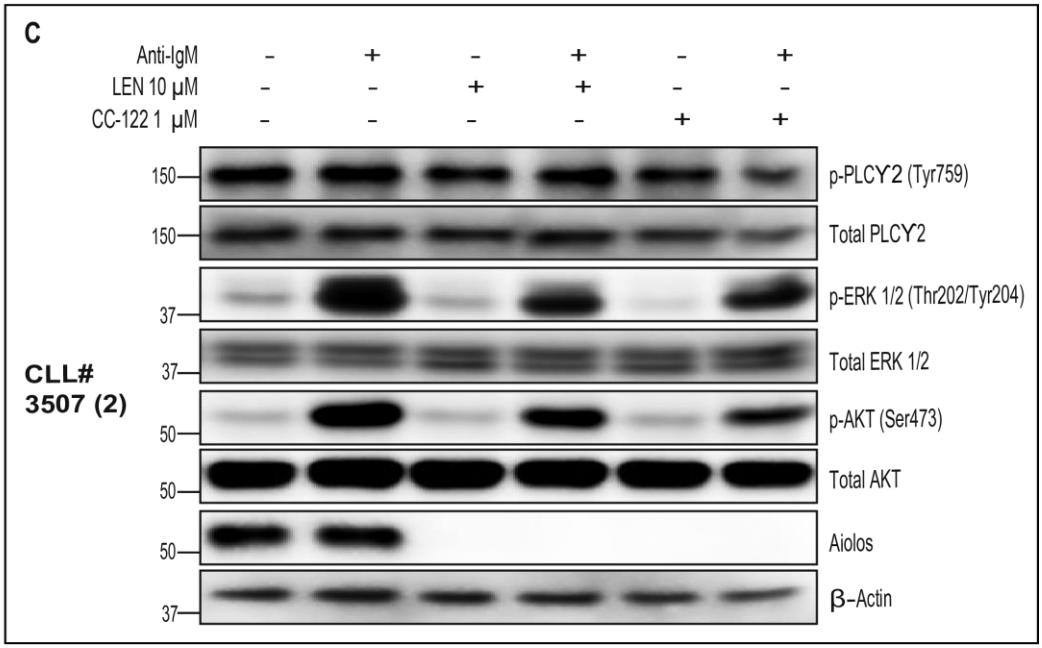
5.3.5 Lenalidomide and CC-122 inhibit Anti-IgM induced p-ERK and p-AKT expression in primary CLL cells

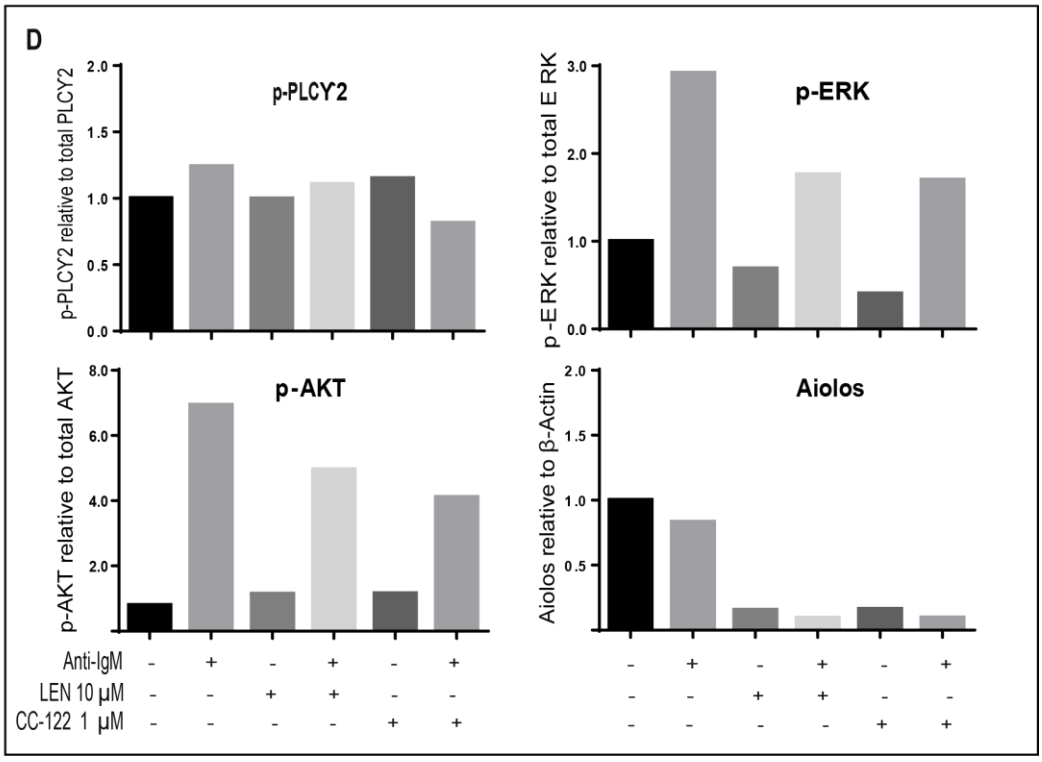
In previous experiments, it was observed that LEN and CC-122 do not inhibit the phosphorylation of BCR signalling intermediate proteins by Anti-IgM stimulation in MAVER-1 or JeKo-1 cells (**Figure 5.3**). We next screened primary CLL cells to study the effects on BCR induced proximal signalling events after drug treatment and Anti-IgM stimulation. Fresh primary CLL cells were seeded on CD154 fibroblasts and treated with LEN (10 µM) and CC-122 (1 µM) for 24 hours. Cells were harvested and stimulated with 20 µg/mL of Anti-IgM for 15 minutes before protein analysis.

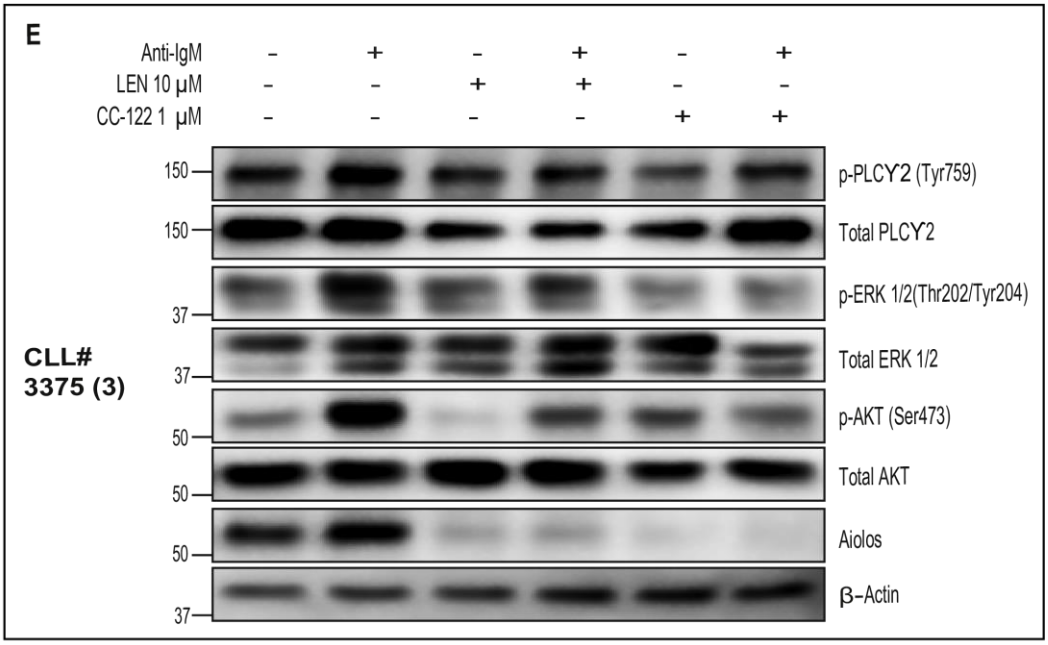
Stimulation of primary CLL cells with Anti-IgM showed differential responses of BCR signalling proteins after LEN or CC-122 treatment (**Figure 5.5**). As expected, primary CLL cells showed higher phosphorylation levels in all studied proteins when they were stimulated with Anti-IgM when compared to unstimulated cells. The treatment with LEN or CC-122 did show some reduction in the expression of p-PLCϒ2 upon stimulation in primary CLL cells (n=6). In contrast, both LEN and CC-122 significantly downregulated BCR induced ERK phosphorylation in all the primary CLL samples investigated

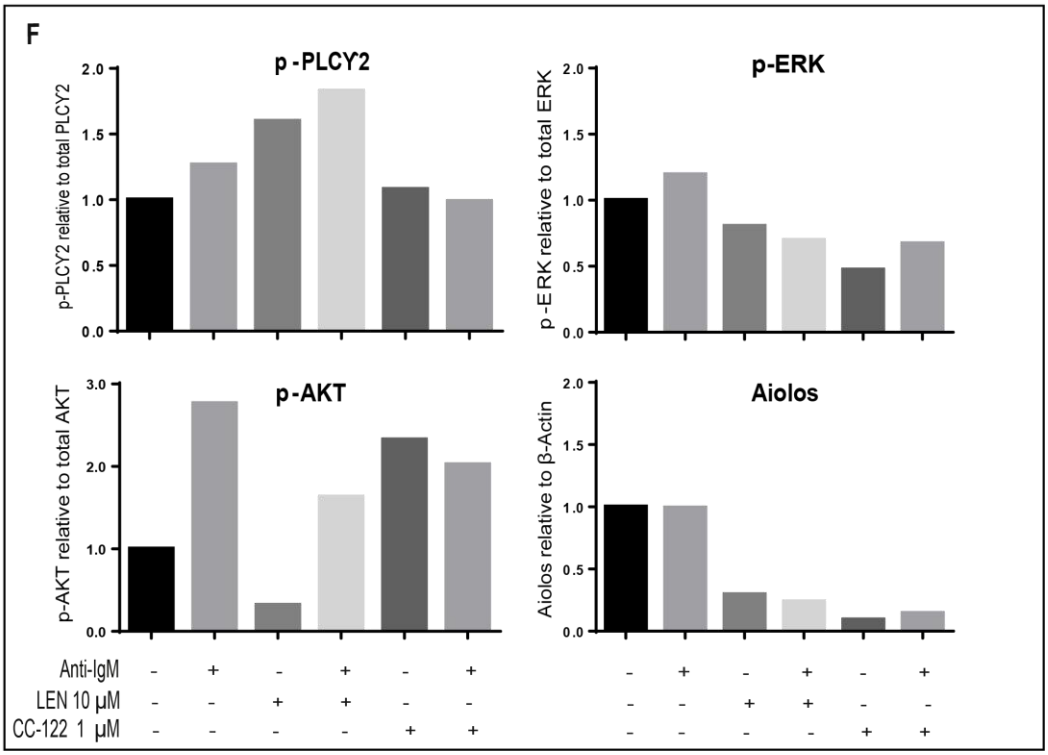
(p< 0.001; paired t-test) (**Figure 5.5 J**). This inhibition was more pronounced with CC-122 than with LEN. The effects on AKT phosphorylation were variable upon LEN and Anti-IgM treatment in individual cases. For instance, both drugs inhibited BCR induced AKT phosphorylation in cases **3473, 3512, 3511, 3507, 3375 and 3367 (Figure 5.5 D)**) whereas in some samples only LEN showed p-AKT inhibition (**3491, 3527 and 3522**). In contrast, neither LEN nor CC-122 showed any effects on p-AKT expression and was similar to untreated (Anti-IgM) cells in some cases (**3484 (A), 3472 and 3424**). As expected, both drugs degraded Aiolos levels irrespective of Anti-IgM stimulation. A total 12 CLL samples were examined and plotted on a scatter graph (**I**). In conclusion, both drugs were efficient at downregulating p-ERK (12/12), but showed variable effects on AKT phosphorylation (6/12) and some reduction in PLCϒ2 phosphorylation in primary CLL cells.

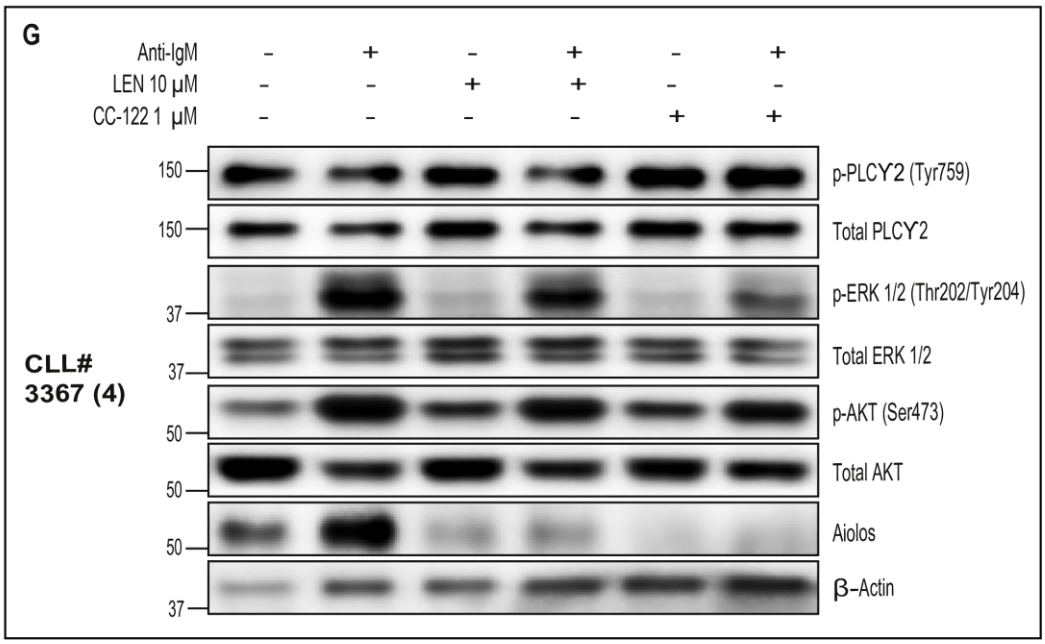

A
Anti-IgM
LEN 10 µM
CC-122 1 µM
CLL# 3484 (1)
150
150
37
37
50
50
50
37
p-PLCγ2 (Tyr759)
Total PLCγ2
p-ERK 1/2 (Thr202/Tyr204)
Total ERK 1/2
p-AKT (Ser473)
Total AKT
Aiolos
β-Actin
B
p-PLCγ2
p-PLCγ2 relative to total PLCγ2
p-ERK
p-ERK relative to total ERK
p-AKT
p-AKT relative to total AKT
Aiolos
Aiolos relative to β-Actin
Anti-IgM
LEN 10 µM
CC-122 1 µM

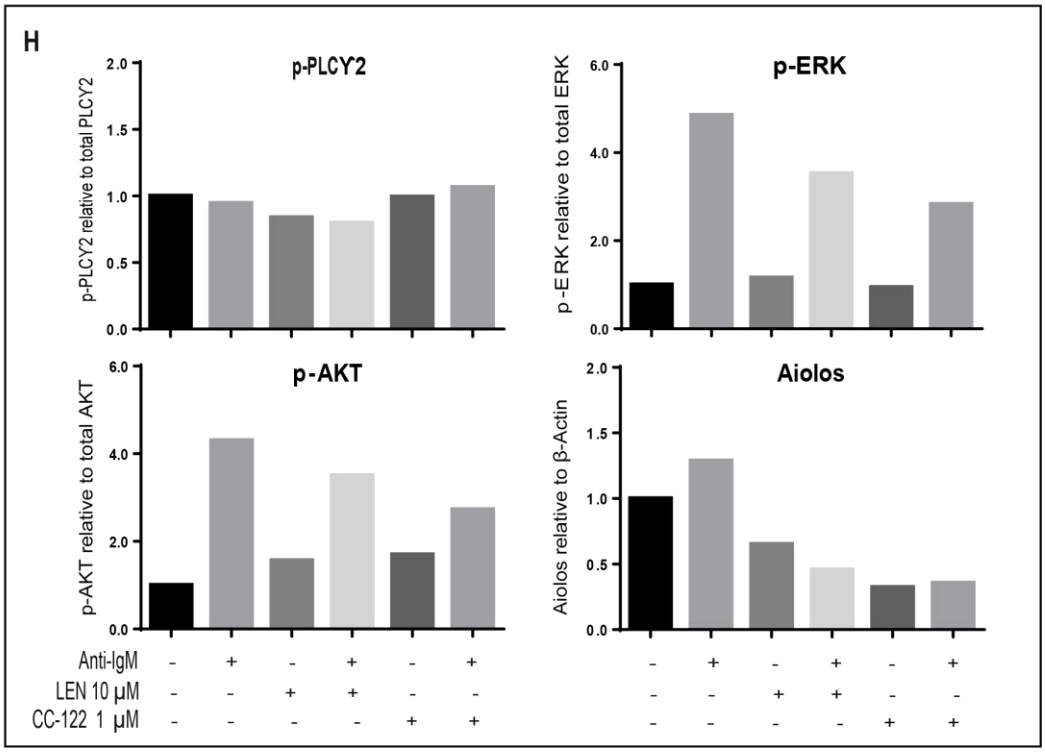

C
Anti-IgM
LEN 10 µM
CC-122 1 µM
CLL# 3507 (2)
150
150
37
37
50
50
50
37
p-PLCγ2 (Tyr759)
Total PLCγ2
p-ERK 1/2 (Thr202/Tyr204)
Total ERK 1/2
p-AKT (Ser473)
Total AKT
Aiolos
β-Actin


D
p-PLCγ2
p-PLCγ2 relative to total PLCγ2
p-ERK
p-ERK relative to total ERK
p-AKT
p-AKT relative to total AKT
Aiolos
Aiolos relative to β-Actin
Anti-IgM
LEN 10 µM
CC-122 1 µM


E
Anti-IgM
LEN 10 µM
CC-122 1 µM
CLL# 3375 (3)
150
150
37
37
50
50
50
37
p-PLCγ2 (Tyr759)
Total PLCγ2
p-ERK 1/2(Thr202/Tyr204)
Total ERK 1/2
p-AKT (Ser473)
Total AKT
Aiolos
β-Actin


F
p-PLCγ2
p-PLCγ2 relative to total PLCγ2
p-ERK
p-ERK relative to total ERK
p-AKT
p-AKT relative to total AKT
Aiolos
Aiolos relative to β-Actin
Anti-IgM
LEN 10 µM
CC-122 1 µM


G
Anti-IgM
LEN 10 µM
CC-122 1 µM
CLL# 3367 (4)
150
150
37
37
50
50
50
37
p-PLCγ2 (Tyr759)
Total PLCγ2
p-ERK 1/2 (Thr202/Tyr204)
Total ERK 1/2
p-AKT (Ser473)
Total AKT
Aiolos
β-Actin


H
p-PLCγ2
p-PLCY2 relative to total PLCY2
p-ERK
p-ERK relative to total ERK
p-AKT
p-AKT relative to total AKT
Aiolos
Aiolos relative to β-Actin
Anti-IgM
LEN 10 µM
CC-122 1 µM

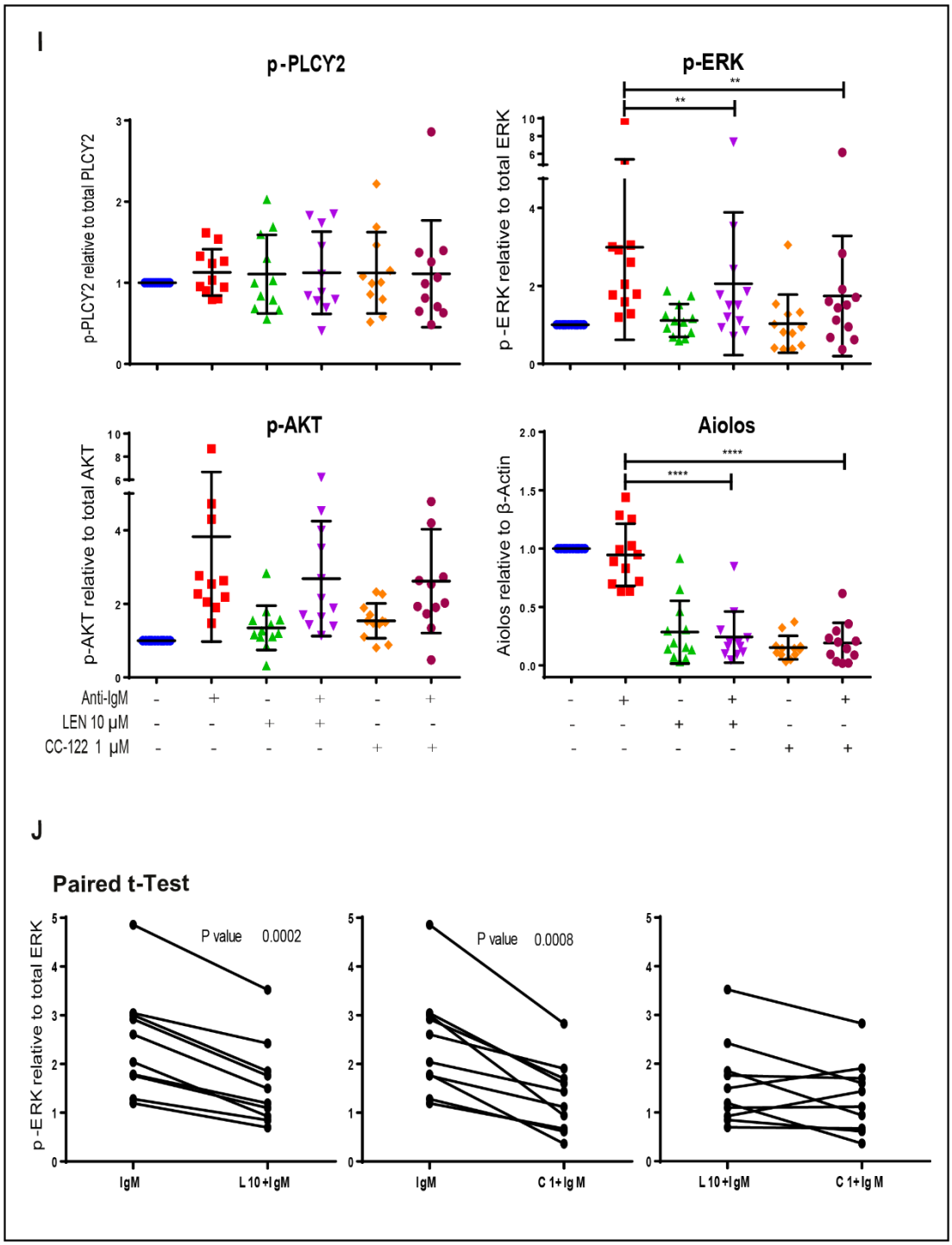

I
p-PLCY2
p-PLCY2 relative to total PLCY2
p-ERK
p-ERK relative to total ERK
**
**
p-AKT
p-AKT relative to total AKT
Aiolos
Aiolos relative to β-Actin
****
****
Anti-IgM
LEN 10 µM
CC-122 1 µM
J
Paired t-Test
p-ERK relative to total ERK
P value 0.0002
P value 0.0008
IgM
L10+IgM
IgM
C1+IgM
L10+IgM
C1+IgM

Figure 5.5: Lenalidomide and CC-122 effects on Anti-IgM induced p-ERK and p-AKT expression in primary CLL cells. Primary CLL cells were thawed, cultured on CD154 fibroblasts and treated with LEN (10 µM) or CC-122 (1 µM) or DMSO for 24 hours. Cells were collected and treated with 20 µg/mL of Anti-IgM for 15 minutes. The lysates were prepared and analysed by western blot. **A, C, E** and **G** are western blots showing phospho- and total protein expression in **representative cases** of CLL cells. **B, D, F,** and **H** illustrate the densitometry analysis showing levels of phosphorylated proteins to total protein. A summary of the densitometry analysis performed on a panel of 12 cases is shown in **I**; Comparison between L10 or C1 with Anti-IgM shown in **J** by paired t-test. ANOVA and Dunnett's multiple comparison tests were used to determine statistically significant differences from UT. ***p*<0.01; ****p*<0.001; [SEM]; n=12).

