Prospective analysis of NF-κB as a Superior prognostic marker in Chronic Lymphocytic Leukaemia

This thesis is submitted in requirement of the University of Cardiff for the Degree of Doctor of Philosophy

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Contents

Publications arising from the work in this thesis

Hewamana S, Alghazal S, Lin TT, Clement M, Jenkins C, Guzman M, Jordan CT, Neelakantan S, Crooks AP, Burnett AK, Pratt G, Fegan C, Rowntree C, Brennan P, and Pepper C. The NF-κB subunit, Rel A, is associated with *in vitro* survival and clinical disease progression in chronic lymphocytic leukaemia and represents a promising therapeutic target. Blood, 2008, 111: 4681-4689.

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Hewamana S, Lin TT, Rowntree C, Karunanithi K , Burnett A, Brennan P, Fegan C and Pepper C. Rel A is a novel prognostic marker in CLL that is independent of V_H gene mutation status, CD38 expression and ZAP-70 expression (Publication, ASH, 2008)

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Oral Presentations

Targeting NF-κB as a therapeutic strategy in CLL

UK CLL Forum, London, 29.04.2008

Targeting NF-κB, a marker of disease progression, for the treatment of chronic lymphocytic leukaemia

Wales Cancer Conference, Cardiff, 01.05.2008

NF-κB in treatment of CLL

Normal and malignant lymphocyte meeting, Cardiff University, 22.01.08

NF-κB in treatment and progression of CLL

Haematology Seminar series, UHW, 17.03.2008

NF-κB, a biomarker for prognosis and target for treatment in chronic lymphocytic leukaemia.

Immunology seminar Series, Cardiff University, 13.11.2007

NF-κB, abiomarker for prognosis and target for treatment in Chronic Lymphocytic leukaemia

Joint Pathology Seminar Series in UHW, 13.09.2007

NF-κB, biomarker for prognosis and target for treatment in CLL

Infection, Immunity and Inflammation (I3 – IRG), An Interdisciplinary research Group (IRG) July 2007

Quantitative and qualitative assessment of constitutive Nuclear Factor kappa B expression in CLL cells and its relationship with *in vitro* spontaneous apoptosis

Joint Haematology / Pathology Seminar Series in UHW February 2007

ABBREVIATIONS

APC	Allophycocyanin
ATM	Ataxia telangiectasia mutated
B2M	β2 microglobulin
Bax	Bcl-2 associated X protein
Bcl-2	B-cell lymphoma protein 2
BCR	B-cell receptor
BH	Bcl-2 sequence homology
BSA	Bovine serum albumin
CD	cluster differentiation
cDNA	complementary DNA
CO_2	Carbon dioxide
CLL	Chronic lymphocytic leukaemia
CLP	common lymphoid precursors
CMP	common myeloid precursors
dATP	deoxy adenine triphosphate
dCTP	deoxy cytosine triphosphate
dGTP	deoxy guanine triphosphate
DMSO	Dimethyl sulfoxide
DNA	deoxyribose nucleic acid
dNTP	deoxy nucleotide triphosphate
dTTP	deoxy thymine triphosphate
ds	double stranded
DTT	dithiothreitol
EBV	Epstein Barr virus
EDTA	ethylene diamine tetra-acetic acid

ELISA	enzyme linked immunosorbant assay
EMSA	electrophoretic mobility shift assay
FACS	Flow assisted cell sorting
FADD	Fas-associated death domain
FCR	Fludarabine, cyclophosphamide and rituximab
FCS	Foetal calf serum
GF	Growth factors
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
HSC	Haemopoiesis stem cells
IgM/IgG	Immunoglobulin M/G
IgV_H	Immunoglobulin heavy chain variable region
IL-1β	Interleukin-1β
LD_{50}	Lethal dose for 50% of the cells
LDT	Lymphocyte doubling time
Mcl-1	Myeloid cell leukaemia sequence 1
M-CSF	monocyte colony stimulating factor
$MgCL_2$	Magnesium chloride
MFI	Mean fluorescence intensity
MPP	multipotent progenitors
mTOR	Mammalian target of rapamycin
NK	Natural killer
NF-κB	nuclear factor kappa β
OS	Overall survival
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

PE	Phycoerythrin
PFS	Progression free survival
PI3K	Phosphoinositol-3 kinase
PIP2	Phosphoinositol-3,4-bisphosphate
PIP3	Phosphoinositol-3,4,5-trisphosphate
PKC	Protein kinase C
RNA	Ribose nucleic acid
RPMI	Roswell Park Memorial Institute culture media
RT-PCR	real time polymerase chain reaction
sCD23	Soluble CD23
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Ser	Serine
TBS	Tris buffered saline
TCR	T cell receptor
Thr	Threonine
TK	Thymidine kinase
TNF	Tumour necrosis factor
TTFT	Time to first treatment
TTSF	Time to subsequent treatment
VEGF	Vascular endothelial growth factor
ZAP-70	Zeta associated protein-70

AIMS OF THE THESIS

1) To perform a qualitative and quantitative analysis of NF-κB in primary CLL cells

2) To evaluate the relative sensitivity of CLL subsets to NF-κB inhibition

3) To investigate potential synergy between NF-κB inhibitors and conventional chemotherapeutic drugs used for the treatment of CLL

4) To establish whether NF-κB is differentially expressed in subsets of patients defined by cytogenetics and IgV_H gene usage

5) To perform longitudinal analysis of NF-κB in serial patient samples to determine whether constitutive NF-κB is altered over time or by therapeutic intervention

6) To investigate the hypothesis that NF-κB is a superior biomarker of prognosis in chronic lymphocytic leukaemia

ABSTRACT

In this study, I characterized basal NF-κB DNA binding in CLL samples and investigated the value of NF-κB as a prognostic marker and therapeutic target in CLL. In contrast to the previous studies, I demonstrated wide heterogeneity in basal NF-κB DNA binding among patients which was associated with *in vitro* survival (P = 0.01) with high white cell count (P = 0.01) and shorter lymphocyte doubling time (P = 0.01). Subunit analysis revealed that in primary CLL cells the principal components were p50, Rel A, and c-Rel. I next investigated the cytotoxicity of a putative NF-κB inhibitor, LC-1, and elucidated its mechanism of action in CLL patient samples. LC-1 induced apoptosis with a mean LD_{50} of 2.9μM after 24 hours; normal B and T-cells were significantly more resistant to its apoptotic effects (P <0.001). Apoptosis was associated with caspase-3 activation that was mediated via the upstream activation of both caspase-8 and caspsase-9. Apoptosis was preceded by a reduction of nuclear Rel A DNA binding and down regulation of the anti-apoptotic NF-κB target genes CFLAR, BIRC5 and BCL2. LC-1 was highly synergistic with fludarabine (mean combination index 0.26). Rel A DNA binding was strongly associated with advanced Binet stage (P<0.0001) but did not correlate with IgV_H mutation status (P = 0.25), CD38 expression (P = 0.87) or ZAP-70 expression (P = 0.55). In addition, it was predictive of time to first treatment (P = 0.02) and time to subsequent treatment (P = 0.0001). Indeed, Rel A was the most predictive marker of survival both from date of diagnosis (hazard ratio 9.1, P = 0.01) and date of entry into the study (hazard ratio 3.9, P = 0.05). Taken together, the data suggests that NF-κB is a promising therapeutic target and prognostic marker in CLL. Prospective clinical trials designed to evaluate these conclusions are clearly now warranted.

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My parents, family, relatives and friends in Sri Lanka who tolerated me being away from their company.

1.0 INTRODUCTION

1.1 Leukaemia

Leukaemogenesis is a clonal process derived from a single cell in the marrow or peripheral lymphoid tissue that has undergone genetic alteration (Pitot *et al.*, 1991). Malignant cells arise due to a 'multi-hit' process requiring several genetic mutations (Vogelstein *et al.*, 1993). In the process of haematopoietic cell proliferation, differentiation, maturation and cell survival errors may occur, resulting in aberrant expression of genes critical in cell development leading to uncontrolled proliferation, prolonged cell survival or blockage of differentiation pathways (Sawyers *et al.*, 1991). These errors may lead to an imbalance between oncogenes and tumour suppressor genes resulting in uncontrolled growth of malignant cells (Hunter *et al.*, 1997).