5.3.6 Lenalidomide and CC-122 increase Anti-IgM induced p-ERK and p-AKT expression in primary MCL cells

Since both LEN and CC-122 consistently inhibit BCR induced phosphorylation of ERK in primary CLL cells but did not have a significant effect in MAVER-1 and JeKo-1 cell lines (**Figure 5.3**), we additionally examined whether or not they had any effect on primary MCL cells. Primary MCL cells were plated on CD154 fibroblasts and treated with LEN or CC-122 for 24 hours and then stimulated with 20 µg/mL of Anti-IgM for 15 minutes.

As shown in **Figure 5.6,** and as with primary CLL cells, induction of phosphorylation of ERK, PLCY2 and AKT was observed in MCL cells stimulated with Anti-IgM. Both LEN and CC-122 resulted in increased phosphorylation of ERK and AKT when compared to untreated (Anti-IgM) in a primary MCL case. The effect was more pronounced with CC-122. As with our panel of CLL cohorts, both drugs induced Aiolos degradation. These results were consistent with the findings in the MCL (MAVER-1 and JeKo-1) cell lines.

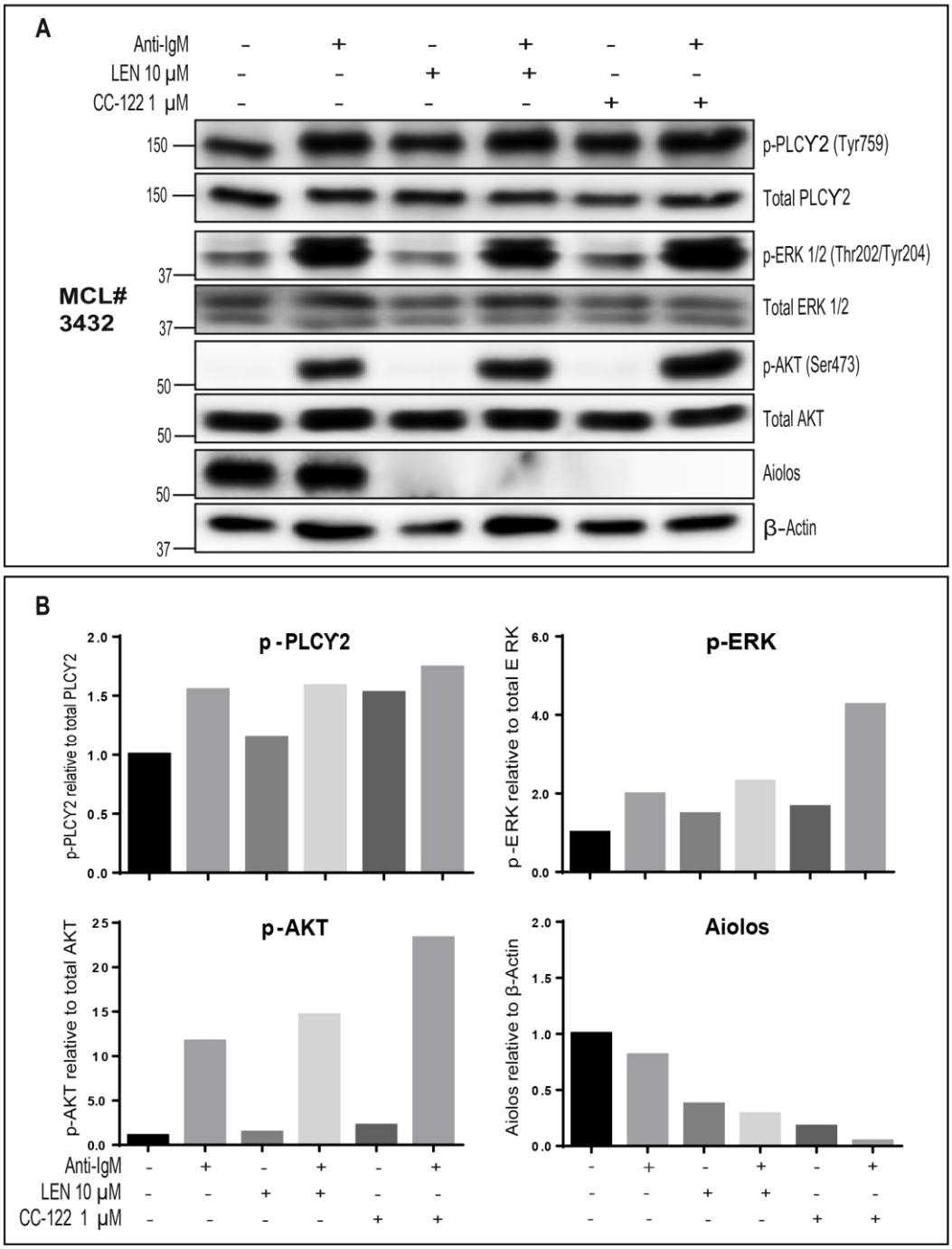


Figure 5.6: Lenalidomide and CC-122 increase Anti-IgM induced p-ERK and p-AKT expression in primary MCL cells. Fresh primary MCL cells

were thawed, cultured on CD154 fibroblasts, and treated with LEN (10 µM) or CC-122 (1 µM) or DMSO for 24 hours. Cells were collected and exposed with 20 µg/mL of Anti-IgM for 15 minutes. The lysates were prepared and analysed by western blot. Western blots showing phospho- and total protein expression in primary MCL cells **(A).** Densitometry analysis showing levels of phosphorylated proteins to total protein **(B).**

5.3.7 Comparison of treatment with Lenalidomide or CC-122 treatment and Anti-IgM and CD40L stimulation in primary CLL cells

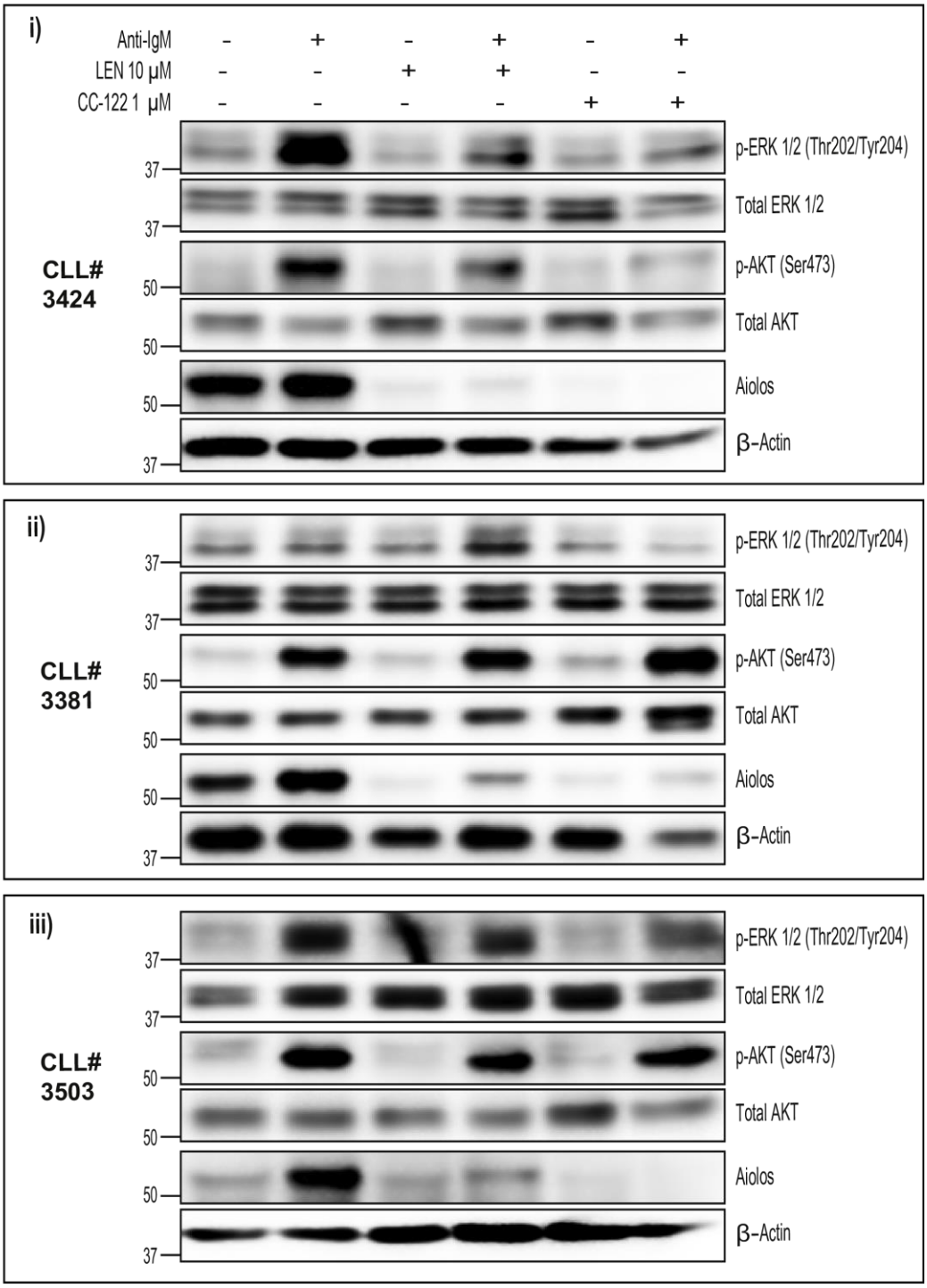
Thus far, our results demonstrate that both LEN and CC-122 inhibit the BCR induced ERK phosphorylation in primary CLL cells but not primary MCL cells. It is well established that Anti-IgM [225] and CD40L [232] play an important role in BCR signalling. Therefore, we sought to examine if the inhibition of phosphorylation is comparable on Anti-IgM or CD40L stimulation. We compared the changes in phosphorylated proteins after Anti-IgM or CD40L stimulation after LEN or CC-122 treatment in primary CLL cells. Primary CLL cells were plated on CD154 fibroblasts and treated with LEN or CC-122 for 24 hours and then were stimulated with Anti-IgM for 15 minutes or CD40L for 2 hours.

Figure 5.7 shows the results of Anti-IgM (**A**) or CD40L (**B**) stimulation on the phosphorylation of a panel of proteins following their treatment with LEN or CC-122. As expected, and shown previously (**Figure 5.5**), Anti-IgM stimulation resulted in induction of phosphorylation of ERK and AKT (**Figure 5.7 A i-iii**). Both LEN and CC-122 inhibit the phosphorylation of ERK and the effect was more obvious with CC-122 than LEN (**3424, 3381** and **3503**). AKT

phosphorylation was also inhibited slightly by both drugs in samples **3424** and **3503**.

As with Anti-IgM, CD40L stimulation induces phosphorylation of ERK and AKT. However, both LEN and CC-122 increased the phosphorylation of ERK and AKT following CD40L stimulation (**Figure 5.7 B i** (**3424**)). However, there were no marked differences between untreated and CD40L treatment in sample **3381**. These results suggest that LEN and CC-122 inhibit the Anti-IgM induced phosphorylation of ERK and AKT in primary CLL cells but not on CD40L stimulation.

A


i)
Anti-IgM - + - + - +
LEN 10 µM - - + + - -
CC-122 1 µM - - - - + +
CLL# 3424
p-ERK 1/2 (Thr202/Tyr204)
Total ERK 1/2
p-AKT (Ser473)
Total AKT
Aiolos
β-Actin
37
37
50
50
50
37
ii)
CLL# 3381
p-ERK 1/2 (Thr202/Tyr204)
Total ERK 1/2
p-AKT (Ser473)
Total AKT
Aiolos
β-Actin
37
37
50
50
50
37
iii)
CLL# 3503
p-ERK 1/2 (Thr202/Tyr204)
Total ERK 1/2
p-AKT (Ser473)
Total AKT
Aiolos
β-Actin
37
37
50
50
50
37

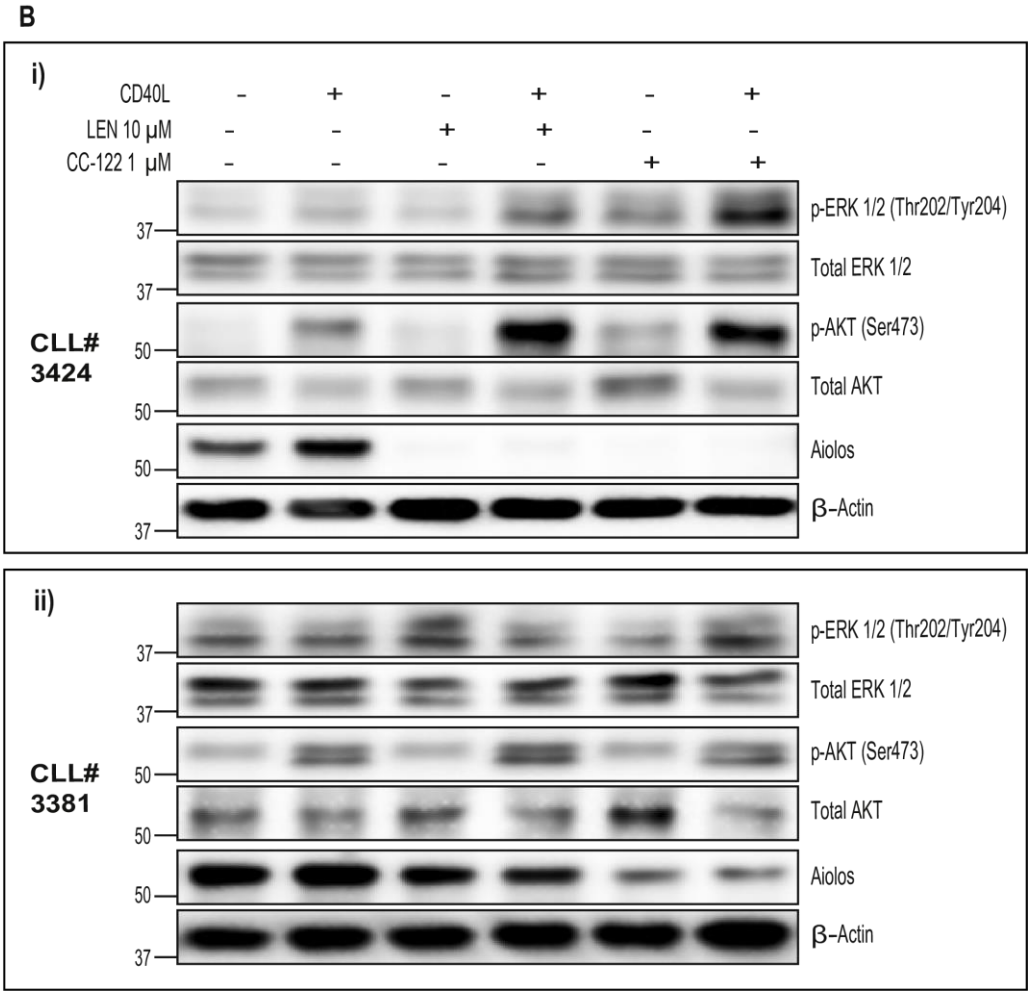
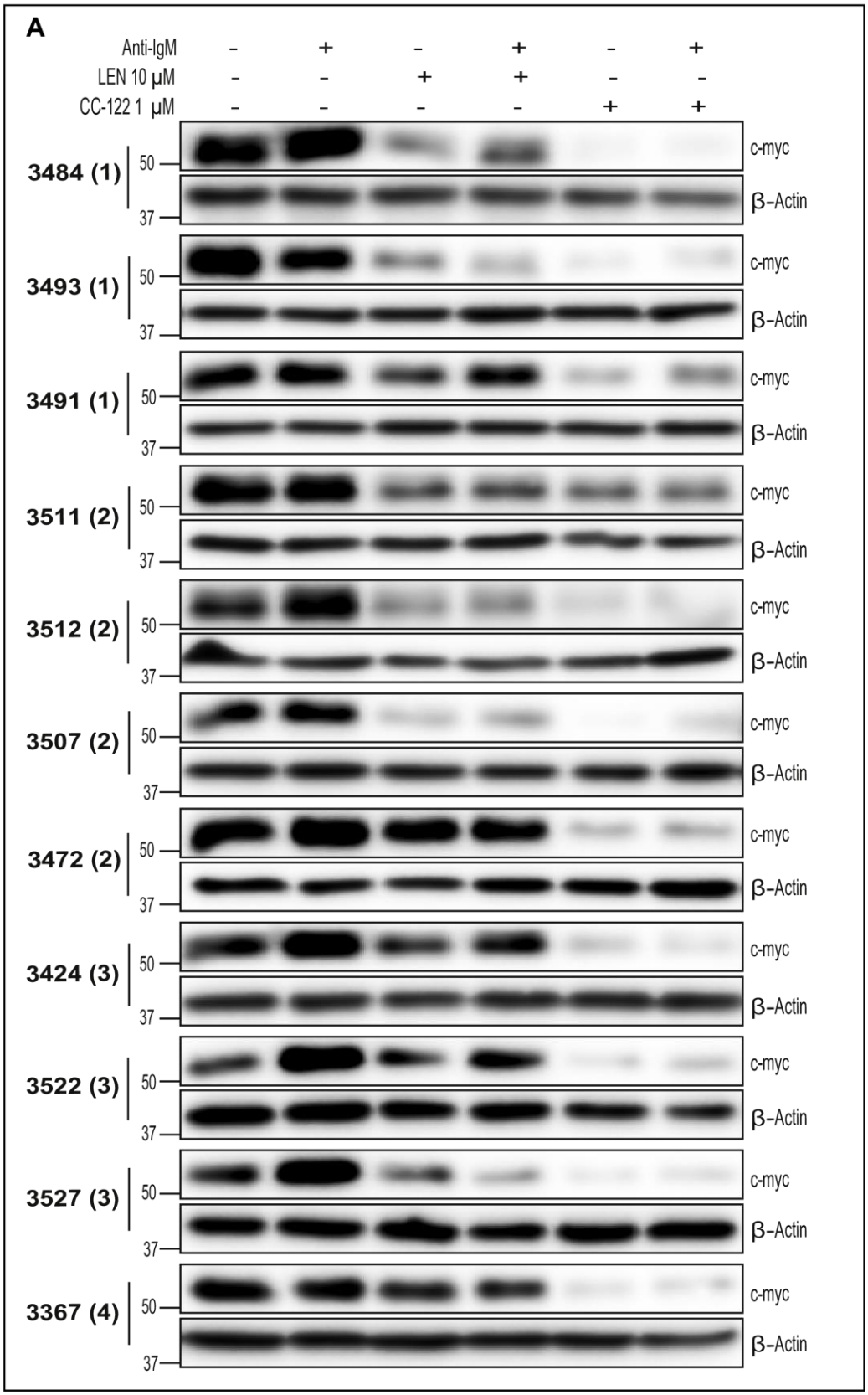


Figure 5.7: Comparison of Anti-IgM or CD40L stimulation after Lenalidomide and CC-122 treatment in primary CLL cells. Fresh primary CLL cells were thawed, cultured on CD154 fibroblasts and treated with LEN (10 µM) or CC-122 (1 µM) or DMSO for 24 hours. Cells were collected and exposed with Anti-IgM and CD40L for 15 minutes and 1 hour respectively. The lysates were prepared and analysed by western blot. Western blots showing phospho- and total protein expression in primary CLL cells; Anti-IgM **A (i-iii)** and CD40L stimulation **B (i & ii);**

5.3.8 Lenalidomide and CC-122 suppress c-Myc expression in primary CLL cells

Previous studies suggest that inhibition of ERK activity is linked with the inhibition of c-Myc expression in HCT116 cells [233]. Hence we sought to determine if there was an association between ERK and c-Myc in response to LEN or CC-122 treatment. Primary CLL cells were exposed to LEN and CC-122 (1 & 10 μM) for 24 hours and subsequently stimulated with Anti-IgM for 15 minutes.