1.2 B-cell Chronic Lymphocytic Leukaemia (CLL)

Chronic lymphocytic leukaemia is a neoplasm of small monomorphic CD19+ B-lymphocytes in the peripheral blood, bone marrow, lymph nodes and spleen usually co-expressing CD5 and CD23 (Jaffe *et al.*, 2001).

1.2.1 Epidemiology of CLL

CLL is 20-30 times more common in Europe, Australia and North America than India, China and Japan (Oscier *et al.*, 2004). The highest incidence is in Australia, the USA, Ireland and Italy (Figure 1.1). The incidence rate in the UK is 3/100,000 per year (Oscier *et al.*, 2004). It represents 30-40% of all

leukaemias and is the commonest leukaemia in the Western world (Cheson *et al.*, 1996; Kali *et al.*, 2000). The incidence of this disease increases dramatically with age (Redaelli *et al.*, 2004) and therefore predominantly affects elderly individuals (Figure 1.2). The median age at diagnosis is between 65 and 70 years but 20-30% of the patients are less than 55 years of age at diagnosis (Oscier *et al.*, 2004). The male:female ratio is approximately 2:1 (Sgambati *et al.*, 2001). The median survival time is approximately 10 years but there is extraordinary heterogeneity in the natural history of CLL (Lee *et al.*, 1987). The median survival of CLL is independent of whether patients present above or below 50-55 years, but younger patients are more likely to die of CLL-related causes while older patients more commonly die of unrelated causes including second primary malignancies (Oscier *et al.*, 2004).

1.2.2 Aetiology of CLL

The cause of CLL is not known and debate about the origin of the leukaemic B-cell is long standing (Ghia *et al.*, 2006). CLL was traditionally considered to be a homogenous disease of mature, immune-incompetent cells that accumulate because of defects in apoptosis (Kern *et al.*, 2004). However, improved understanding of the immunobiology of CLL has led to substantial changes in the current view of this disease; CLL is now viewed as at least two related entities (Schoeder *et al.*, 1994; Ghia *et al.*, 2006). The presence of somatic mutations of IgV_H gene indicates that at least a proportion of CLL cases have encountered an antigen during the natural history of the disease and have subsequently transitted the germinal centre (Walsh *et al.*, 2005; Ghia *et al.*, 2006). However, cases without IgV_H mutation are distinct from

navie B-cells and are most similar to mutated CLL cases except for their IgV_H status (Schroeder *et al.*, 1994; Fais *et al.*, 1998).

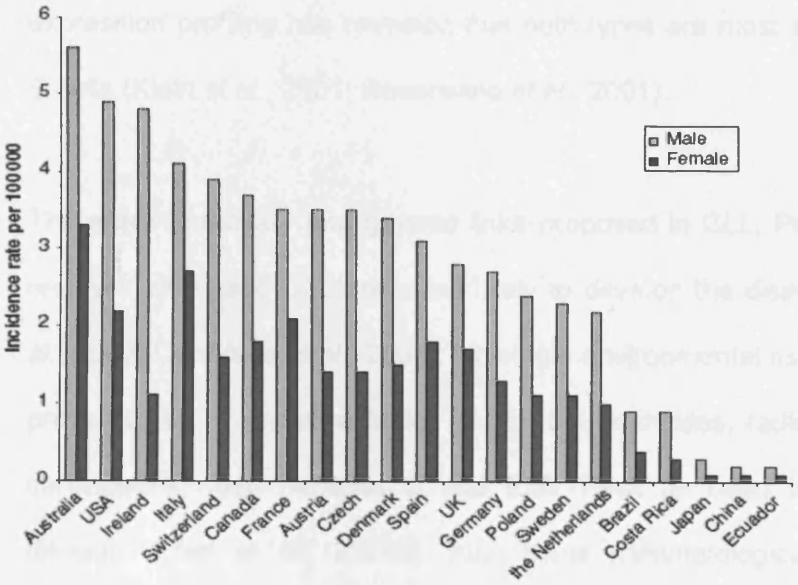

Figure 1.1 Age-standardized world incidence of chronic lymphocytic leukaemia (adapted from Redaelli *et al.*, 2004).

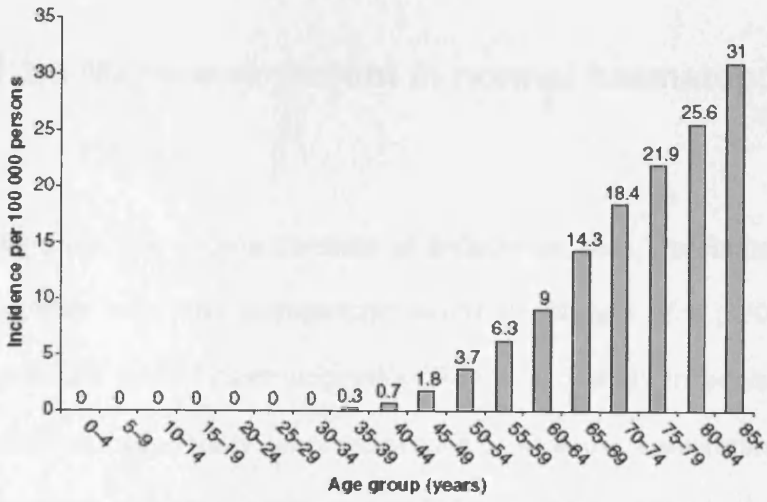

Figure 1.2 Age-specific incidence of chronic lymphocytic leukaemia (adapted from Redaelli *et al.*, 2004)

Furthermore, both CLL subtypes have a surface phenotype of activated B-cells; expressing CD23, CD25, CD69, and CD79 and under expression of CD22 (Schroeder *et al.*, 1994; Damle *et al.*, 2002). In addition, gene expression profiling has revealed that both types are most similar to memory B-cells (Klein *et al.*, 2001; Rosenwald *et al.*, 2001).

There are hereditary and genetic links proposed in CLL. Persons with close relatives who have CLL are more likely to develop the disease (Rawstron *et al.*, 2002; Caporaso *et al.*, 2004). No single environmental risk factor has been proven to be a causative factor in CLL but pesticides, radiation, an array of carcinogens, diet, hepatitis C and EBV have all been implicated in this disease (Linet *et al.*, 2004a). Also some rheumatological conditions are associated with a higher risk of lymphoproliferative disorders including CLL (Mellemkjaer *et al.*, 1996; Royer *et al.*, 1997).

1.2.3 Microenvironment in normal haematopoiesis and in CLL

Bone marrow stroma consists of endothelial cells, fibroblasts, macrophages together with their extracellular matrix (Hoffbrand *et al.*, 2005) and it is an important part of haematopoiesis (Roy *et al.*, 1999). In addition, the germinal centres of secondary lymphoid organs provide microenvironmental support for developing B-cells (Ghia *et al.*, 2002). Despite long life *in vivo*, CLL cells undergo spontaneous apoptosis *in vitro* under conditions that support the growth of normal B-cell lines (Lagneaux *et al.*, 1998; Burger *et al.*, 1999; Yamauchi *et al.*, 2001). This suggests a specific requirement for *in vivo*

signalling for the survival of CLL cells (Ghia *et al.*, 2002; Ghia *et al.*, 2005; Gorgun *et al.*, 2005; Nishio *et al.*, 2005).

Both cell contact and soluble factors are implicated in CLL cell survival. T-cells epithelial cells and Nurse like cells (NLC), differentiated from blood mononuclear cells (Nishio *et al.*, 2005) are thought to play a major role in survival signalling in CLL (Tsukada *et al.*, 2002; Nishio *et al.*, 2005; Planelles *et al.*, 2004; Endo *et al.*, 2007). Also follicular dendtritic cells in the lymph nodes and bone marrow provide signals for malignant B-cell proliferation and survival through CD40 ligation and up regulation of BCL2 family proteins (Chilosi *et al.*, 1985; Petrasch *et al.*, 1994; Choe *et al.*, 2000; Caligaris-Cappio *et al.*, 2003). Interleukin 6 (IL-6), B-cell activating factor belonging to the TNF family (BAFF) and a proliferation-inducing ligand (APRIL) are among other soluble factors identified in B-cell survival. These factors are increased in CLL and are thought to play a major role in cell survival via activation of NF-κB and over expression of anti-apoptotic proteins including Mcl-1 (Tsukada *et al.*, 2002; Nishio *et al.*, 2005).