As shown in **Figure 5.8,** both LEN and CC-122 downregulate c-Myc expression in examined primary CLL cells. The level of c-Myc protein was similar in unstimulated and with Anti-IgM stimulated cells. c-Myc expression increased upon 24 hours culture of primary CLL cells on CD154 fibroblasts with IL21. Low levels of c-Myc were observed in 10/11 samples at T0 (data not shown). This suggests that the combination of CD154 fibroblasts and IL21 stimulates CLL cell proliferation *in vitro.* Interestingly, treatment with CC-122 showed significant downregulation of c-Myc expression ($p<0.0001$) in all examined samples (11/11). However, LEN ($p<0.05$) showed less inhibition and also variable responses in c-Myc expression compared to CC-122. We suggest that the downregulation of c-Myc expression is likely due to inhibition of ERK phosphorylation especially with CC-122 treatment.


A
Anti-IgM
LEN 10 µM
CC-122 1 µM
3484 (1)
3493 (1)
3491 (1)
3511 (2)
3512 (2)
3507 (2)
3472 (2)
3424 (3)
3522 (3)
3527 (3)
3367 (4)
50
37
c-myc
β-Actin

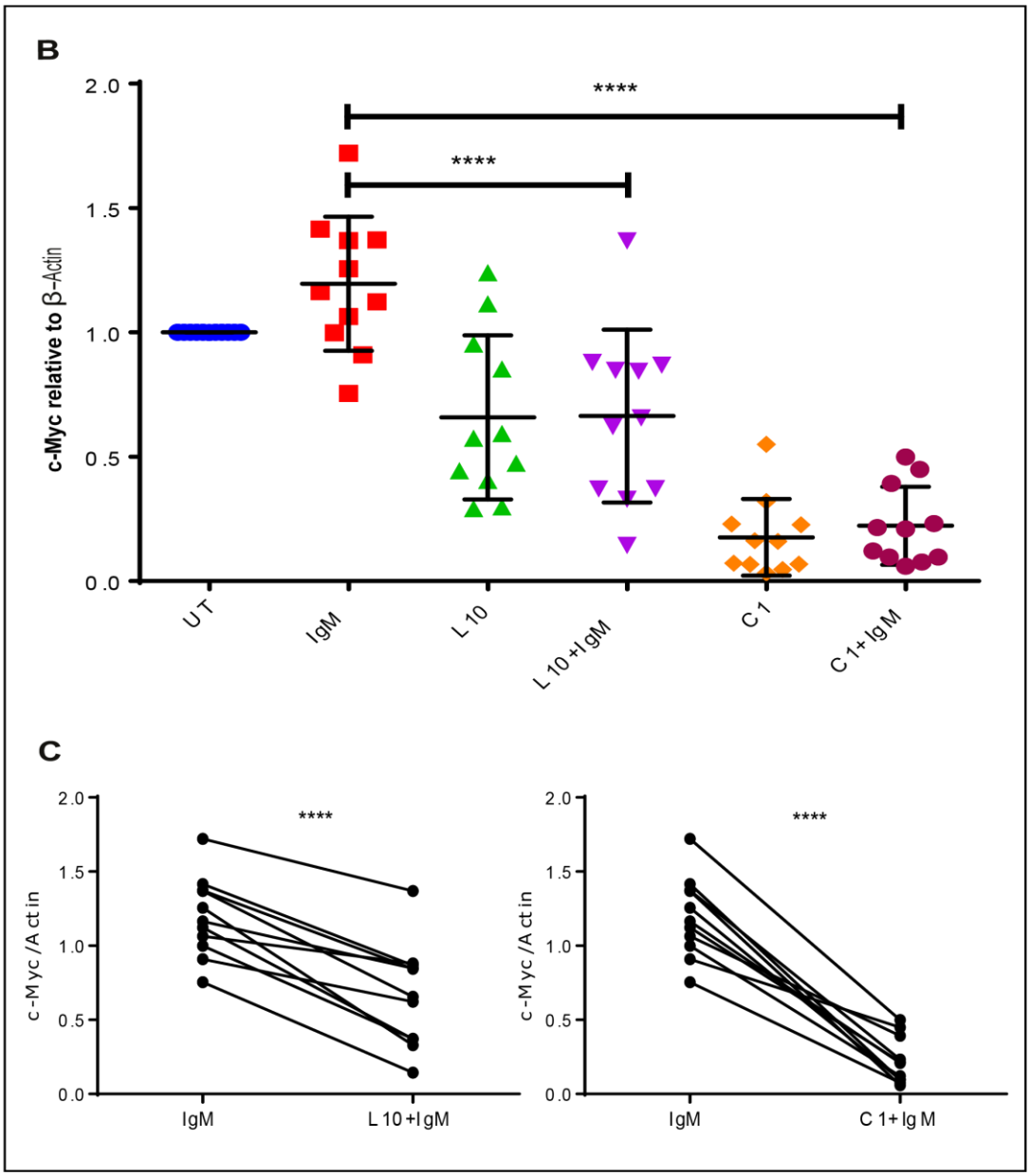


Figure 5.8: Lenalidomide and CC-122 suppress c-Myc expression by inhibiting p-ERK levels in primary CLL cells. Fresh primary CLL cells were thawed, cultured on CD154 fibroblasts and treated with LEN (10 µM) or CC-122 (1 µM) or DMSO for 24 hours. Cells were collected and exposed to Anti-IgM for 15 minutes. The lysates were prepared and analysed by western blot. Western blots showing phospho- and total protein expression in primary CLL cells; **A.** Western blot; **B.** Densitometry; **C.** Paired t-test analysis; Dunnett's multiple comparison test was used to determine statistically significant differences from UT. ****p<0.0001; [SEM]; n=9).

5.3.9 Lenalidomide and CC-122 alter p21 expression in primary CLL cells

Both LEN and CC-122 likely inhibit cell proliferation by promoting down regulation of phospho-ERK and c-Myc in primary CLL cells. Previous studies indicate that decreased ERK phosphorylation results in induction of p21 level in HUVEC [234] and HCT116 cell lines [233]. We sought to confirm the relationship between LEN and CC-122 induced anti-proliferative activity and activation of CDKi ($p21^{WAF1/CIP1}$ and p27).

Figure 5.9 shows the both LEN and CC-122 change the expression of $p21^{WAF1/CIP1}$ in primary CLL cells. After 24 hours, both LEN and CC-122 upregulate p21 expression in all examined primary CLL cells (n=6). Not surprisingly, the degree of induction was higher with CC-122 than LEN. As expected, untreated cells showed very low level of p21 expression. In contrast, upon treatment p27 levels remained unchanged when compared to untreated cells. The results suggest that inhibition of p-ERK and c-Myc up-regulates p21 protein expression but not p27 in primary CLL cells and may be an explanation for the anti-proliferative effects.

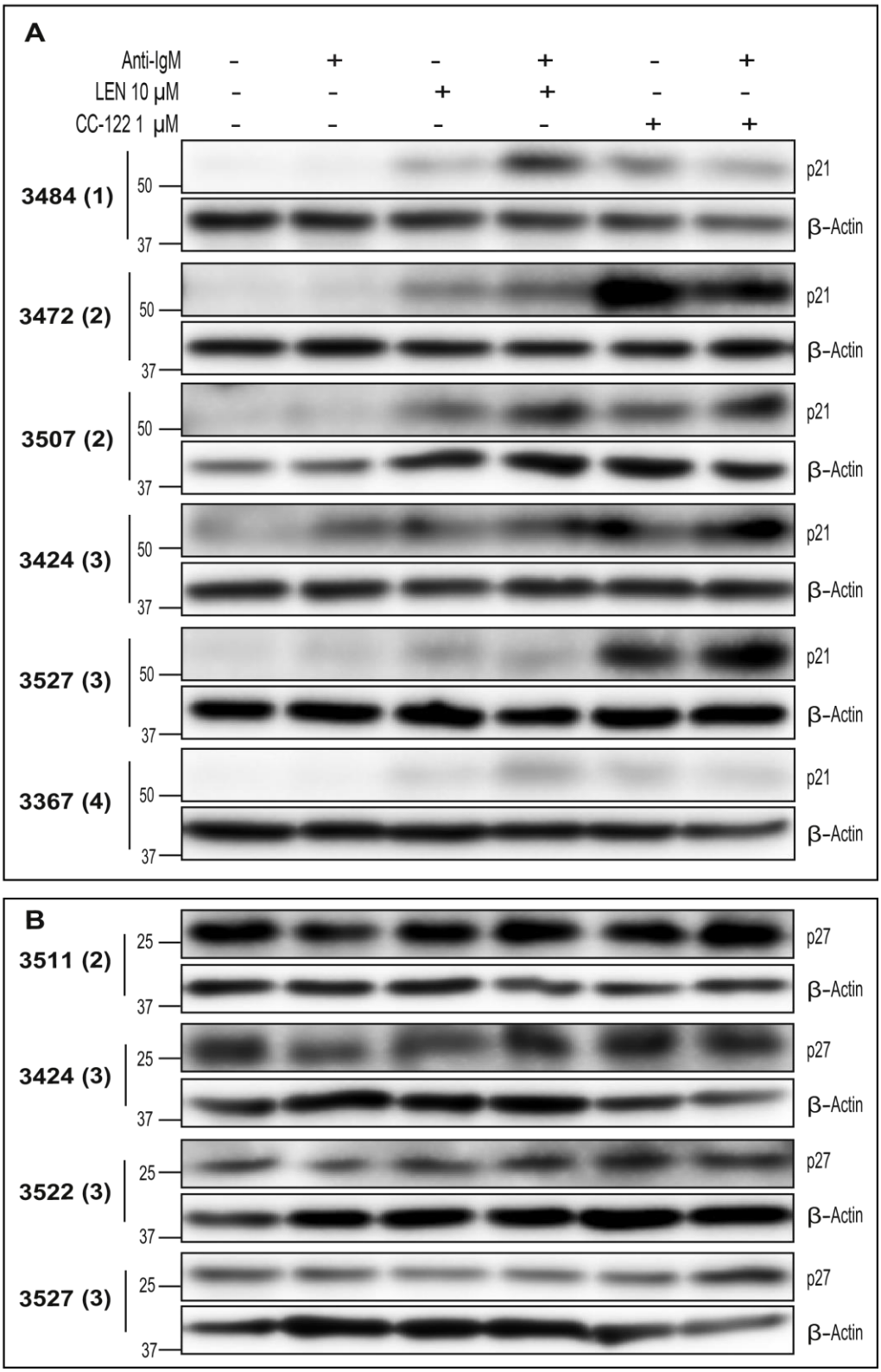

A
Anti-IgM - + - + - +
LEN 10 µM - - + + - -
CC-122 1 µM - - - - + +
3484 (1)
3472 (2)
3507 (2)
3424 (3)
3527 (3)
3367 (4)
50
37
p21
β-Actin
B
3511 (2)
3424 (3)
3522 (3)
3527 (3)
25
37
p27
β-Actin

Figure 5.9: Lenalidomide and CC-122 alter p21 expression in primary CLL cells. Fresh primary CLL cells were thawed, cultured on CD154 fibroblasts and treated with LEN (10 µM) or CC-122 (1 µM) or DMSO for 24 hours. Cells were collected and exposed to Anti-IgM for 15 minutes. The lysates were prepared and analysed by western blot; **A.** p21 and **B.** p27;

5.3.10 Treatment with IMiDs results in a reduction of p-ERK and c-Myc levels which causes an induction of p21

Having established the effects of IMiDs on BCR signalling pathway proteins in primary CLL cells, we next combined all the results to address the possible mechanism of action of IMiDs. **Figure 5.10** provides evidence that both LEN and CC-122 downregulate p-ERK and c-Myc protein expression in all examined cases with Aiolos degradation serving as a control for the action of the drugs. Interestingly, p21 was upregulated following treatment. Such a pattern may help to explain the reason for inhibition of proliferation of the primary CLL cells after treatment that was observed in **Chapter 3.**

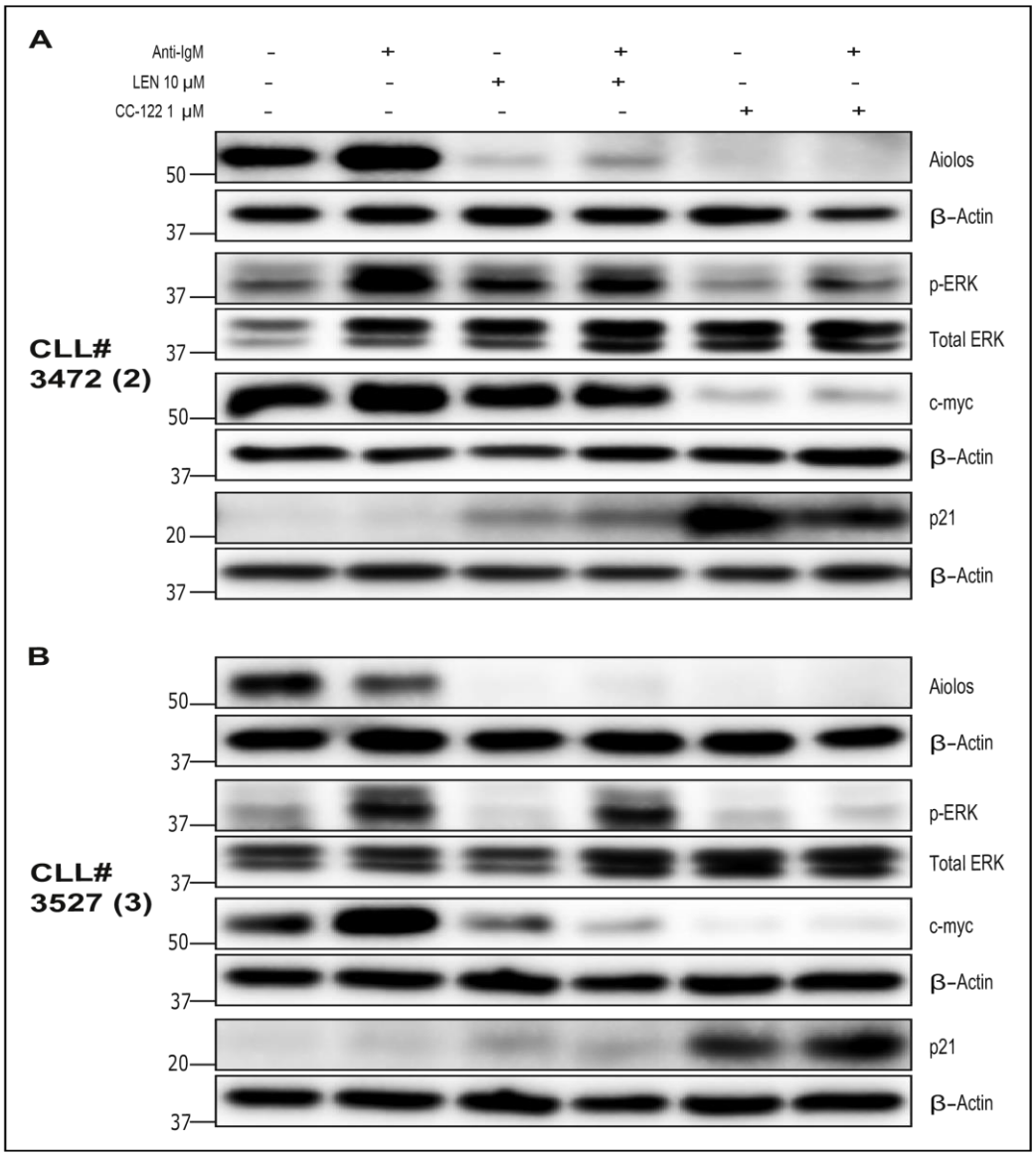


Figure 5.10: Treatment with IMiDs result in a reduction of p-ERK and c-myc levels which results in the induction of p21. CLL cells were treated with Lenalidomide (10 µM) or CC-122 (1 µM) for 24 hours and then BCR-cross linking for 15 minutes using soluble Anti-IgM antibody. Whole cell lysates were analysed for the change in expression of proteins detailed above. Representative primary CLL cases; **A. 3472; B. 3527.**

5.4 Discussion

The major aim of this chapter was to investigate if LEN and CC-122 have an effect on the BCR signalling pathway.

In CLL, BCR signalling is critical for malignant cell survival and also disease biology [82]. Several studies have reported that BCR signalling proteins (Lyn, Syk, ERK, AKT and NF*k*B) are constitutively phosphorylated in CLL [82, 225]. LEN is currently used in the clinic and has therapeutic activity in relapsed/refractory CLL patients [200, 201, 235]. CC-122 is a non-pthalimide analogue of LEN and has been shown to have an anti-tumour activity in DLBCL cell lines [180]. Our results indicate that CC-122 is superior to LEN at inhibiting the proliferation of CLL cells but like LEN does not induce apoptosis.

First, we optimised the methods to achieve successful stimulation of the BCR pathway by Anti-IgM using MAVER-1 and JeKo-1 cells. MEC-1, a CLL cell line is known to harbour constitutive phosphorylation of ERK1/2 [225]. We then tested a broad panel of B-CLL cell lines with Anti-IgM stimulation. Our results suggested that MEC2, HG3, EHEB and MEC1 exhibit ERK1/2 phosphorylation even without stimulation. MAVER-1 and JeKo-1 cells exhibited induction of phosphorylation of BCR pathway proteins (p-PLCϒ2, p-ERK and p-AKT) upon Anti-IgM stimulation (**Figure 5.3**), as shown by other groups [236, 237]. LEN (10 µM) and CC-122 (1 µM) treatment did not alter the phosphorylation levels of these BCR signalling proteins in MAVER-1 or JeKo-1 cells.

Withdrawal of serum inactivates the Raf-MEK-ERK signalling pathway [238] and results in decreased basal phosphorylation levels. Upon serum starvation MAVER-1 cells treated with LEN or CC-122 and Anti-IgM stimulation did not show any changes in the phosphorylation status (**Figure 5.4**). Interestingly, regardless of the serum conditions, both LEN and CC-122 mediate the degradation of Aiolos expression. It seems that these drugs do not impact the BCR pathway in MAVER-1 or JeKo-1 cells.

Like MAVER-1 cells, primary CLL cells were sensitive to BCR stimulation by Anti-IgM (**Figure 5.5**). Interestingly, both LEN and CC-122 significantly inhibit the phosphorylation of ERK upon BCR engagement in all screened primary CLL cells (**Figure 5.5 I**). Notably, CC-122 induced greater inhibition of p-ERK than LEN in 50% of samples tested (**Figure 5.5 A & G**). In contrast, PLCϒ2 phosphorylation was constant even with LEN or CC-122 treatment in most samples (10/12) (**Figure 5.5)**.

The results in a primary MCL sample mirrored those in the cell lines (**Figure 5.3**). Our findings suggest that the inhibition of phosphorylation by LEN and CC-122 is specific to primary CLL but not MCL cells.

Both Anti-IgM and CD40L play important roles in the signal propagation through BCR and activate multiple downstream pathways [239, 240]. We sought to investigate the specific route or mechanisms through which LEN and CC-122 impact BCR signalling proteins. Our data suggests that both drugs inhibit Anti-IgM induced ERK phosphorylation (**Figure 5.7 A**). Although CD40L stimulation increased the phosphorylation levels with drug treatment

(**Figure 5.7 B**), our studies indicate that LEN and CC-122 inhibit BCR signalling on Anti-IgM, but not CD40L, induced ERK phosphorylation in primary CLL cells. Lastly, we examined downstream effects of ERK phosphorylation changes upon LEN and CC-122 treatment. Recent studies revealed that suppression of p-ERK and c-Myc induces p21 expression in human colorectal carcinoma HCT116 cells [233]. Both LEN and CC-122 showed significant downregulation of c-Myc (**Figure 5.8**) and induction of p21 expression (**Figure 5.9**). We show that CC-122 is superior to LEN in its effects on c-Myc and p21.

To summarise this chapter, we show that LEN and CC-122 downregulate the phosphorylation of p-ERK caused by Anti-IgM stimulation in primary CLL cells. In addition, there does seem to be a clear correlation between p-ERK and p21 levels. These observations may explain why these drugs inhibit primary CLL cell proliferation as described in **Chapter 3.**

Chapter 6 : Priming of B-CLL cell lines and primary CLL cells with epigenetic drugs sensitise them to the effects of Lenalidomide and CC-122

6.1 Introduction

We have established that IMiDs have anti-proliferative effects in the majority of CLL cases (**Figure 3.7**). In some cases, however, CLL cells are less sensitive to the effects of LEN and CC-122 and in others completely resistant to both agents. Based on emerging reports, a better therapeutic response to these drugs could potentially be achieved upon priming with epigenetic drugs.