1.2.4 Clinical manifestations of CLL

Most of the patients with CLL are asymptomatic hence more than 50% are diagnosed following a routine blood test. However, some patients present with fatigue, autoimmune haemolytic anaemia, infections, hepatomegaly, splenomegaly, lymphadenopathy or extra nodal infiltrates (Keating *et al.*, 1998; Hoffbrand *et al.*, 2001; Hoffbrand *et al.*, 2005). The term small lymphocytic lymphoma (SLL), consistent with CLL, is restricted to cases with

the tissue morphology and immunophenotype of CLL, but these cases do not have a frank leukaemia i.e. the disease is confined to the lymphoid organs.

1.2.5 Diagnosis of CLL

CLL is characterised by a morphologically normal B-lymphocytosis of $\geq 5x10^9/L$ with confirmed clonality in the peripheral blood (Figure 1.4). In addition, CLL is invariably associated with bone marrow infiltration; the bone marrow infiltration pattern may be nodular, interstitial, diffuse or a combination of the three (Figure 1.5). Diagnosis is aided by the CLL immunophenotyping score which includes assessment of CD5 and CD23, FMC7, CD79b and surface immunoglobulin (SmIg) (Matutes *et al.*, 1994) (Table 1.2 and Figure 1.6). Lymph nodes are affected in some cases by diffuse infiltration of small lymphocytes identical to low-grade SLL. Also there could be hypogammaglobulinemia and associated serum monoclonal protein (Oscier *et al.*, 2004). The list of tests proposed by the National Cancer Institute Working Group (NCI-WG) for the diagnosis of CLL is summarised below (Hallek *et al.*, 2008).

1) **Clonal B-lymphocytosis of $\geq 5x10^9$ / L.**

   Clonality of circulating lymphocytes is confirmed by flow cytometry.

2) **Immunophenotype features**

   Co-expression CD5 and B-cell surface antigens CD19, CD20 and CD23. Low level expression of CD20, CD79b and surface immunoglobulin. Each clone of leukaemia cells is restricted to expression of kappa or lambda immunoglobulin light chains.

3) **Other tests**

Molecular genetics, ZAP-70, CD38 and IgV_H, Serum markers, Marrow examination

CD5	Positive
CD23	Positive
CD79b	Weak
SmIg	Weak
FMC-7	Negative

Table 1.2 CLL Score based on immunophenotyping (Matutes *et al.*, 1994)

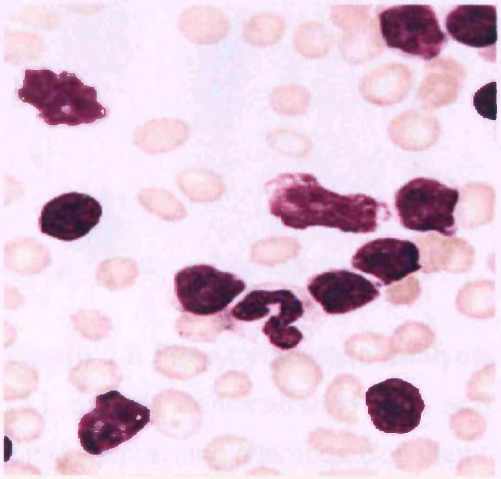

Figure 1.4 Blood film appearance of CLL (X40)

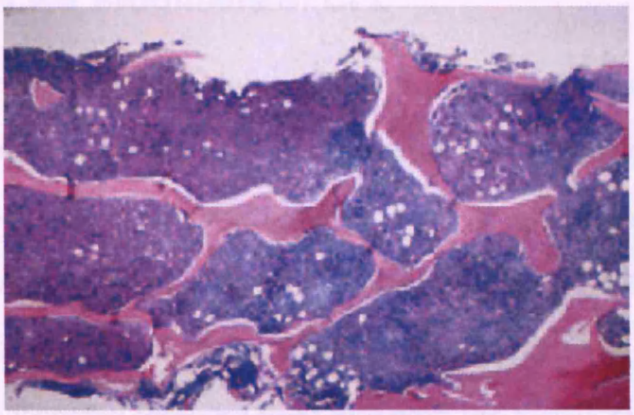

Figure 1.5 Bone marrow appearance showing nodular infiltration by CLL cells (X10) (adapted from Postgraduate Haematology; Hoffbrand *et al.*, 2005)