Over the last 10 years, considerable improvements have been achieved in the management of CLL, and yet the disease remains incurable. Introduction of an anti-CD20 monoclonal antibody, Rituximab, to chemotherapy has been highly effective in both relapsed and untreated patients [129, 241]. Also, a combination of Rituximab with chemotherapeutic drugs such as Fludarabine and Cyclophosphamide (FCR) prolonged overall survival in CLL [242]. Conversely, this combination leads to greater toxicity when compared to chemotherapy alone [243]. LEN has been shown to have activity in CLL patients with unfavourable characteristics such as 17p-, 11q-, and unmutated

IgVH [244]. Subsequent studies report that LEN in combination with Rituximab is superior compared to LEN alone [245]. Based on the promising activity of LEN, combinations with Fludarabine and Rituximab where investigated but show that the combination was not tolerable due to significant toxicity [246]. Interestingly, addition of LEN in a sequential manner following initial treatment with Fludarabine and Rituximab, showed better tolerability and less toxicity [247]. Several independent studies report that a combination of 5-Azacytidine and LEN has promising activity in high risk MDS and also previously treated elderly patients with acute myeloid leukaemia (AML) [248-253].

Despite the recent advances in development of new drugs, for example those that target the BCR signalling pathway CLL remains incurable with a high incidence of relapse [254]. There is a clear need to develop therapeutic regimens with novel drug combinations to achieve better efficacy, and reduce the possibility of relapse and drug resistance.

We hypothesized that epigenetic drugs could sensitize CLL cells to LEN or CC-122 treatment. If so, this may help in developing combinations (that address dose and schedule) to improve responses and develop personalized approaches.

In this chapter, we utilized model B-CLL cell lines (EHEB, MEC2, MEC1 and HG3) as well as primary CLL cells to examine their responsiveness to LEN or CC-122 after sensitization by epigenetic drugs. For these studies, we chose, a DNA methyltransferase (DNMT) inhibitor 5-Azacytidine (or 5-Aza) and, a

Histone deacetylase (HDAC) inhibitor Romidepsin (or Romi) as epigenetic priming drugs to combine with IMiDs.

The **aim** of this chapter, therefore, is:

1. To study the effects and relevance of epigenetic priming ahead of LEN or CC-122 treatment in B-CLL cell lines and primary CLL cells.

6.2 Methods

6.2.1 Priming of B-CLL cell lines with 5-Azacytidine and Romidepsin prior to treatment with LEN or CC-122

EHEB, MEC2, MEC1 and HG3 cell lines were cultured at 0.3-1×10^4 cells per well in 96 well plates and were incubated with escalating concentrations of 5-Azacytidine (0.062-1 µM) or Romidepsin (0.062-1 nM) (gifts from Celgene, USA) or an equal volume of DMSO. The cells were pre-treated for 24, 48 and 72 hours and then incubated with LEN or CC-122 (0.62-10 µM) or an equal volume of DMSO for additional 72 hours. Cell proliferation was measured using the previously described BrdU incorporation assay. Data is represented as percentage of cell proliferation relative to the untreated.

6.2.2 Treatment of primary CLL cells by LEN and CC-122 after sensitization with 5-Azacytidine and Romidepsin

Primary CLL cells were plated on CD154 fibroblast cells at a density of 0.5×10^6/mL, supplemented with rhIL-21 (25 ng/mL), and were incubated with 5-Azacytidine (4 µM) and Romidepsin (0.5 nM) or an equal volume of DMSO for 48 hours. The cells were then treated with varying concentrations of LEN or CC-122 (0.125-10 µM) or an equal volume of DMSO for additional 72 hours. Following this, 50% of the media was replaced with fresh complete media containing LEN or CC-122, rhIL-21 and BrdU reagent and incubated for another 48 hours. Cell proliferation was estimated by quantification of BrdU incorporation. Data is presented as percentage of cell proliferation relative to the untreated.

6.3 Results

6.3.1 Optimization of epigenetic drug priming prior to LEN and CC-122 in MEC1 cells

To assess the priming effect of 5-Azacytidine and/or Romidepsin in presence of LEN or CC-122, the first step was to optimize the concentration of both epigenetic drugs in MEC1 cells. It was previously demonstrated **(Figure 3.1 C)** that MEC1 cells show only a slight inhibition of proliferation when treated with CC-122 and are resistant to LEN. MEC1 cells were examined to check if 5-Azacytidine or Romidepsin impart a priming effect for IMiD sensitivity. After treatment of MEC1 cells with 5-Azacytidine (0.062-1 µM) or Romidepsin (0.062-1 nM) for 24, 48 and 72 hours and a fixed concentration of LEN or CC-122 (1 µM) was added and cells were incubated for additional 72 hours. Cell proliferation was assessed by BrdU incorporation.

On pre-treatment with 5-Azacytidine or Romidepsin MEC1 cells showed variable proliferation in response to the combination with LEN or CC-122. 5-Azacytidine alone did not show any effect up to 1 µM for 6 days**.** However, approximately 25-50% inhibition of proliferation was achieved at 1 µM of 5-Azacytidine pre-treatment followed by 1 µM LEN or CC-122 for 72 hours **(Figure 6.1 A)**. CC-122 showed greater inhibition than LEN at all time points.

Interestingly, MEC1 cells were sensitive to Romidepsin alone and showed inhibition of proliferation at the range of concentrations investigated. A dose dependent response was observed in combination with LEN or CC-122 at all-time points **(Figure 6.1 B)**. These results suggest that 1 µM 5-Azacytidine

may sensitize cells to IMiD treatment. This concentration was chosen for further experiments.

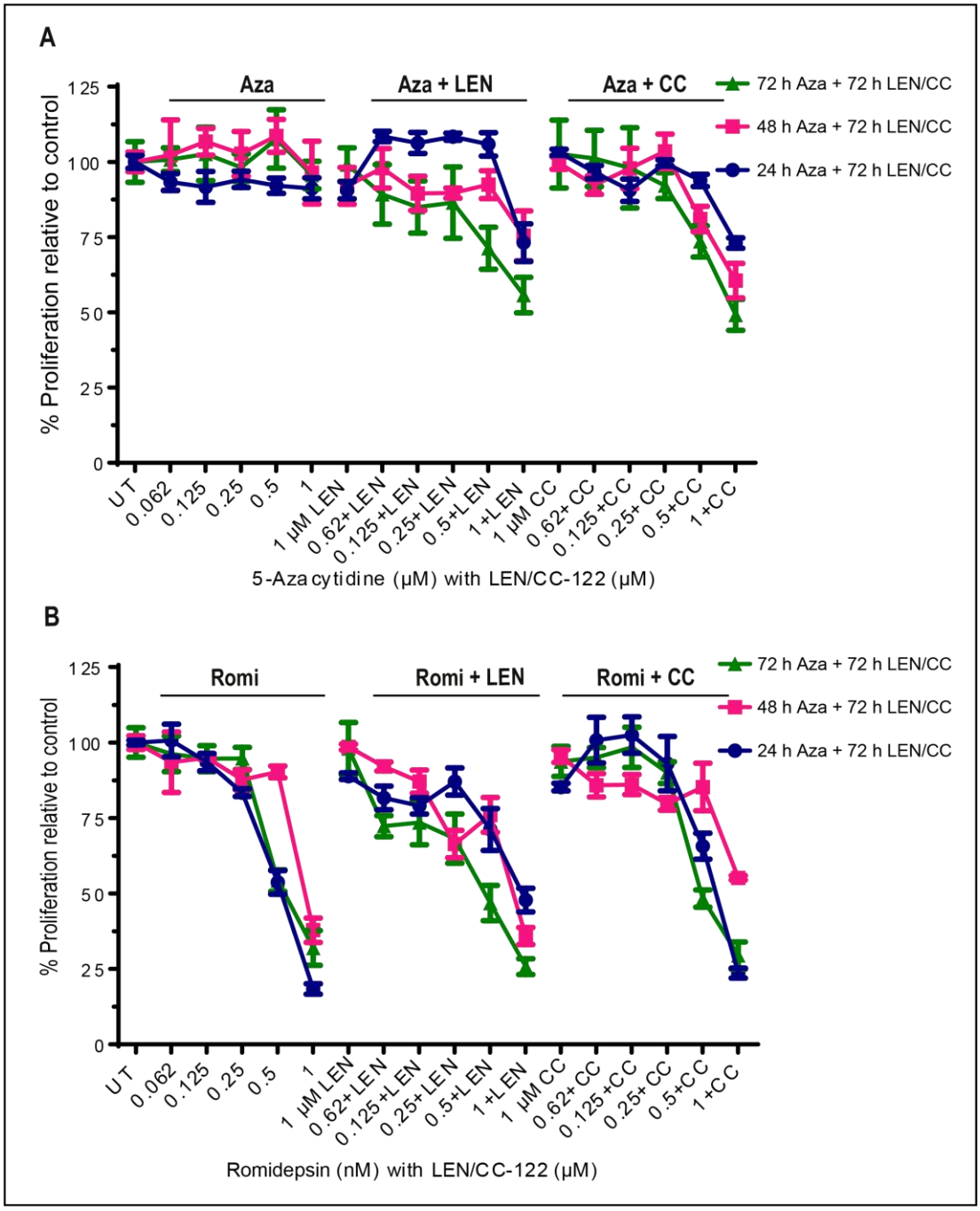


Figure 6.1: Optimization of the concentration of epigenetic drugs as sensitisers to LEN or CC-122 in MEC1 cells. MEC1 cells were pre-treated with 5-Azacytidine (0.062-1 µM) or Romidepsin (0.062-1 nM) for 24, 48 or 72 hours. After each time point, cells were exposed to LEN or CC-122 at 1 µM for additional 72 hours. A BrdU cell proliferation assay was performed at days 4, 5 and 6. Data is represented as percentage of proliferation relative to the untreated control and plotted as a line graph. **A.** 5-Aza with LEN & CC-122; **B.** Romi with LEN & CC-122.

6.3.2 Concentration-dependent inhibition of proliferation by LEN and CC-122 after sensitization with 5-Azacytidine in B-CLL cell lines

Having demonstrated that MEC1 cells are sensitized to IMiD treatment after 5-Azacytidine pre-treatment (**Figure 6.1**), we sought to examine other B-CLL cell lines. Based on above results various B-CLL cell lines were examined by two different approaches as 5-Azacytidine potentiated the anti-proliferative effect with IMiD after 48 and 72 hours. The first was to sensitize B-CLL cells by 1 µM 5-Azacytidine for 48 hours and then treat the cells with LEN or CC-122 for additional 72 hours. The second was to sensitize cells for 72 hours followed by exposure to LEN or CC-122 for additional 72 hours.

Figure 6.2 suggests that priming with 5-Azacytidine induces a higher inhibition of proliferation when combined with either LEN or CC-122 in all B-CLL cell lines. In **Chapter 3** (**Figure 3.3**), we have shown differential responses of EHEB, MEC2, MEC1 and HG3 cells to LEN or CC-122 alone. EHEB cells were relatively sensitive to IMiD alone treatment (proliferation inhibition >50%) at 10 µM (**Figure 3.3 A**). However, 48 and 72 hours pre-treatment with 5-Azacytidine potentiated the inhibition of proliferation (~25%) compared to either drug as single agents (**Figure 6.2 A-D**). MEC2 and MEC1

cells were less responsive and showed only 20-30% inhibition with IMiD treatment. Interestingly, 5-Azacytidine pre-treatment showed 30% and 40% proliferation inhibition to LEN and CC-122 after both time points. The effects were superior with CC-122 compared to LEN, (~10%) (**Figure 6.2 A-D**). Similarly, the least responsive HG3 cells also showed higher inhibition of proliferation following pre-treatment compared to either LEN or CC-122 alone (~ 25-30%). Notably, both drugs were almost equally effective to the combination at 48 and 72 hours (**Figure 6.2 A-D**)**.** These results suggests that pre-treatment with 5-Azacytidine sensitizes all B-CLL cell lines to LEN and CC-122 treatment.

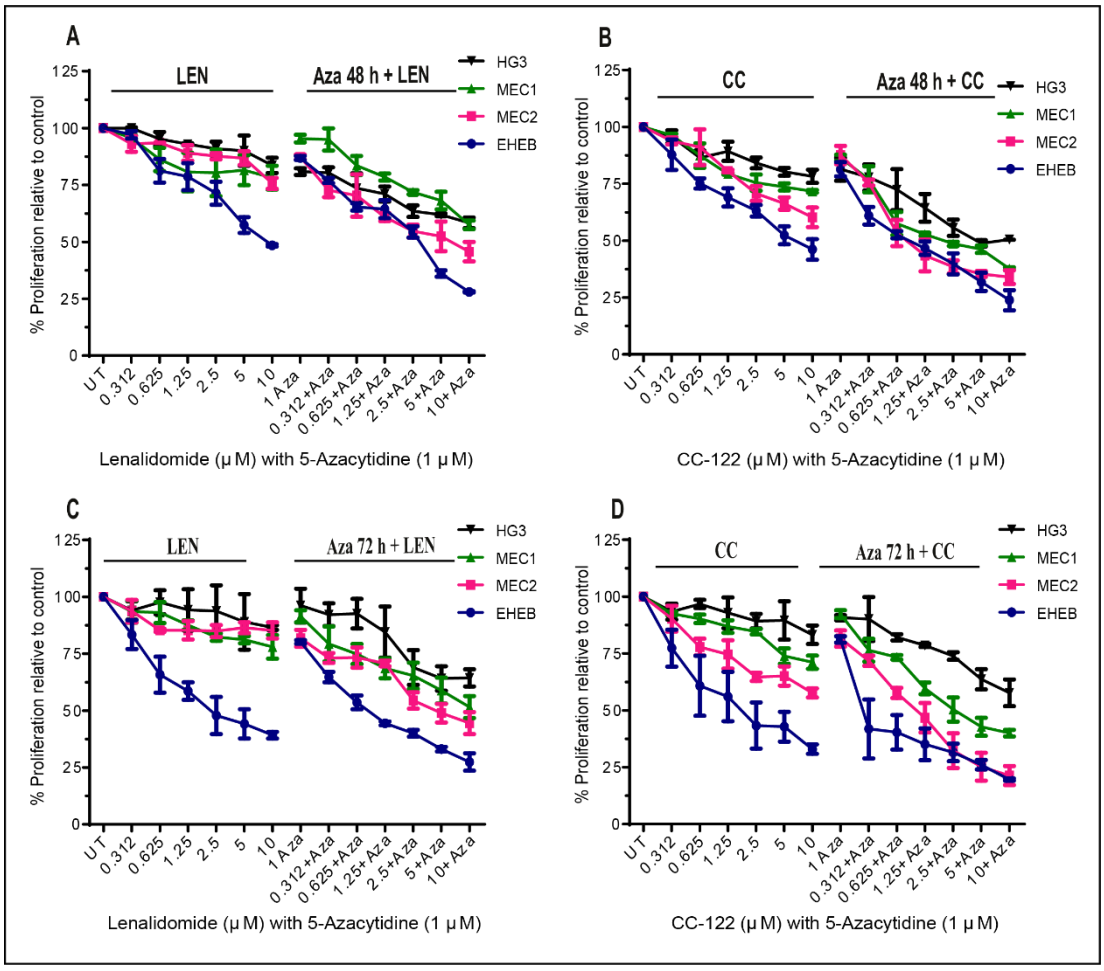


Figure 6.2: Concentration dependent inhibition of proliferation in B-CLL cell lines with 5-Azacytidine sensitization and LEN or CC-122 treatment. B-CLL cell lines were pre-treated with 5-Azacytidine (1 µM) for 48 hours **(A & B)** and 72 hours **(C & D).** Cells were then treated with different concentrations (0.312 to 10 µM) of LEN **(A & C)** or CC-122 **(B & D)** for additional 72 hours. Cell proliferation was measured on day 5 or 6 in a BrdU incorporation assay. Data is presented as percentage of cell proliferation relative to the untreated control.

6.3.3 Optimization of the concentration of 5-Azacytidine and Romidepsin for priming in primary CLL cells prior to IMiD treatment.

We have shown that epigenetic priming with 5-Azacytidine prior to LEN and CC-122 treatment results in a greater inhibition of proliferation in B-CLL cell lines (**Figure 6.2**). From these results, we hypothesized that epigenetic priming could also sensitize primary CLL cells to IMiDs. It was essential to optimize the method to examine the sensitivity of primary CLL cells to 5-Azacytidine or Romidepsin alone prior to treatment with IMiDs. Cells were treated with varying concentrations of 5-Azacytidine (0.12 to 8 µM) and Romidepsin (0.12 to 8 nM) for up to 7 days. Cell proliferation was measured after 1, 4 and 7 days in a BrdU assay.

Figure 6.3 showed that both 5-Azacytidine and Romidepsin inhibit proliferation at high concentrations in primary CLL cells even after 24 hrs. 5-Azacytidine inhibited proliferation only at 4 µM concentration. However, Romidepsin had an effect on CLL cell proliferation at very low concentrations (<1 nM). Based on these results, we chose 4 µM for 5-Azacytidine and 1 nM

for Romidepsin as priming concentrations for further experiments with LEN or CC-122 in primary CLL cells.

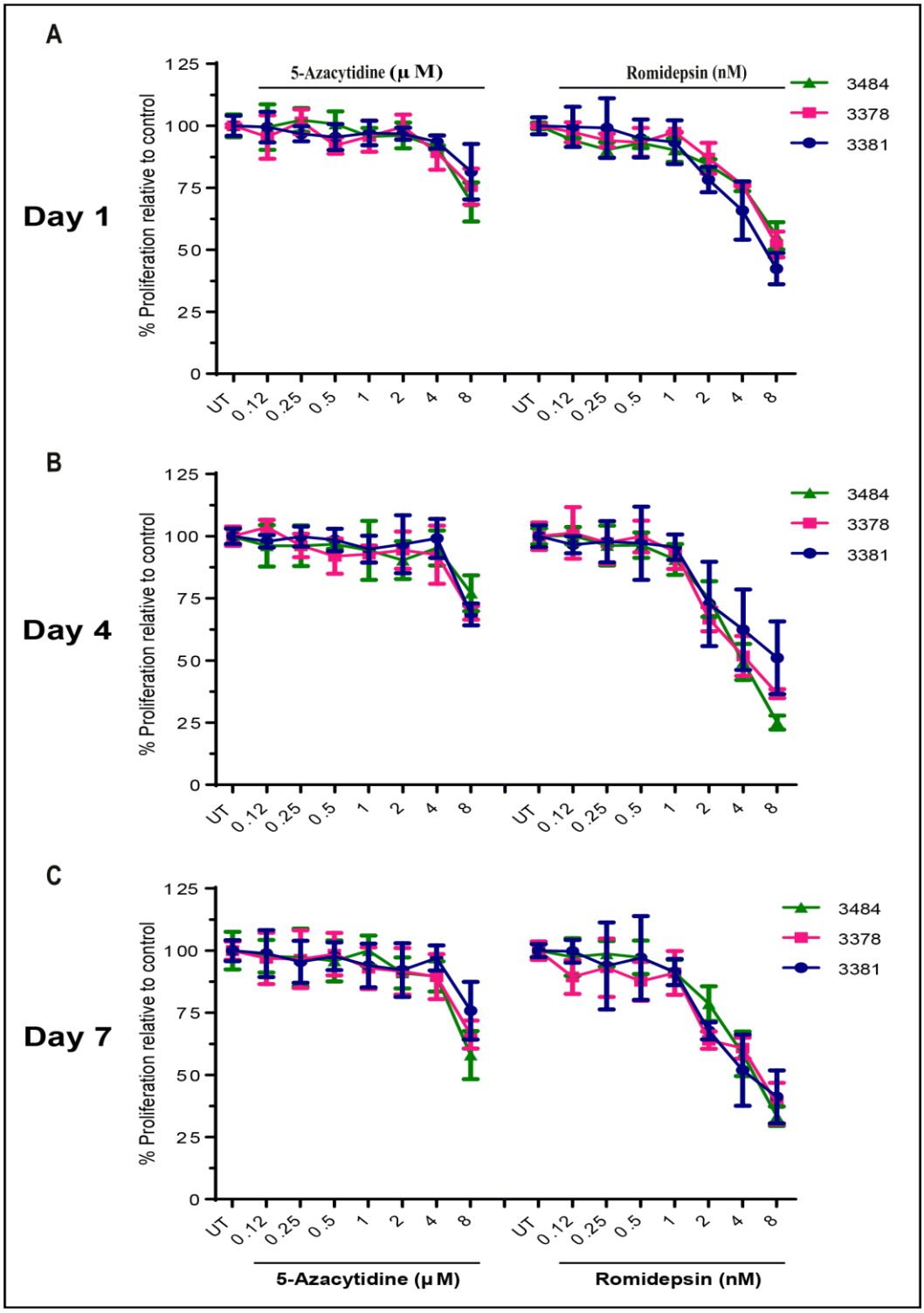


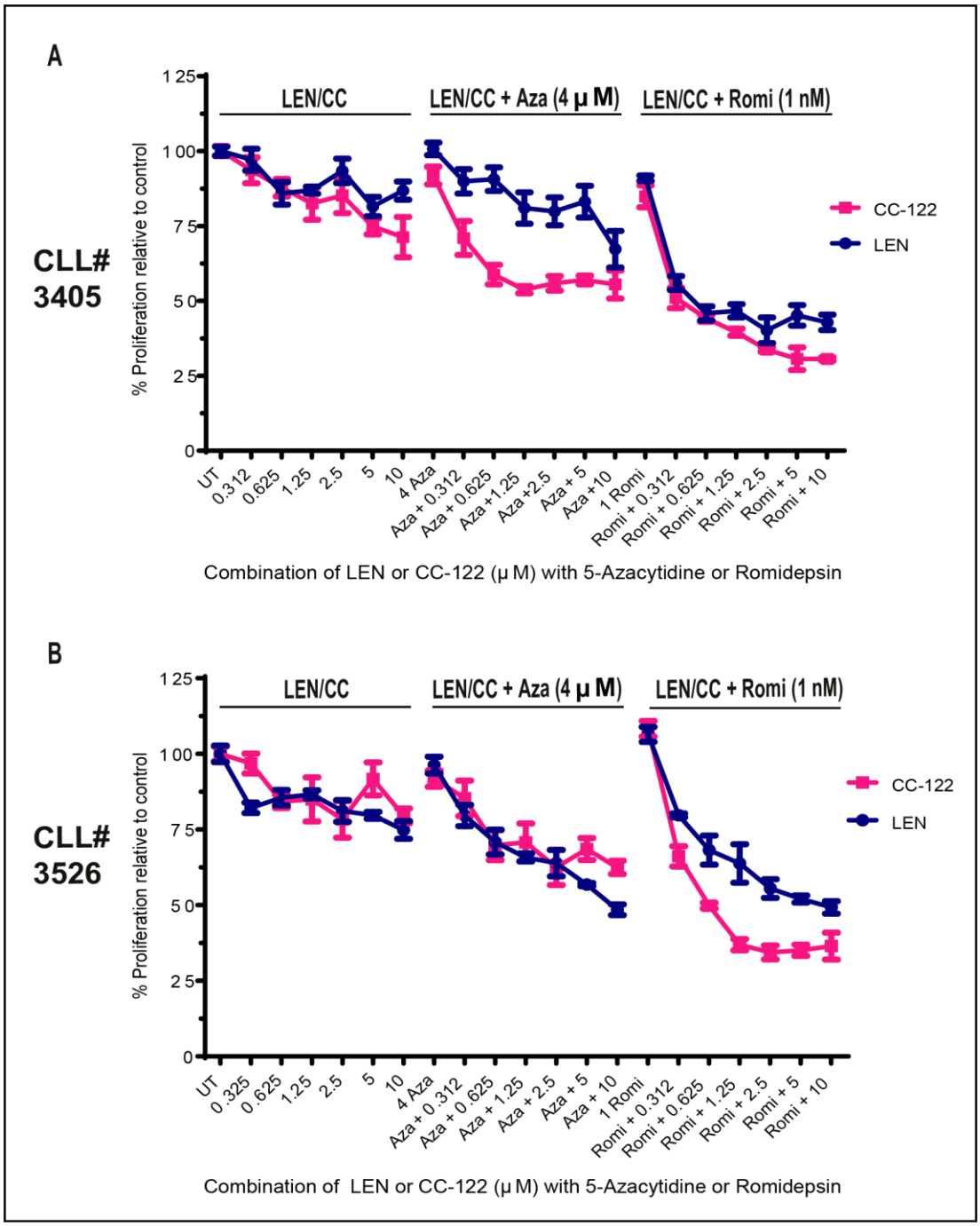
Figure 6.3: Optimization of 5-Azacytidine and Romidepsin priming concentration in primary CLL cells. Primary CLL cells were co-cultured with CD154 fibroblasts supplemented with rhIL-21 and then treated with 5-Azacytidine (0.12-8 µM) or Romidepsin (0.12-8 nM) for up to 7 days. Cell proliferation was measured by BrdU incorporation after **1 (A), 4 (B) and 7 (C)** days.

6.3.4 Concentration dependent inhibition of proliferation by LEN and CC-122 in primary CLL cells following sensitization with 5-Azacytidine or Romidepsin

Having established priming concentrations of 5-Azacytidine and Romidepsin in primary CLL cells (**Figure 6.3)**, we next examined whether this primed CLL cells for better response to LEN or CC-122. For this study, we have chosen to study primary CLL cells that showed inferior responses to LEN or CC-122 alone. Freshly thawed primary CLL cells were co-cultured with CD154 fibroblasts, pre-treated with 5-Azacytidine or Romidepsin for 48 hours and then with LEN or CC-122 for an additional 72 hours. Cell proliferation was measured on day 8.

Figure 6.4 shows that priming with 5-Azacytidine or Romidepsin prior to LEN or CC-122 treatment potentiates the inhibition of proliferation in primary CLL cells. Both LEN and CC-122 alone inhibit proliferation by approximately 20-40% (variable between cases) in the examined primary CLL cases **(**n=4). Interestingly, prior incubation with 5-Azacytidine and then treatment with IMiDs, inhibited proliferation of by a further 20% (3405), 15-20% (3526), 30% (3491) and ~25% (3394). Similarly, Romidepsin pre-treatment has a greater sensitizing effect on the primary CLL cells when combined with LEN and CC-122 treatment. Approximately, 40% (3405), 30-40% (3526), 10-20% (3491),

10-25% (3394) of inhibition was achieved in **(Figure 6.4. A-B).** In most CLL samples, both 5-Azacytidine and Romidepsin pre-treatment showed higher inhibition of proliferation with CC-122 than LEN. Furthermore, when we combined 5-Azacytidine and Romidepsin ahead of exposure to IMiDs, there was a superior response. **(Figure 6.4. C & D).** Intriguingly, LEN showed slightly greater inhibition when combined with both drugs **(Figure 6.4. C)**. From these observations, we confirmed that priming with epigenetic drugs can sensitise primary CLL cells to LEN and CC-122 treatment.


A
CLL# 3405
LEN/CC
LEN/CC + Aza (4 µ M)
LEN/CC + Romi (1 nM)
% Proliferation relative to control
125
100
75
50
25
0
CC-122
LEN
UT
0.312
0.625
1.25
2.5
5
10
4 Aza
Aza + 0.312
Aza + 0.625
Aza +1.25
Aza +2.5
Aza + 5
Aza +10
1 Romi
Romi + 0.312
Romi + 0.625
Romi + 1.25
Romi + 2.5
Romi + 5
Romi + 10
Combination of LEN or CC-122 (µ M) with 5-Azacytidine or Romidepsin
B
CLL# 3526
LEN/CC
LEN/CC + Aza (4 µ M)
LEN/CC + Romi (1 nM)
% Proliferation relative to control
125
100
75
50
25
0
CC-122
LEN
UT
0.325
0.625
1.25
2.5
5
10
4 Aza
Aza + 0.312
Aza + 0.625
Aza + 1.25
Aza + 2.5
Aza + 5
Aza + 10
1 Romi
Romi + 0.312
Romi + 0.625
Romi + 1.25
Romi + 2.5
Romi + 5
Romi + 10
Combination of LEN or CC-122 (µ M) with 5-Azacytidine or Romidepsin

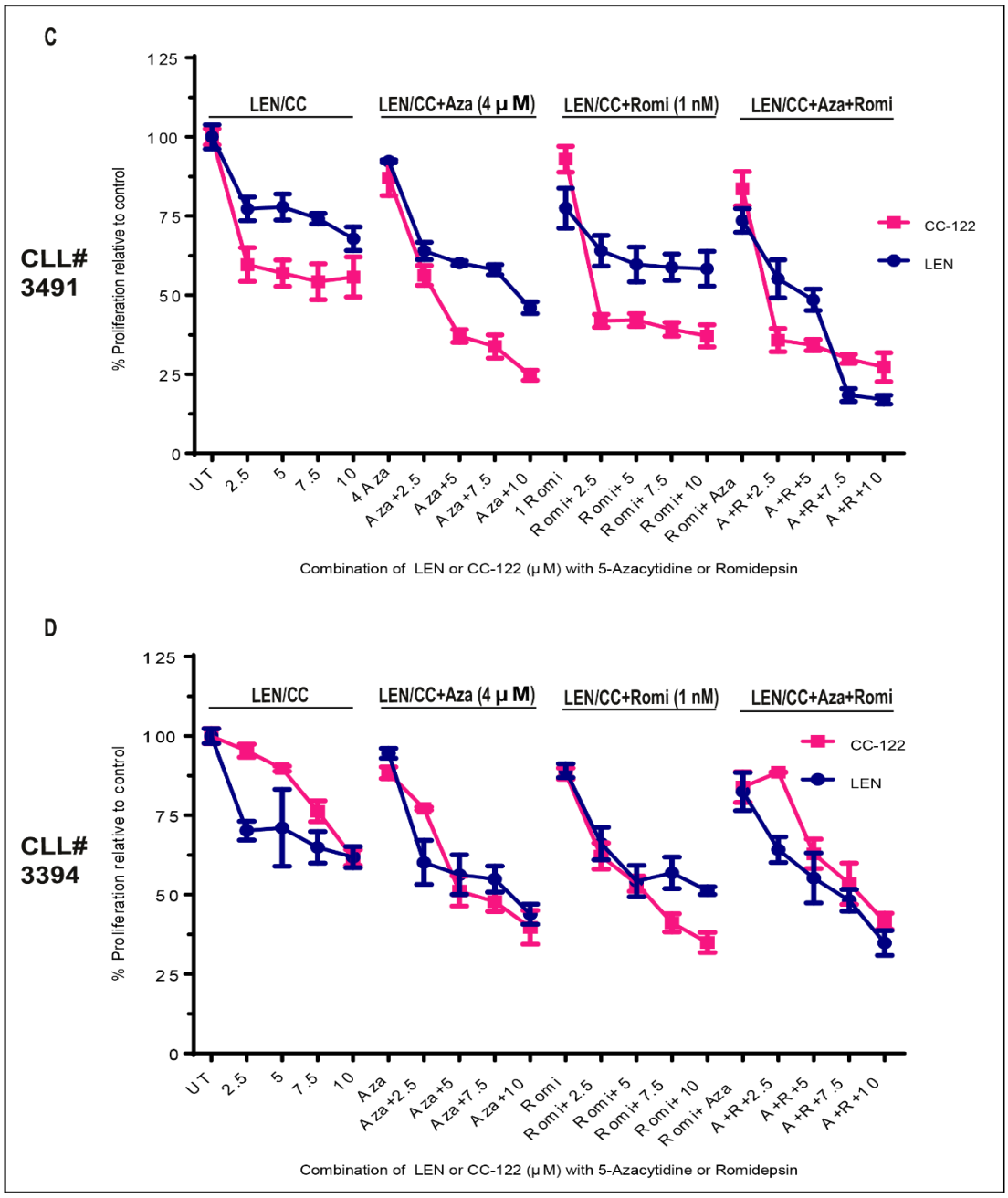


Figure 6.4: Concentration dependent inhibition of proliferation by LEN and CC-122 after 5-Azacytidine or Romidepsin sensitization in primary CLL cells. Fresh primary CLL cells were co-cultured with CD154 fibroblasts, supplemented with rhIL-21, and then treated with 5-Azacytidine (4 µM) or Romidepsin (1 nM) for up to 48 hours. CLL cells were then treated with different concentrations (0-10 µM) of LEN or CC-122 for 72 hours. Cell proliferation was measured by BrdU assay on day 8. Data is presented as percentage of cell proliferation relative to untreated control. Primary CLL samples; **A. 3405; B. 3526; C. 3491; D. 3491.**

6.4 Discussion

The aim of this chapter was to examine if the priming with epigenetic drugs (5-Azacytidine or Romidepsin) improved the effectiveness of LEN and CC-122 in CLL. To do so, we optimized the dosage and scheduling of the priming drugs in both B-CLL cell lines and primary CLL cells and analysed their effects on proliferation as a read-out.

In recent years, LEN has been shown to have an activity in relapsed and refractory CLL [244] and better therapeutic responses in high-risk patients are observed in combination with Rituximab [245]. Previous studies report that re-expression of epigenetically silenced tumour suppressor genes may improve outcomes with conventional chemotherapy [255]. 5-Azacytidine, a demethylating agent, restores p16INK4a, a cell cycle regulator that is epigenetically silenced in adult T-cell leukaemia/lymphoma (ATLL) [256]. Similarly, the 5-Azacytidine and Lenalidomide combination has been shown to be very effective in MDS and MM patients [248, 253]. Furthermore, Cosenza et al., (2016) reported that the combination of Romidepsin and Lenalidomide act synergistically and induce cell death in T-cell lymphoma cell lines [257].

Studies to optimize the dosage and scheduling of both drugs were first conducted in order to improve the effects of LEN or CC-122 on proliferation of CLL cells. Interestingly, proliferation was quickly inhibited with 1 µM of 5-Azacytidine combined with LEN or CC-122 after 72 hours, whereas, the same concentration of 5-Azacytidine alone did not show any effect indicating

a priming effect in MEC1 cells (**Figure 6.1 A**). In contrast, MEC1 cells were very sensitive to Romidepsin alone but did exhibit proliferation inhibition in combination with LEN and CC-122. However, an alternative explanation for this phenomenon could be induction of apoptosis rather than solely proliferation inhibition (**Figure 6.1 B**).

In our data generated in the B-CLL cell lines (EHEB, MEC2, MEC1 and HG3), there is support for the hypothesis that pre-treatment with 5-Azacytidine potentiates the anti-proliferative effect of LEN and CC-122 (**Figure 6.2**). In the cell lines that were less responsive to LEN or CC-122 alone, prior exposure with 5-Azacytidine greatly enhanced the proliferation inhibition in a dose-dependent manner.

In addition, primary CLL cells showed similar responses to priming effects observed in the B-CLL cell lines. The concentrations of the epigenetic drugs were chosen to ensure a 'priming effect' ahead of treatment with LEN or CC-122. We were able to demonstrate that primary CLL cells, following priming with 5-Azacytidine or Romidepsin for 48 hours, showed enhanced effects of LEN or CC-122 treatment to a greater degree (15-40%) than either IMiD treatment alone (**Figure 6.4 A-D**) in all examined CLL cases. In all samples CC-122 showed a greater anti-proliferative effect than LEN. Further studies are required to understand the mechanisms that mediate the observed synergistic responses.

To summarize, these results taken together confirm that epigenetic priming sensitizes CLL cells to LEN and CC-122 treatment in both B-CLL cell lines and primary CLL cells.

Chapter 7 : Conclusions and Future work

The novel outcomes resulting from this thesis are:

1. The novel IMiD analogue CC-122 has greater activity when compared to LEN. Both drugs are cytostatic but not cytotoxic to CLL cells.
2. LEN and CC-122 show differential anti-proliferative effects in B-CLL cell lines and primary CLL cases (**Chapter 3**).
3. Although analysis of the effects of treatment with IMiDs on individual anti- and pro-apoptotic proteins did not suggest any actionable correlations in B-CLL cell lines and primary CLL cases, the ratios of certain family members did suggest there would be a benefit to combining CC-122 or LEN in specific patients with agents such as ABT-199 that are currently in use in the clinic (**Chapter 4**).
4. Both LEN and CC-122 inhibit BCR induced ERK phosphorylation and this may partially account for the inhibition of proliferation of cell lines and CLL cases in response to treatment. There was a clear correlation between downregulation of c-Myc expression and upregulation of p21 (**Chapter 5**).
5. Epigenetic priming potentiates the anti-proliferative effects of LEN and CC-122 in B-CLL cell lines and primary CLL cells (**Chapter 6**).

Complex cellular signalling pathways relay extracellular stimuli into the nucleus to impact gene expression and tightly control biological processes. In

CLL, the BCR signalling pathway is critical in determining the fate of the cell. The activation of ITAMs induces cell proliferation and survival by activating AKT, ERK and other downstream pathways [258].

The results presented suggest that LEN and CC-122 predominantly inhibit proliferation by inhibiting ERK phosphorylation and impacting c-Myc levels, thereby leading to the induction of p21 in primary CLL cells, with CC-122 showing superior activity. Our experimental approach compared the effects of LEN and CC-122 in their ability to inhibit proliferation of CLL cell lines and primary CLL cells and then expanded this to investigate their effects on relevant pathways. Specifically, we investigated the effect of these compounds on the cell cycle (**Chapter 3**), the changes in expression of pro- and anti-apoptotic family members (**Chapter 4**), and the BCR signalling pathway (**Chapter 5**). Furthermore, to determine novel suitable combinations with other therapeutic agents to potentiate the action of LEN and CC-122, the effects of epigenetic priming prior to treatment with IMiDs were explored (**Chapter 6**). The present chapter attempts to integrate our experimental findings and in doing so, provide a unifying explanation for the effects of both LEN and CC-122 in CLL.

It has been previously shown that Lenalidomide inhibits proliferation by upregulating p21 in B-lymphoma and myeloma cell lines and also in primary CLL cells [196, 199]. In considering the extent to which our findings support the hypothesis, firstly, it can be noted that both LEN and CC-122 inhibit proliferation of B-CLL cell lines and primary CLL cells and cause a change in

expression in some cell cycle variables and these responses were generally more pronounced with CC-122 (**Figure 3.3 & 3.7**).

Both drugs significantly altered cyclin A and B expression in B-CLL cell lines and this was significant with EHEB and also associated with proliferation inhibition (**Figure 3.4**). In contrast, other B-CLL cell lines and primary CLL cells did not change cyclin levels to any significant degree (**Figure 3.4 & 3.8**). Interestingly, levels of the CDK inhibitor, p21 were upregulated in all primary CLL cases (**Figure 3.9**) and there was clear association between its expression and proliferation. In summary, we have produced evidence that both LEN and CC-122 inhibit the proliferation of cell lines and primary CLL cells, with CC-122 having a superior activity. In addition, we have data to suggest that one way the IMiDs exert their effect, is by altering the expression of proteins involved in cell cycle regulation, namely, p21.

In addition to the inhibition of proliferation, it has been shown that LEN and CC-122 induce apoptosis in multiple myeloma and DLBCL [160, 180]. In our study, we show that treating B-CLL cell lines (up to 5 days) and primary CLL cells (up to 3 days) with LEN and CC-122 did not induce apoptosis even in those with profound inhibition of proliferation (**Figure 4.1 & Figure 4.3**). Hence, and not surprisingly, the levels of BH3 domain containing pro- and anti-apoptotic proteins did not show any significant changes (**Figure 4.2 & Figure 4.4**). Notably, we did find that the ratios of Bcl-2: Bax and Mcl-1: Bax were altered with IMiD treatment (**Figure 4.4**), suggesting that it may be possible to predict which patients may benefit from combination of IMiDs with

drugs such as ABT-199 that target the apoptotic pathway. In summary, we have shown that LEN and CC-122 do not induce apoptosis in CLL and this is different from their effects in other haematological cancers [160, 180].

We have also demonstrated that investigating the effects of the IMiDs on the B-cell receptor pathway contribute to elucidating the mechanism of action of both drugs in CLL. This work provides novel insights into this particular field given the finding that the activation of ERK phosphorylation (and other members of the pathway), by Anti-IgM stimulation, was inhibited by both LEN and CC-122 (**Figure 5.5**). Since all B-CLL cell lines that were used in this study are anergic to BCR stimulation (data not shown), it was essential to work with MCL cell lines (MAVER-1 and JEKO-1) to optimize the time and dosage of Anti-IgM antibody treatment for crosslinking studies. Our results showed that the phosphorylation state of PCLƳ2, ERK, and AKT, which are involved in BCR signalling, remain unchanged in response to treatment. This is reassuring because no inhibition of proliferation was observed in these cell lines (data not shown).

To elucidate the role of the BCR pathway, after stimulation with Anti-IgM, in the response to the IMiDs, we used a cohort of cases that showed variable inhibition of proliferation following treatment. The phosphorylation of proteins in the BCR pathway (ERK and AKT) was inhibited by LEN and CC-122 after Anti-IgM stimulation (**Figure 5.5**). This indicates perturbation of BCR signal transduction and likely contributes to the decrease in proliferation following drug exposure. In addition, all primary CLL cases tested, there was a greater

reduction in p-ERK after CC-122 treatment than with LEN (**Figure 5.5 I**), and could account for greater proliferation inhibition with the former drug. In contrast, only 50% of the cases showed inhibition of AKT phosphorylation (**Figure 5.5 I**), and the level of inhibition was similar for both treatments. Before we could propose a mechanism of this observed ERK and AKT phosphorylation inhibition, we had to confirm that the inhibition is specific to Anti-IgM stimulation. Primary CLL cells that were stimulated by soluble CD40L, showed an increased ERK and AKT phosphorylation but this was not inhibited by LEN and CC-122 treatment (**Figure 5.7 B**). However, BCR stimulation, by Anti-IgM alone, shows a similar pattern like the one observed previously (**Figure 5.7 A**). This indicates that LEN and CC-122 inhibit the phosphorylation of ERK and AKT via the BCR signalling pathway. This observation led us to investigate further downstream targets of ERK in CLL. Our study showed a significant ($p < 0.0001$) downregulation of c-Myc and increased p21 levels in all primary CLL cases following treatment (**Figure 5.8** & **5.9 A**). Again, this effect was more pronounced with CC-122 than LEN. In contrast, p27 levels remained the same in all examined cases (**Figure 5.9 B**). In summary, we suggest a mechanism whereby LEN and CC-122 inhibit ERK phosphorylation, leading to downregulation of c-Myc expression and upregulation of p21 expression in primary CLL cells. Although both LEN and CC-122 are from the same drug family, they likely have different modes of action that make CC-122 more efficient in the majority of CLL cases (in-house data; data not shown). To further confirm that these results are specific to CLL, we examined MCL cell lines and primary MCL samples and

found contrasting results. This provides a degree of confidence that the results that we obtain in primary CLL samples are true and suggest distinct effects of CC-122 and LEN on CLL cells.