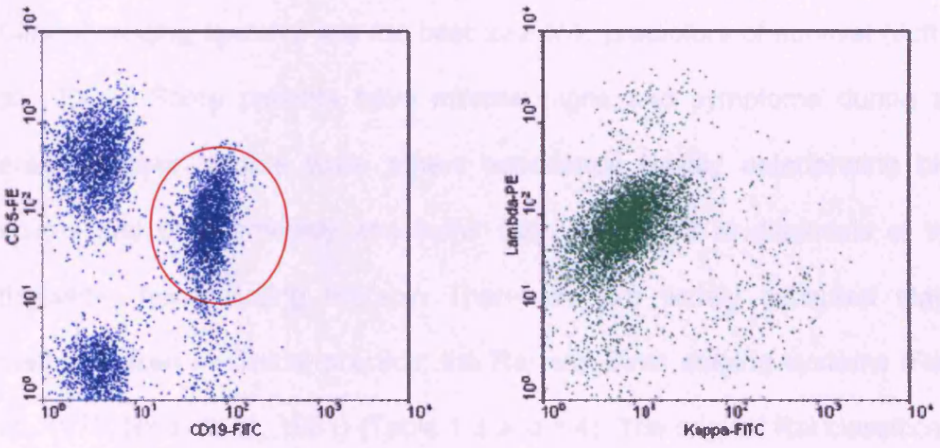

Figure 1.6 Flow cytometric analysis of CLL. Flow cytometric analysis of a CLL sample demonstrating CD5 and CD19 double positive CLL cells (red circle) expressing a lambda clone light chain restriction.

1.2.6 Prognosis of CLL

CLL manifests a very heterogeneous clinical course with some patients having normal age-adjusted survival whereas the median survival for those patients with advanced stage disease is only 3 years (Lee *et al.*, 1987). According to recent studies absolute 5 year survival in CLL is 50% - 60% (Catovsky et al., 2007; Brenner *et al.*, 2008). Clinical parameters as well as a wide array of laboratory tests are used to predict the tumour burden and natural course of the disease at diagnosis (Grever *et al.*, 2007). An overview of these parameters is out lined below.

1.2.6.1 Clinical Stage

Clinical staging systems are the best available predictors of survival (Jaffe *et al.*, 2001). Some patients have minimal signs and symptoms during their entire disease course while others experience rapidly deteriorating blood counts and organomegaly and suffer from symptoms at diagnosis or soon thereafter necessitating therapy. There are two widely accepted staging methods used in clinical practice; the Rai and Binet staging systems (Rai *et al.*, 1975; Binet *et al.*, 1981) (Table 1.3 and 1.4). The original Rai classification was subsequently modified to limit the number of prognostic groups (Rai *et al.*, 1987). These systems define early (Rai 0, Binet A), intermediate (Rai I/II, Binet B) and advanced (Rai III/IV, Binet C) stage disease with median estimated survival times of >10 years, 5-7 years, and 1-3 years respectively. Clinical staging solely relies on physical examination and standard laboratory tests and is simple, inexpensive and can be applied worldwide. These staging systems remain the cornerstone on which decisions regarding medical follow-

up and treatment are built, but one of their major problems is their failure to predict progression of the disease particularly in early stages and hence there has been continual effort to identify other prognostic factors.