It is important to appreciate that a minority of CLL cases are resistant to the IMiD compounds and some showed a weaker response than others (in-house data). We hypothesised that pre-treatment of these cells with, for example, epigenetic priming drugs, may increase the sensitivity of such cases to IMiDs. There is a growing interest in sensitizing cells to re-express epigenetically silenced genes by demethylation [255]. Of interest, our results reveal that treating B-CLL cell lines and primary CLL cases with 5-Azacytidine prior to IMiD exposure, showed greater inhibition of proliferation than IMiDs alone especially in CLL cases that were less sensitive to single agents alone (**Figure 6.2 & 6.4**). 5-Azacytidine alone has no effect on proliferation (**Figure 6.3**), but appears to make the cells more susceptible to proliferation inhibition by LEN and CC-122, even at low concentrations (**Figure 6.4**). Similarly, Romidepsin sensitized primary CLL cells to IMiDs and showed greater degree of proliferation inhibition (**Figure 6.4 A & B**). Furthermore, we attempted combining both 5-Azacytidine and Romidepsin as a priming combination prior to LEN and CC-122 treatment and found encouraging results (**Figure 6.4 C & D**). In summary, our observations could inform future studies to develop personalized treatment regimens in CLL that incorporate LEN or CC-122.

In conclusion, this study has demonstrated strong linkage for a mechanism of inhibition of proliferation and the following: inhibition of ERK phosphorylation, downregulation of c-Myc, as well as enhancement of p21 expression in CLL (**Figure 7.1**). The findings of this thesis could ultimately improve the therapeutic strategies used in the management of CLL and also help to develop potential combinational regimens that improve outcomes for individual patients.

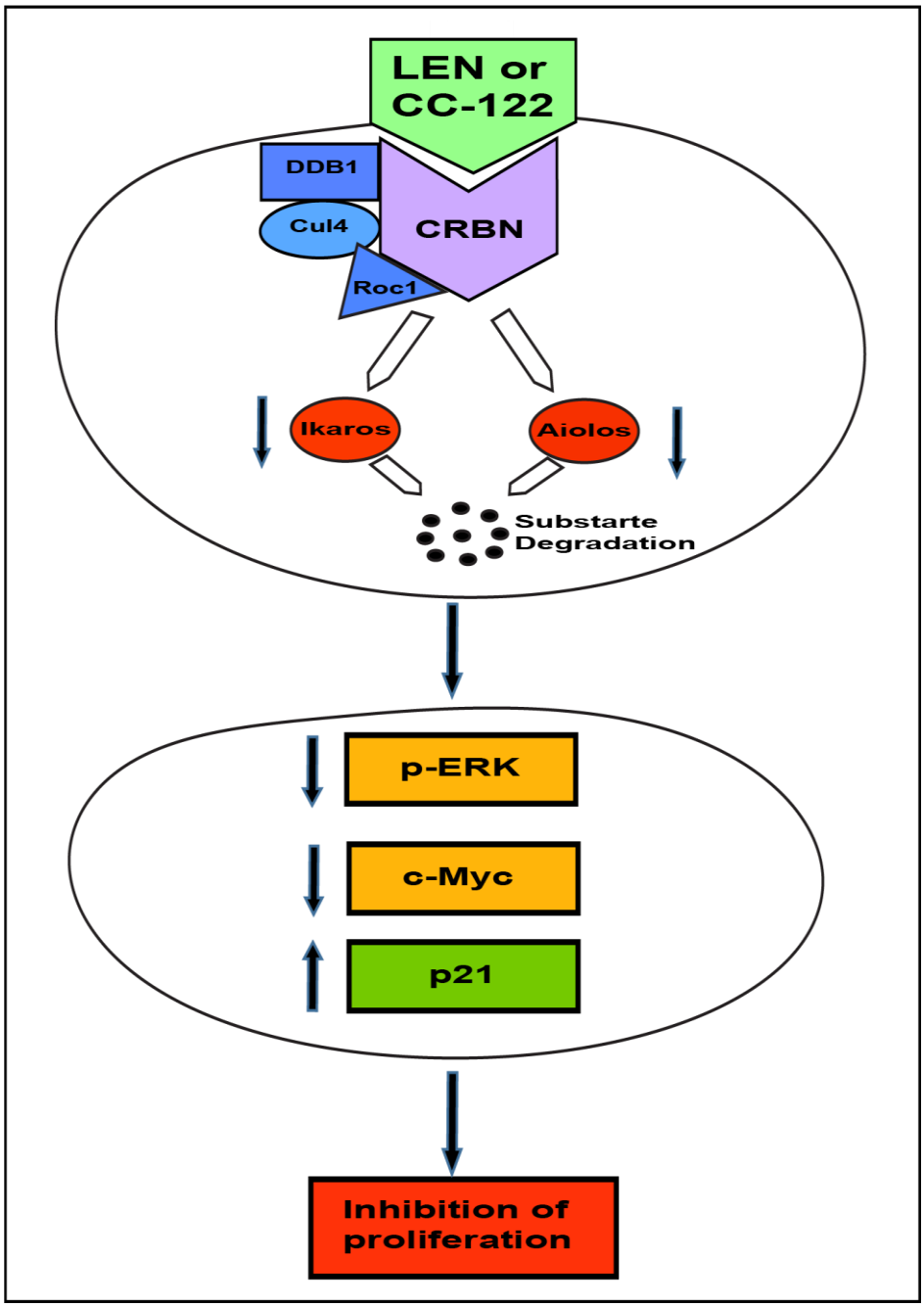


Figure 7.1: Proposed mechanism for the effect of IMiDs in CLL. Upon BCR stimulation by Anti-IgM, the signal is transiently propogated through the ERK pathway and the signal is sustained through c-Myc and leads to proliferation in CLL. However, IMiDs inhibit of proliferation likely due to a decrease in ERK phosphorylation and c-Myc activity. The inhibition of proliferation seems to be mediated through the upregulation of p21.

Future Research

As stated in previous chapters, this study has largely compared the immunomodulatory drug Lenalidomide with a novel analogue CC-122 in CLL and we have conclusively shown that CC-122 has superior activity. We have identified possible mechanisms of IMiDs mediated inhibition of CLL cell proliferation *in vitro*. However, the relevance of this study can be further improved with following work proposed for future.

1. Investigation of the impact of ERK upstream targets by Lenalidomide and CC-122 in primary CLL cells

In chapter 5, we found that both LEN and CC-122 significantly lower the ERK phosphorylation in primary CLL cells. It will be important to examine the proteins that control ERK regulation. The Ras protein is a GDP/GTP binding protein that acts as an intracellular signal transducer mainly controlling cell proliferation and apoptosis [259]. Previous studies reported that elevated expression of the Ras family of oncogenes is a frequent event and plays a crucial role in the development of disease [260]. In such a study, we could use a Ras-GTP and Ras-GDP assay to see the activity of Ras in treated and untreated primary CLL cells. This assay could be based on differential affinity of Ras-GTP and Ras-GDP for Ras binding domain (RBD) of Raf-1. This will provide additional information on the Ras-MAPK pathway in CLL.

2. Evaluation of a gene expression profile with epigenetic drugs and IMiDs in primary CLL cells

In chapter 6, we investigated the dose and scheduling of epigenetic priming drugs used in combination with LEN and CC-122. Results promisingly suggested a benefit to using such a combination. Therefore, this study can be followed up to see if any epigenetic changes are co-ordinated with changes in gene expression. The current hypothesis is that the epigenetic priming with IMiDs leads to increased efficacy of proliferation inhibition. Therefore, primary CLL cells that are sensitized with 5-Azacytidine or Romidepsin may potentially alter a 'resistance specific gene expression' profile and cell cycle regulators that helps in enhancing the effects of IMiDs. This will also aid in other approaches utilising epigenetic drugs (alone or in combination) for the management of CLL.

3. Validation of predicted combination in primary CLL cells

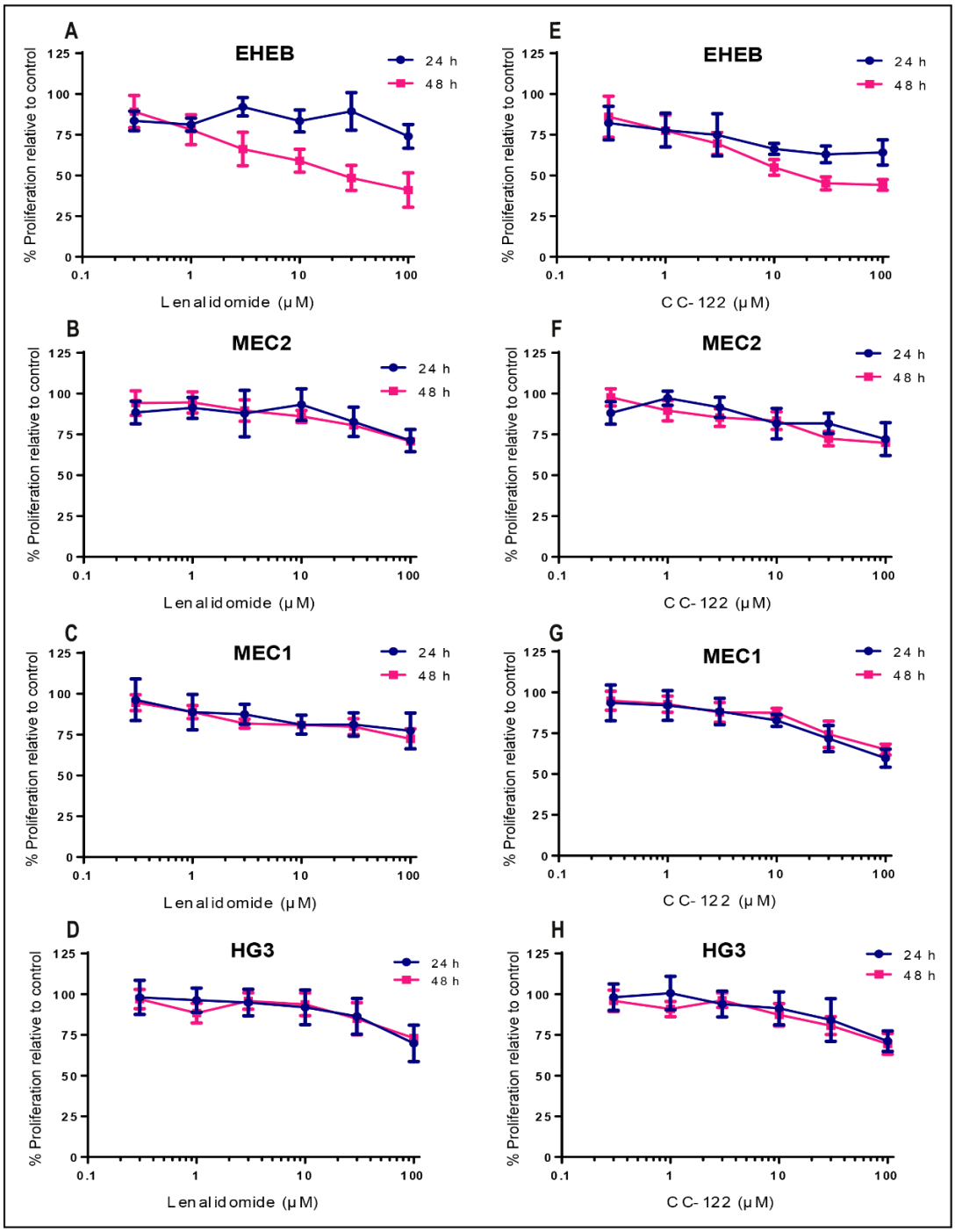
In chapter 4, we found different ratios, after treatment, for the apoptotic proteins Bcl-2: Bax and Mcl-1: Bax in primary CLL cases. It will be important to examine if the specific Bcl-2 inhibitor (ABT-199) and Mcl-1 inhibitor (S63845) in combination with LEN or CC-122 change individual protein expression, or the ratios, in primary CLL cells. Such ratios may allow us to tailor specific combinations of treatments to each patient and would help to develop new therapeutic strategies.

4. Explore the mechanisms that may explain the superior activity of CC-122 when compared to LEN in CLL

Our results demonstrate that CC-122 is consistently superior to LEN in inhibiting proliferation of CLL cells. Both drugs seem to equally inhibit Aiolos expression. This implies that CC-122 may impact the expression of genes and proteins that are distinct from LEN activity. Further characterisation of such differential activity will require comprehensive gene expression and proteomic studies to better understand the underlying mechanisms and may inform future drug design.

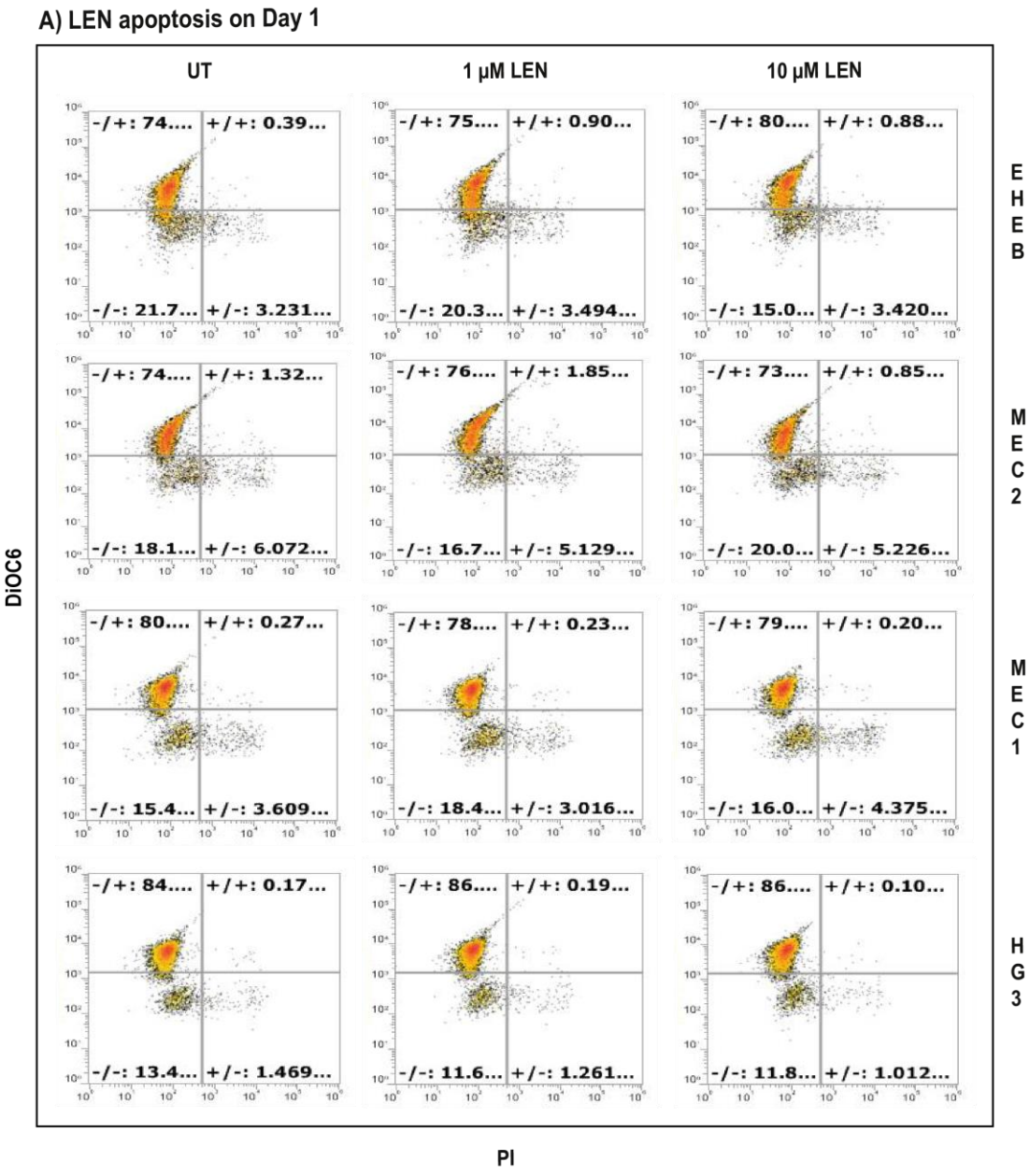
Appendix A

Appendix A. Figure .1: Effect of LEN and CC-122 on proliferation in B-CLL cell lines at 24 & 48 h.

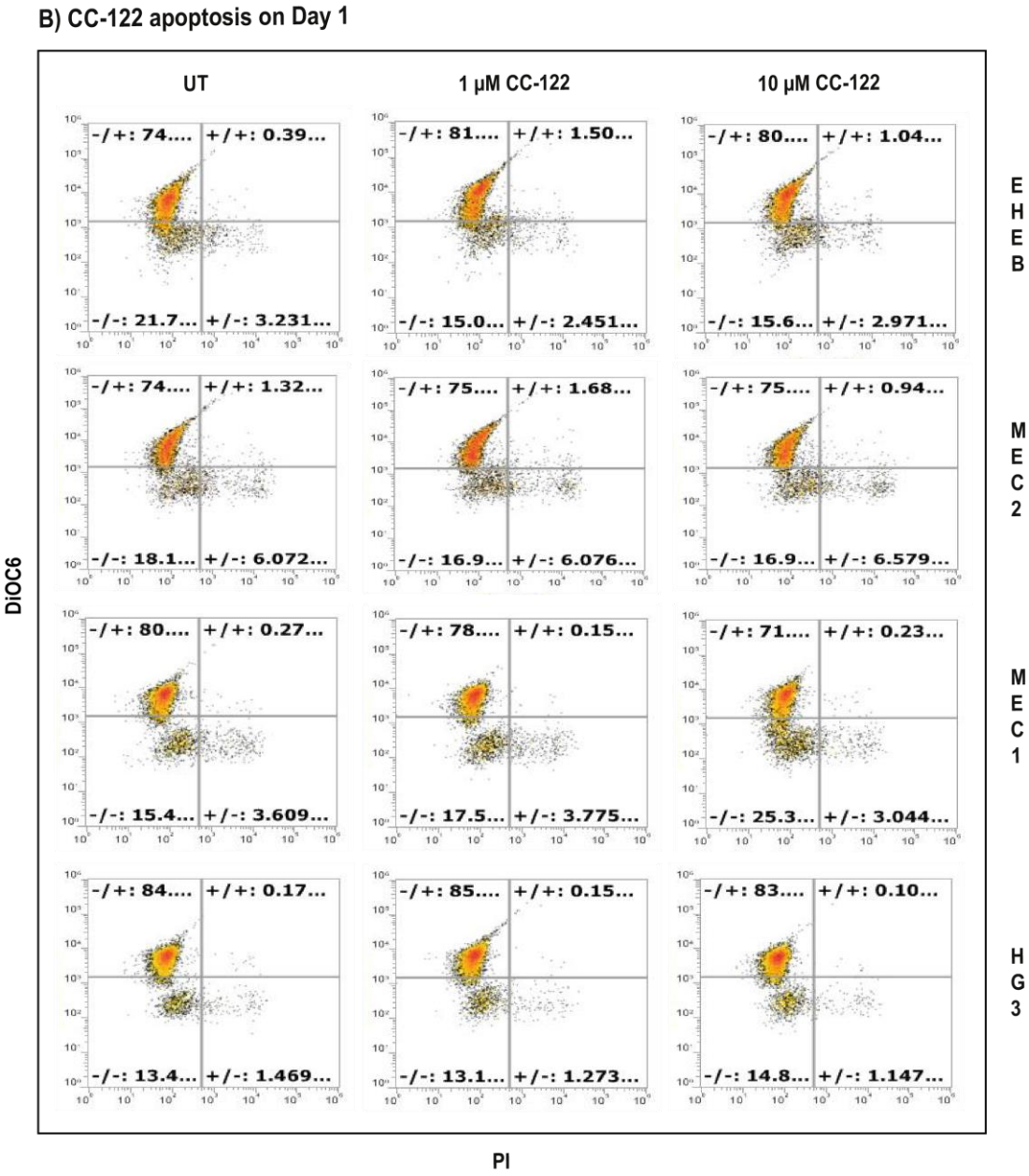


Appendix B

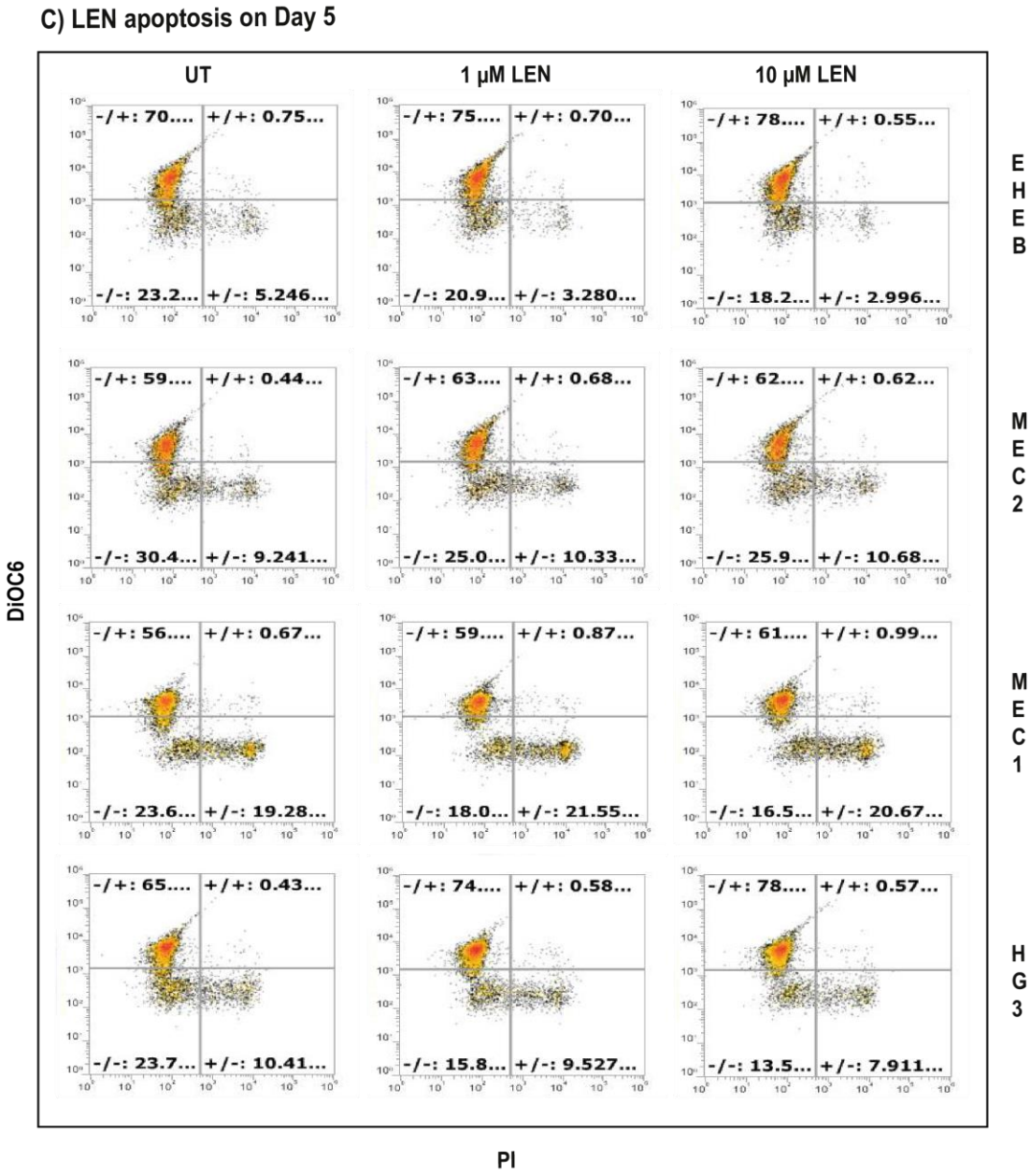
Appendix B. Figure 1: FACS based measurement of apoptosis in B-CLL cell lines following treatment with Lenalidomide on Day 1.