Low Risk (Former Rai 0)	Leukaemia cells in blood and/ bone marrow
Intermediate Risk (Former Rai I and II)	Lymphocytosis, lymphadenopathy, splenomegally and/ or hepatomegally
High Risk (Former Rai III and IV)	Haemoglobin <11g/dL or Platelets < 100 $x10^9$ /L

Table 1.3 Modified Rai Staging system

Stage A	≤2 of involved lymph node areas*
Stage B	>2 of involved lymph node areas
Stage C	Haemoglobin <10g/dL or Platelets <100 $x10^9$/ L

Table 1.4 Binet Staging system

*Lymph node areas: head and neck, including Waldeyers ring, axillary, groin, including superficial femoral, palpable spleen, liver (clinically enlarged)

1.2.6.2 Lymphocyte doubling time (LDT)

LDT, a clinical measure that addresses the kinetics of cell growth, is calculated by determining the number of months the absolute lymphocyte count (ALC) takes to double. Patients with an LDT of less than 12 months (6 months in some studies) have been shown to have a shorter survival (Montserrat *et al.*, 1986; Figure 1.7). Furthermore, LDT has been confirmed as an independent predictor of disease progression (Molica *et al.*, 1987; Vinolas *et al.*, 1987)

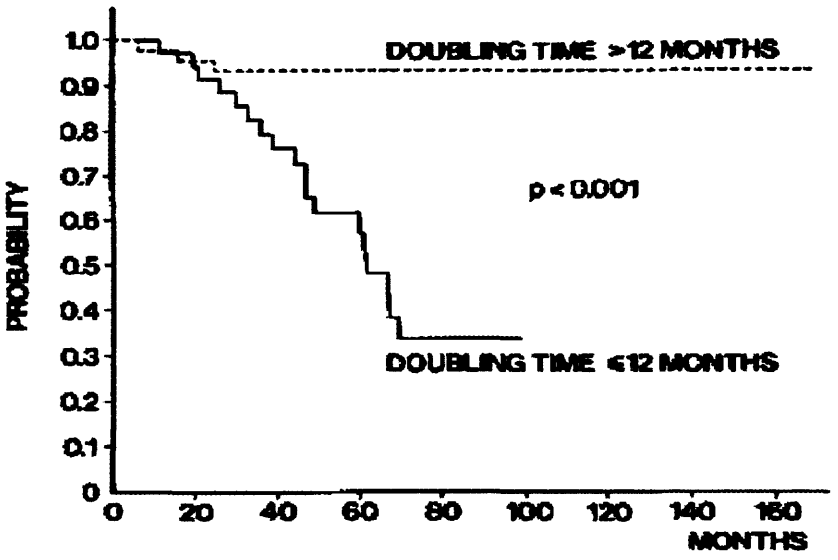

Figure 1.7 LDT and survival in CLL (adapted from Montserrat *et al.*, 1986)

1.2.6.3 Chromosomal aberrations

Fluorescence *in-situ* hybridization (FISH) can identify genomic aberrations in approximately 80% of CLL cases (Dohner *et al.*, 2000; Zenz *et al.*, 2007). The most frequent aberrations are deletions in 13q14, 11q or 17p and trisomy 12

(Dohner *et al.*, 2000; Krober *et al.*, 2002, Mellstedt *et al*, 2005). The 17p and 11q deletions occur almost exclusively in unmutated sub-groups and are associated with inferior prognosis in CLL patients. Apart from providing insight into the pathogenesis of the disease and defining prognostic subsets, genomic aberrations identify subgroups of patients with distinct clinical features for example 11q- is associated with lymphadenopathy. Deletions in 11q and particularly 17p are associated with rapid disease progression and inferior survival (Grever *et al.*, 2007) and there is increasing evidence that detection of certain chromosomal deletions denote resistance to standard chemotherapy. Patients with a deletion in 17p show resistance to standard chemotherapy regimens using alkylating drugs and purine analogues (Dohner *et al.*, 1995; Grever *et al.*, 2007). Therefore detection of these cytogenetic abnormalities has obvious prognostic value and may even influence therapeutic decisions.

1.2.6.4 Novel biological and molecular markers

Recent technological advancements have enabled investigators to examine a wider array of biological and molecular markers in an attempt to identify differences in disease biology that may predict those patients most likely to develop advanced-stage disease and who may benefit from alternative treatment strategies. Studies have shown that ZAP-70 expression, CD38 expression, mutational status of IgV_H genes and cytogenetic abnormalities can predict the prognosis in CLL.