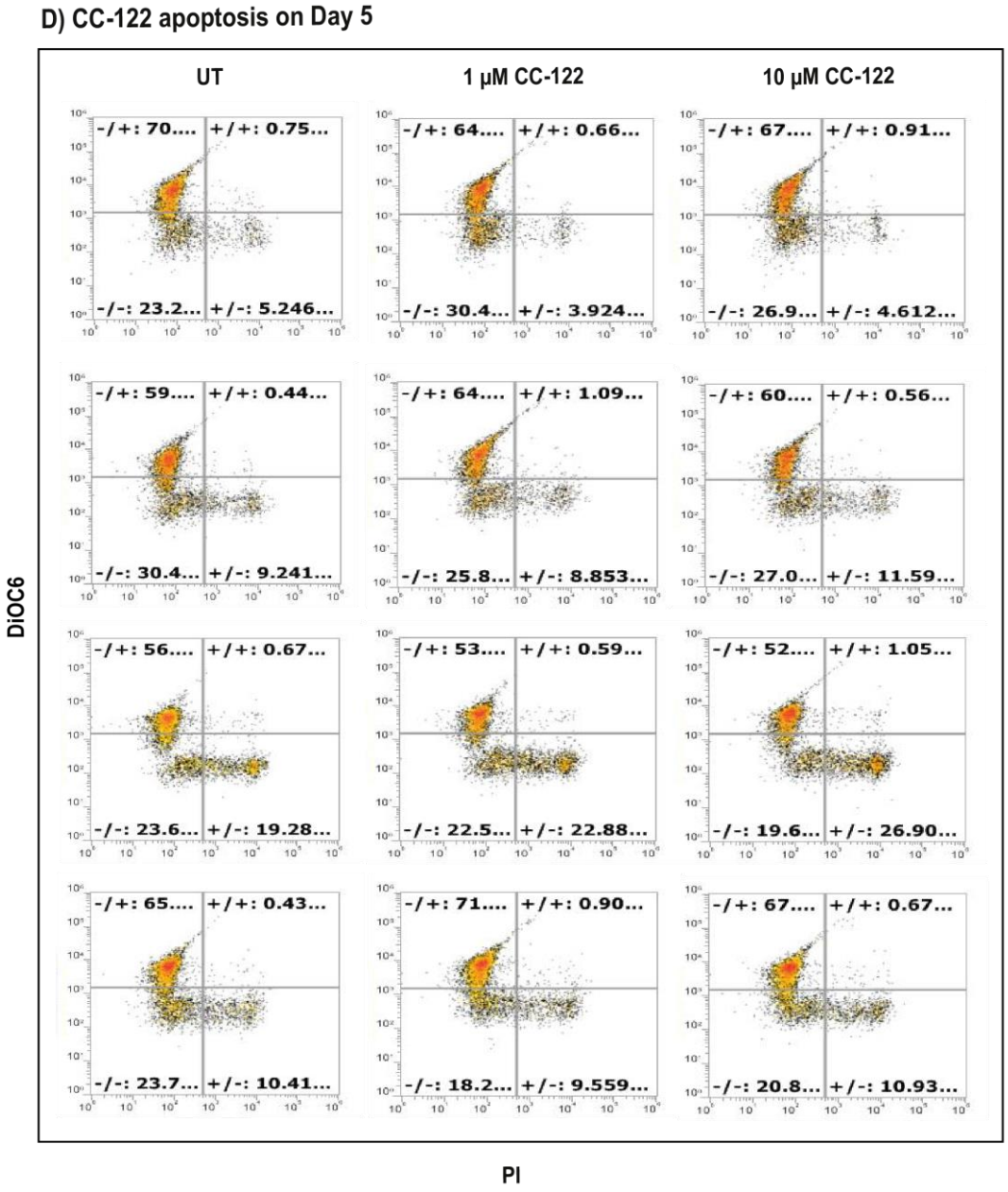
Appendix B. Figure 2: FACS based measurement of apoptosis in B-CLL cell lines following treatment with CC-122 on Day 1.



Appendix B. Figure 3: FACS based measurement of apoptosis in B-CLL cell lines following treatment with Lenalidomide on Day 5.

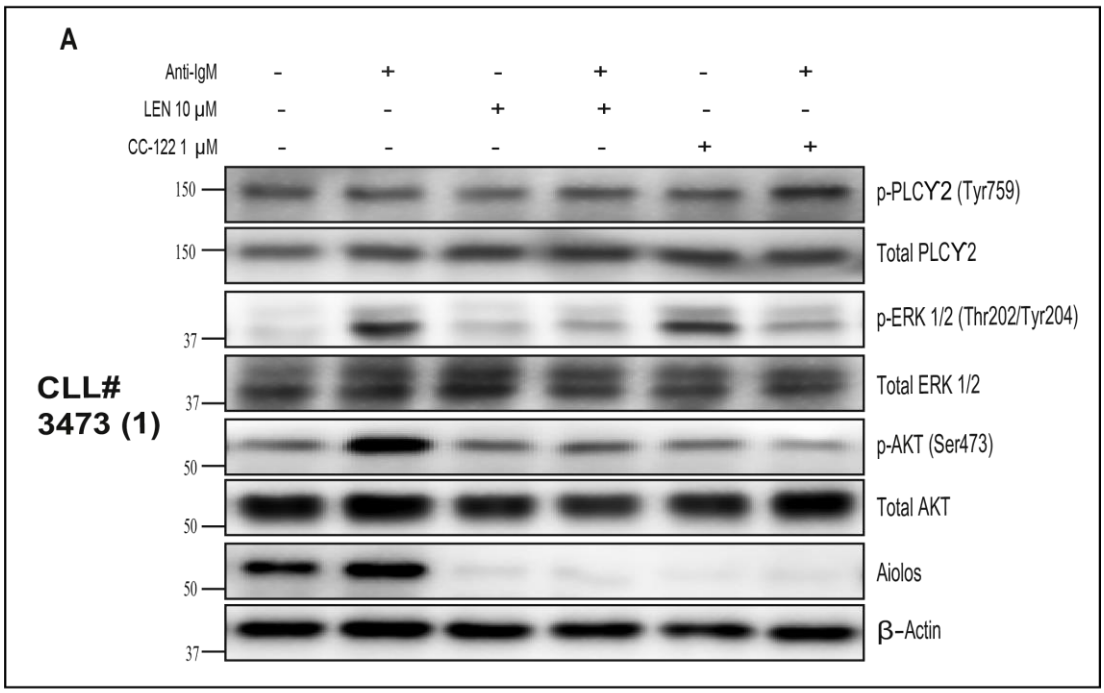


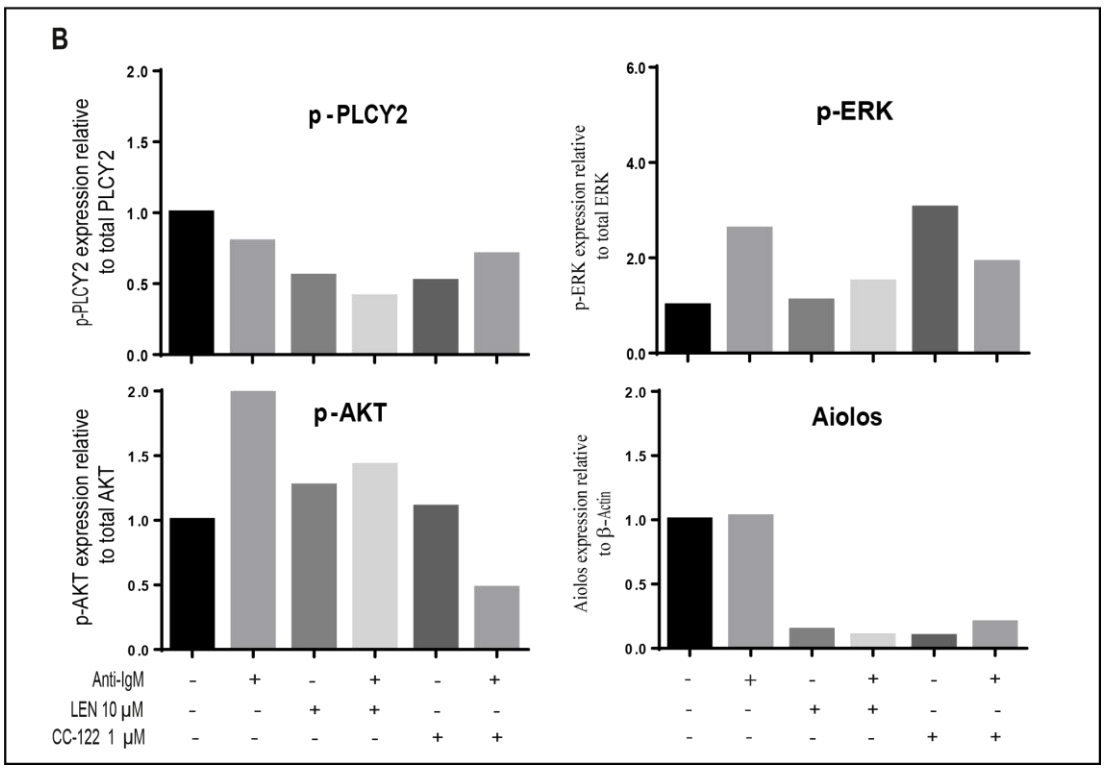
Appendix B. Figure .4: FACS based measurement of apoptosis in B-CLL cell lines following treatment with CC-122 on Day 5.



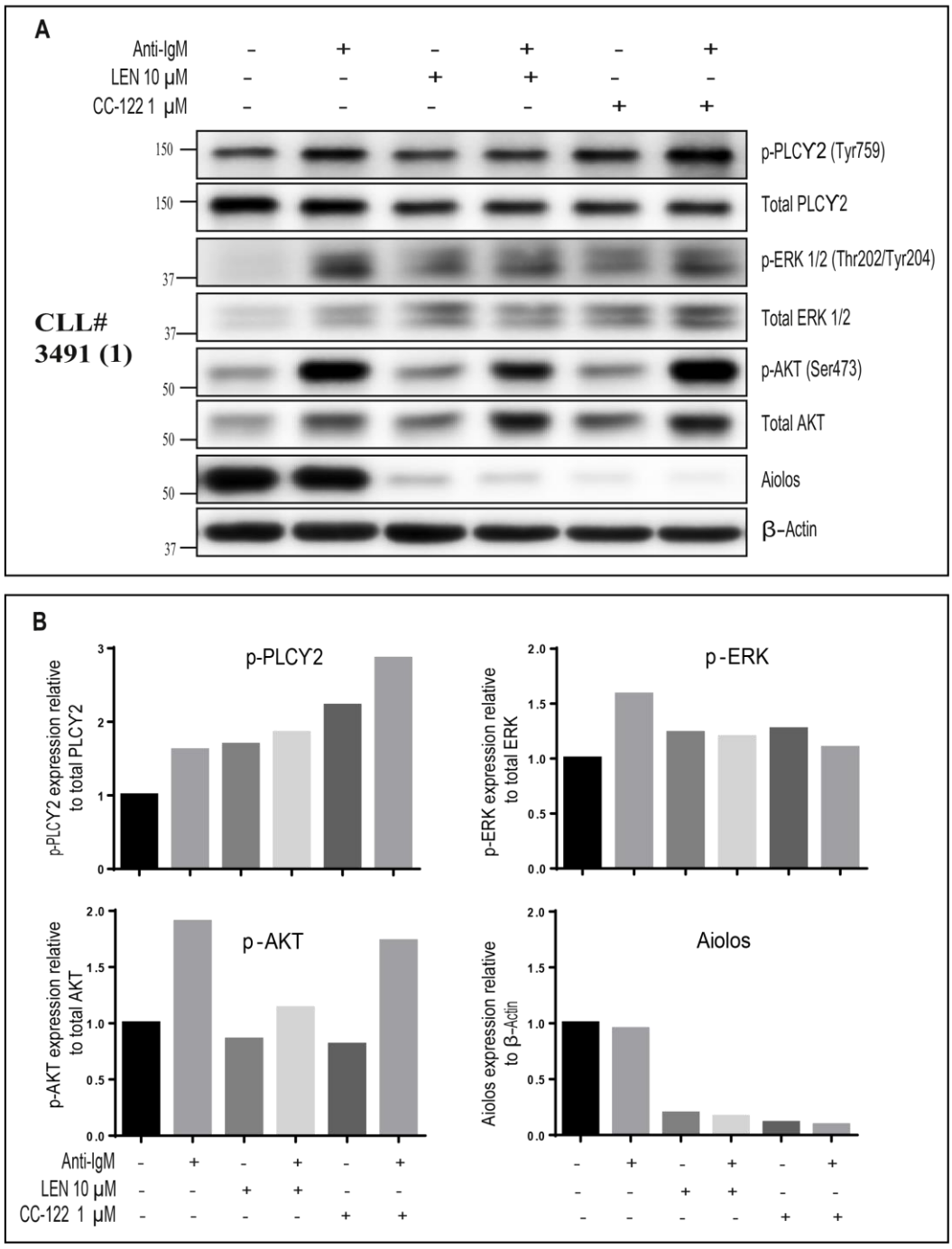
Appendix C

Appendix C. Figure .1: LEN and CC-122 inhibit Anti-IgM induced p-ERK and p-AKT expression in primary CLL cells.

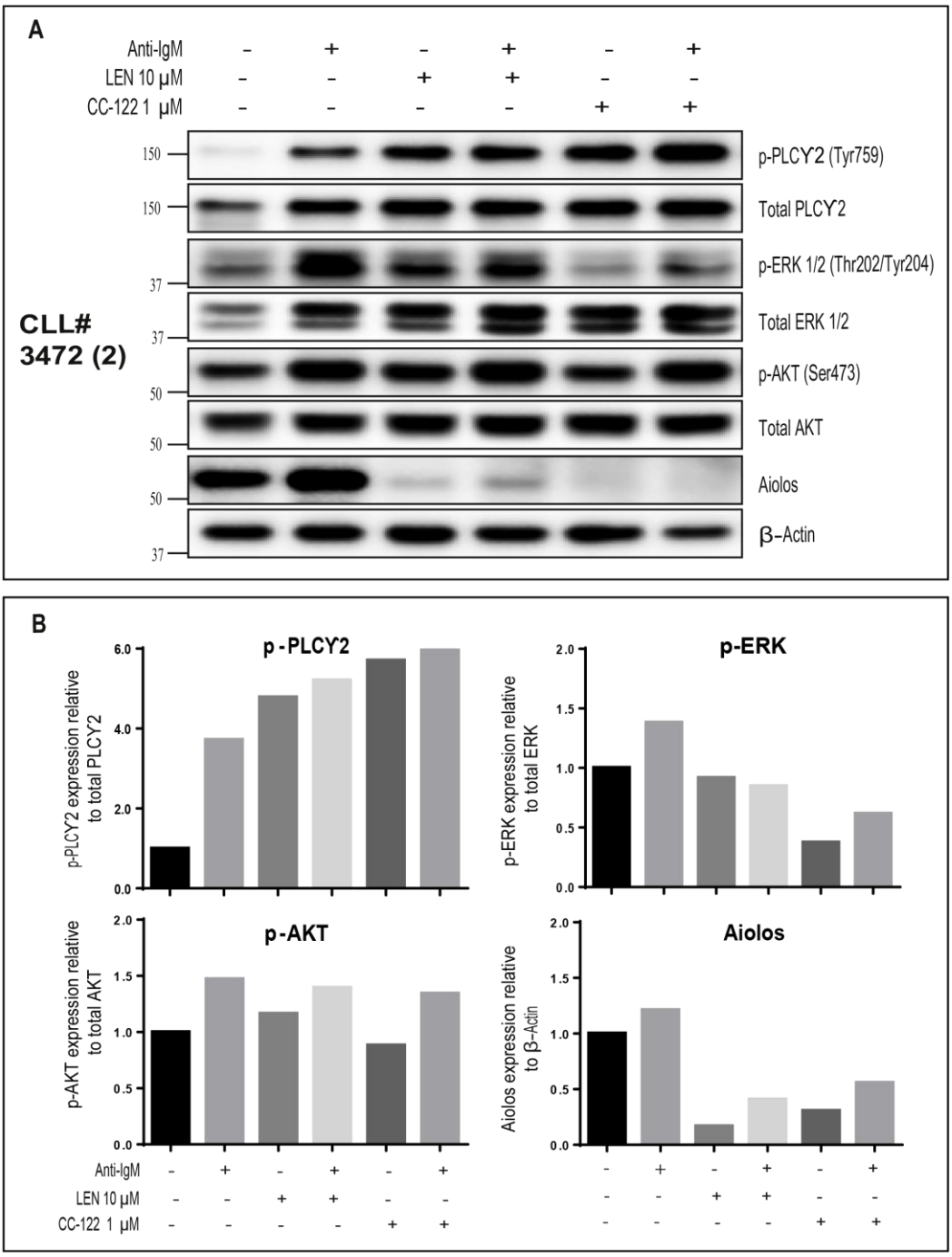




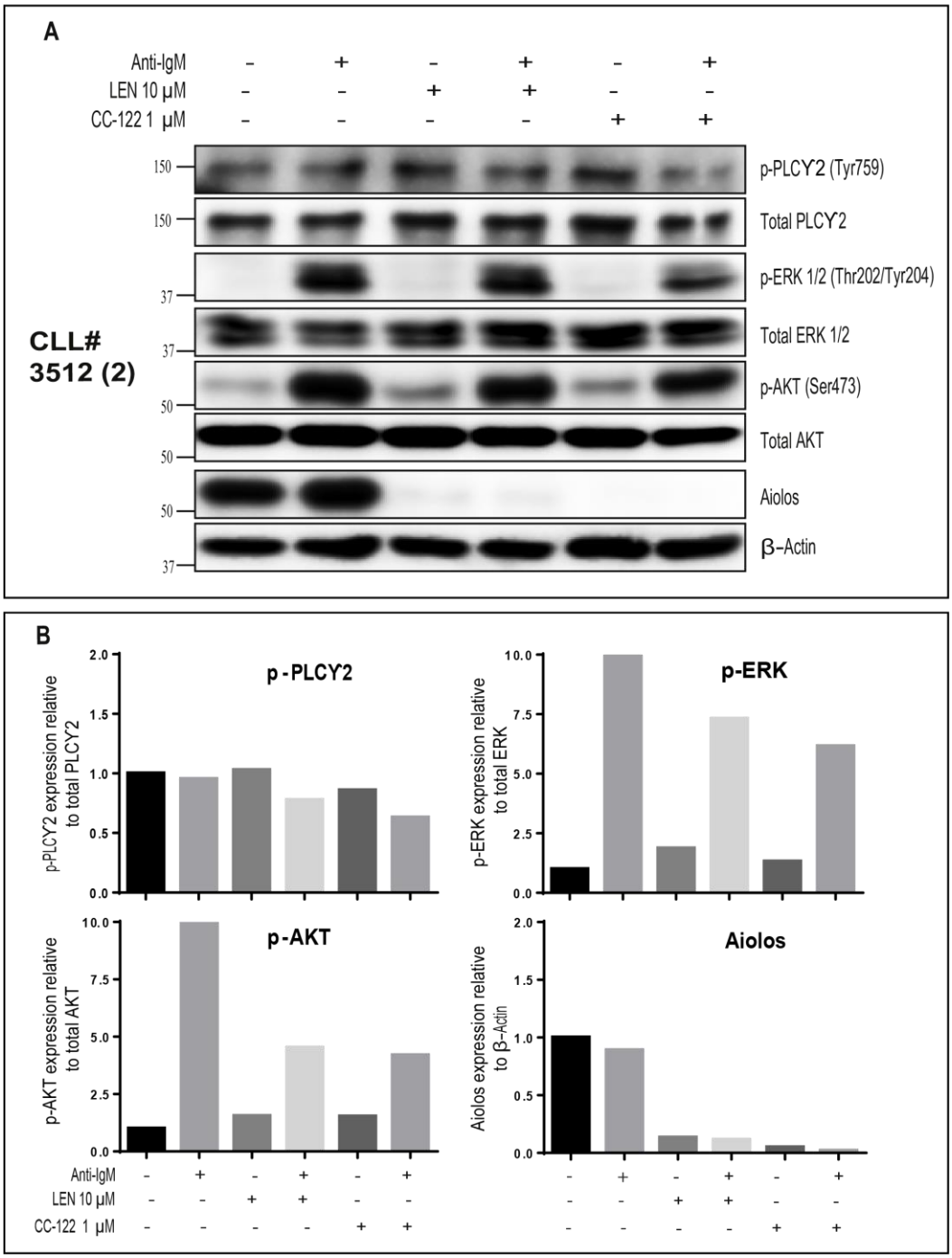
Appendix C. Figure .2: LEN and CC-122 inhibit Anti-IgM induced p-ERK and p-AKT expression in primary CLL cells.



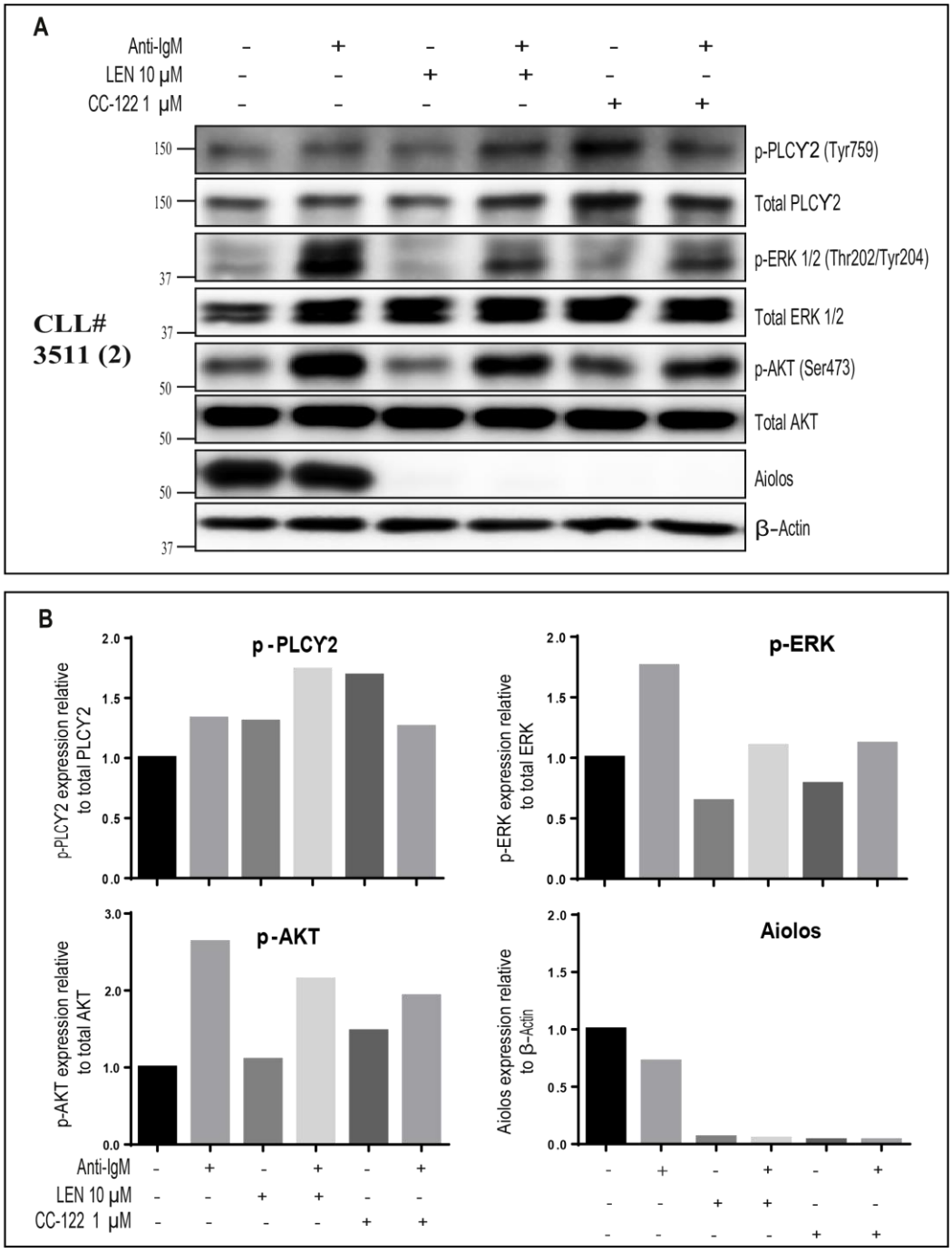
Appendix C. Figure .3: LEN and CC-122 inhibit Anti-IgM induced p-ERK and p-AKT expression in primary CLL cells.



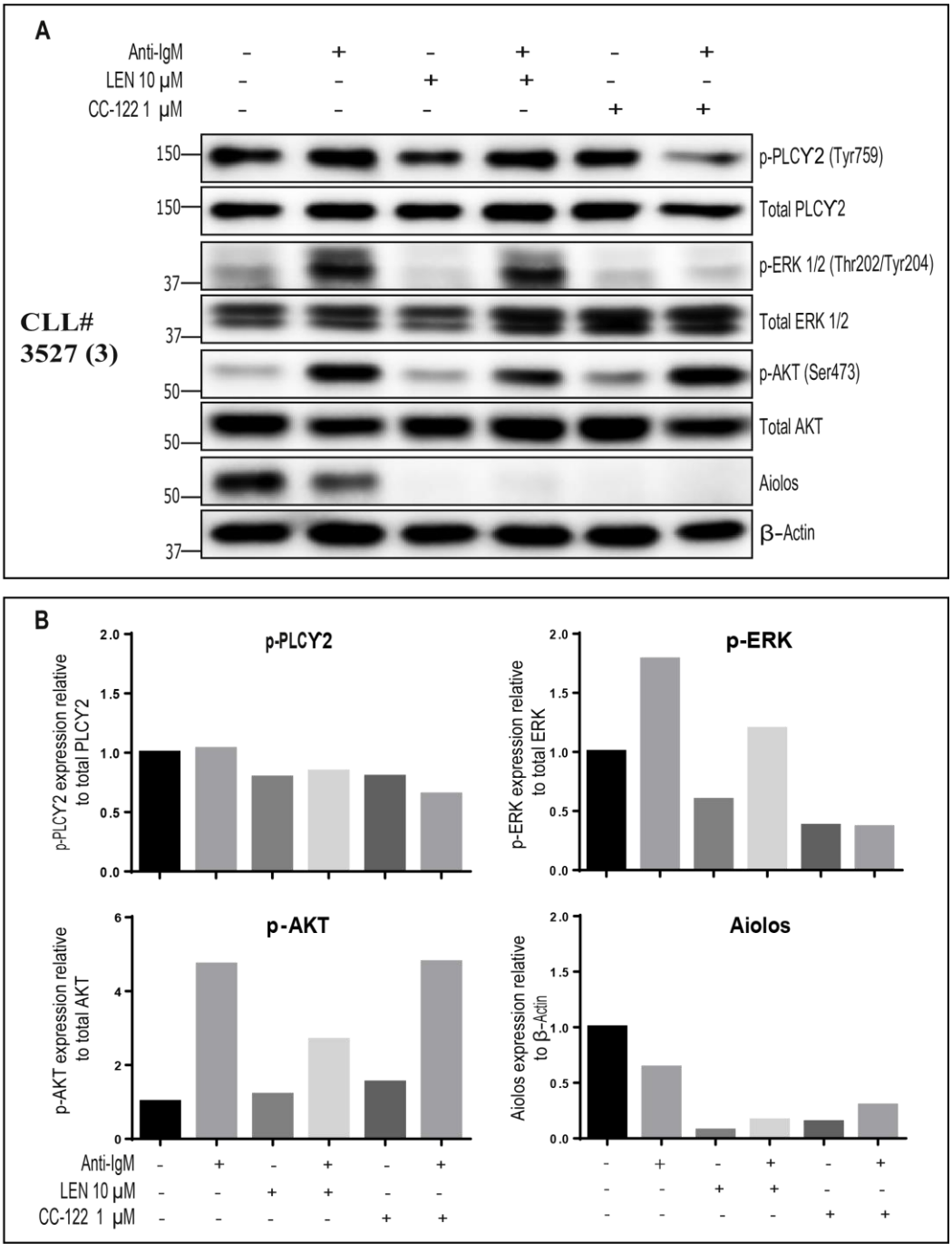
Appendix C. Figure .4: LEN and CC-122 inhibit Anti-IgM induced p-ERK and p-AKT expression in primary CLL cells.



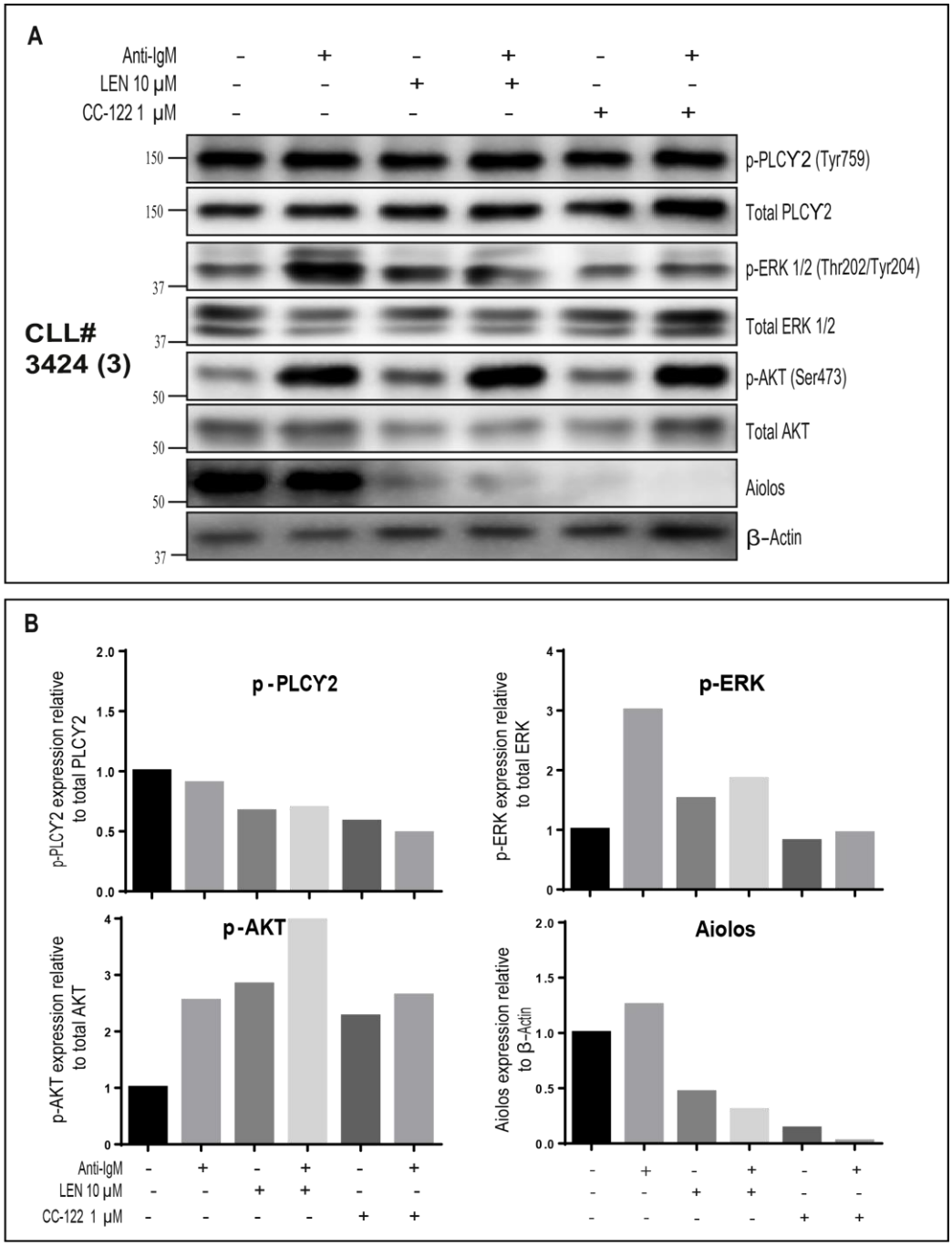
Appendix C. Figure .5: LEN and CC-122 inhibit Anti-IgM induced p-ERK and p-AKT expression in primary CLL cells.



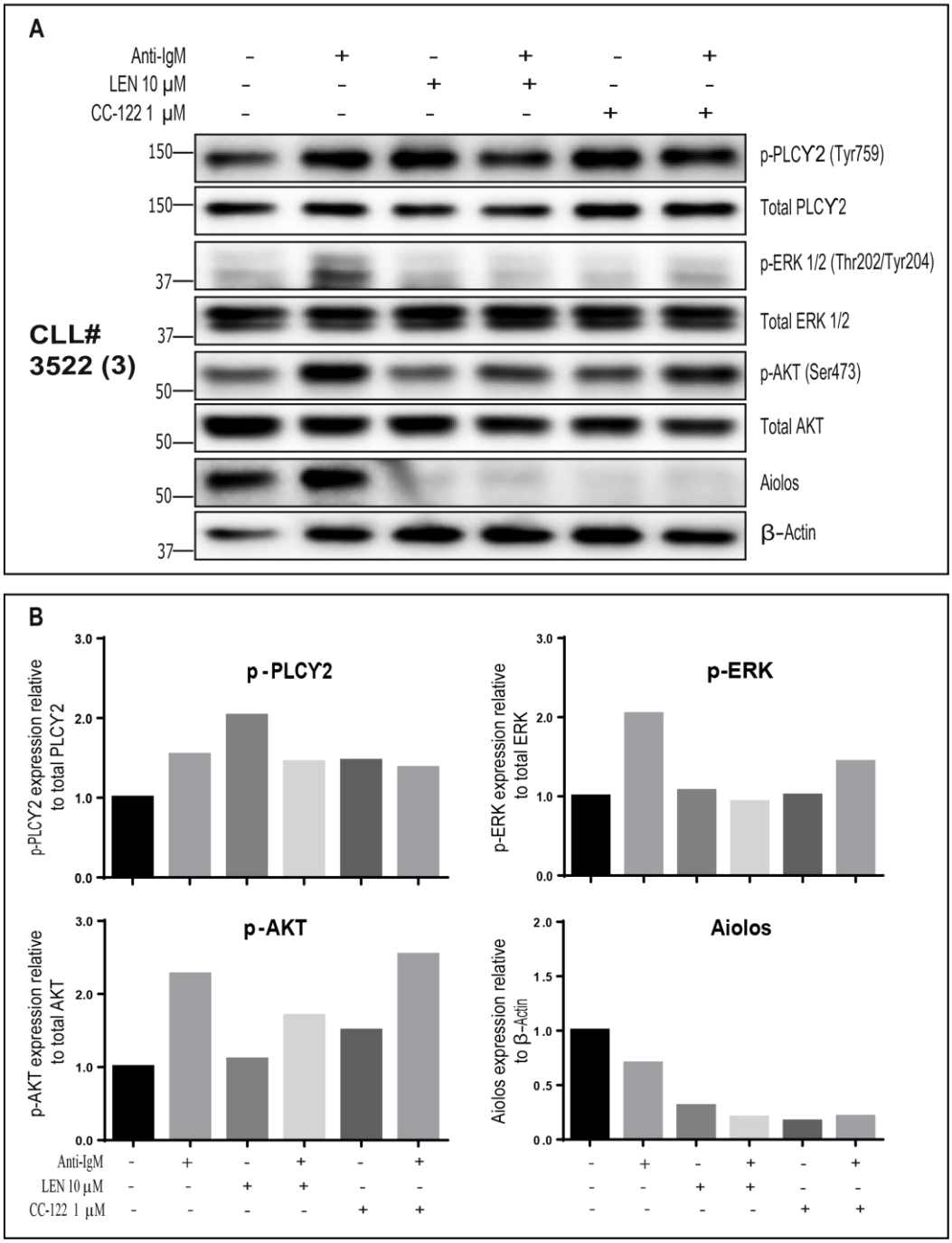
Appendix C. Figure .6: LEN and CC-122 inhibit Anti-IgM induced p-ERK and p-AKT expression in primary CLL cells.



Appendix C. Figure .7: LEN and CC-122 inhibit Anti-IgM induced p-ERK and p-AKT expression in primary CLL cells.



Appendix C. Figure .8: LEN and CC-122 inhibit Anti-IgM induced p-ERK and p-AKT expression in primary CLL cells.



Appendix D

Appendix D. Table .1: Buffers used in this study.

Buffer	Components	Purpose
1x SDS-PAGE running buffer	25 mM Tris, 192 mM Glycine, 0.1% (w/v) SDS, pH=8.3	Western blot
Transfer buffer	25 mM Tris, 192 mM Glycine, pH=6.8	Western blot
TBS	25 mM Tris pH=7.6, 150mM Nacl	Western blot
TBS-T	TBS with 0.1% Tween-20	Western blot
Blocking buffer	5% w/v low fat milk in TBST	Western blot
Clear sample buffer	1% SDS, 125 mM Tris, pH 6.8, 5 mM EDTA, 10% glycerol	Lysate preparation
5 x loading buffer	5% SDS, 625 mM Tris pH 6.8, 50% glycerol, 5% β-mercaptoethanol and bromophenol blue	Lysate preparation
Ponceau staining	0.2% w/v Ponceau S 5% glacial acetic acid	Western blot
Stripping buffer	10% SDS, 0.5 M Tris HCl, β-mercaptoethanol and dH_2O	Western blot
PBS	Powder dissolved in dH_2O, pH7.4	Cell culture
Trypsin-EDTA	0.25% trypsin in PBS, Na_2-EDTA	Cell culture (Adherent cells)
BrdU		Cell proliferation

Appendix D. Table .2: Drugs used in this study

Drug	Catalog no	Manufacturer
Lenalidomide	Gift	Celgene
CC-122	Gift	Celgene
5-Azacytidine	A2385	Sigma
Romidepsin	Gift	Celgene

Appendix D. Table .3: Growth media used in this study

Media	Composition	Cells	Manufacturer
RPMI	10% FBS, L-Glutamine, P/S	EHEB, HG3, Primary CLL	Sigma
IMDM	10% FBS, L-Glutamine, P/S	MEC1, MEC2	Sigma
DMEM	10% FBS, L-Glutamine, P/S	Fibroblast	Sigma

Appendix D. Table .4: Software used for data analysis in this study

Software	Purpose
Aida image analyzer	Densitometry
Alliance	Capture images of western blots
ImageJ	Densitometry
Graphpadv6	Analysis & plots the graphs
Adobe illustrator	Create images
Gene5	Protein determination & BrdU cell proliferation assay

Appendix D. Table .5: General Reagents used

All the reagents were purchased from Sigma-Aldrich unless listed here.

Supplier	Reagents
Bio-Rad Laboratories	Precision plus protein standard, DC ™ Protein assay,
Fisher scientific	Glycine, Tris base, SDS, Tween® 20, Methanol, DMSO
Geneflow	Acrylamide, Resolving and Stacking buffer, Tris glycine electro blotting buffer, WESTAR supernova HRP detection substrate (ECL)
Life technologies	rhIL-21 Recombinant human protein, Fetal bovine serum
Stratech scientific Ltd	AffiniPure F(ab')$_2$ fragment Goat Anti-Human IgM,

Appendix E

Appendix E. Table .1: Clinical data of CLL cases used within this thesis.

No.	Sample	WBC (10^9/L)	Rai	Binet	p53	status	Ig Isotype	Karyotype	Treatment
1	3405	364.5						11q-	
2	3490	193.1						13q-	Ib
3	3491	82.7					IgM	11q-, 13q-	Ritux or Bend, Of, Id
4	3492	67.6						11q-	FCR, AdMIRe
5	3493	72.8		A				13q-	
6	3484						IgM	13q-	Of, Clb
7	3473							Normal	UT
8	3378							13q-	
9	3472	149.9						13q-	Of or Clb
10	3511							13q-	UT
11	3507	33		A					
12	3512							13q-	FCx6 (2006) FCR
13	3526	234						Trisomy 12	
14	3381	4.3		A					Of or Clb, R-Clb
15	3429	291.6						17p	Ib
16	3459	49.2						17p-, 13q-	Clb, Flu, CP, Of, Len, Dex
17	3375	168.4					IgM	17p-	
18	3424						IgM	13q-	Ritu or Bend, Of, Ritux / Id, Ib
19	3522						IgM	11q-, 13q-	FC, Flu, FCR, Ritux or Bend, Id or Ritux, Ib
20	3527			A				normal	
21	3394		III					17p	Flu/CP, Of, Campath
22	3458		0	A				17p,-, 13q-	Flu/CP, Clb, to start Ib
23	3500	226.7						Trisomy 12	FC, FCR, Ritux/Bend, Ib
24	3367	400						17p-	CLL210
25	3503								

Ib: Ibrutinib; **Ritux:** Rituximab; **Dex:** Dexamethasone **Flu:** Fludarabine; **Id:** Idelalisib;

CP: Cyclophosphamide; **Pred:** Prednisolone **FCR:** Flu/CP/Ritux; **Clb:** Chlorambucil

Bend: Bendamustine **IFRT:** Involved field radiation therapy; R-Clb: Ritux+Clb

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