1.2.6.5 Somatic mutation in the immunoglobulin heavy chain variable region (IgV_H)

The mutational status of the immunoglobulin variable region genes (IgV_H) has been shown to have a prognostic value in CLL (Damle *et al.*, 1999; Hamblin *et al.*, 1999). In addition, differential gene expression patterns characteristic for these genetically defined CLL subtypes have been reported (Haslinger *et al.*, 2004). Cases with somatically mutated IgV_H genes have been consistently shown to have a better prognosis than those without mutations in IgV_H genes (Krober *et al.*, 2002; Oscier *et al.*, 2002,). Initial reports indicated the median survival of mutated cases to be 293 months while those with unmutated genes was 117 months. However, cases that utilise segments V_H 3-21 may confer a poor prognosis regardless of mutation status (Thorselius *et al.*, 2006; Tobin *et al.*, 2004b).

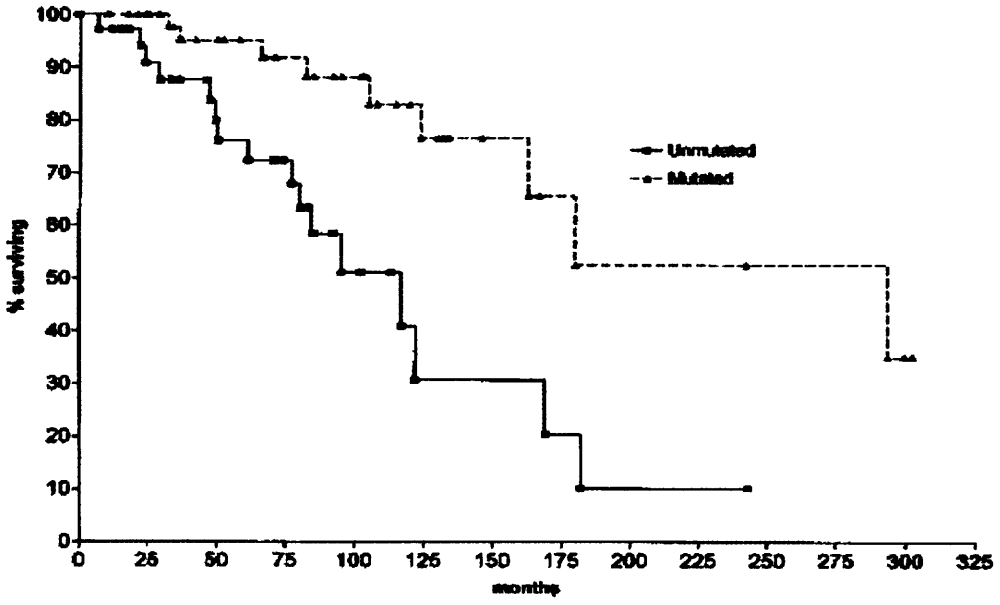

Figure 1.8 Overall survival according to IgV_H mutation status (adapted from Hamblin *et al.*, 1999)

1.2.6.6 CD38 expression

The percentage expression of CD38 has been shown to be an independent prognostic marker and correlates with advanced stage of disease, poor responsiveness to chemotherapy, a shorter time to first treatment, progression rate following first-line therapy and a shorter survival time (Ibrahim *et al.*, 2001; Morabito *et al.*, 2002; Ghia *et al.*, 2003). It is considered to be a surrogate marker of IgV_H mutation status although this is controversial (Hamblin *et al.*, 2002) and is widely used for prognostication in its own right. However, there is an association between CD38 and soluble CD23, β2 microblobulin (β2M) and lymphocyte doubling time (LDT) (Heintel *et al.*, 2001) and more recently a number of groups have shown an association between CD38 and proliferation markers (Damle *et al.,* 2007; Lin *et al.,* 2008). The best cut-off point for CD38 expression is still debatable. The most widely used cut-off points in clinical studies are 7%, 20% and 30% (Thornton *et al.*, 2004; Ibrahim *et al.*, 2001; Damle *et al.*, 1999). Also there some studies indicating predictive value of CD38 expression is enhanced by measurement of antigen density in terms of antibody binding capacity rather than as the percentage of cells expressing the antigen (Mainou-Fawler *et al.*, 2004).

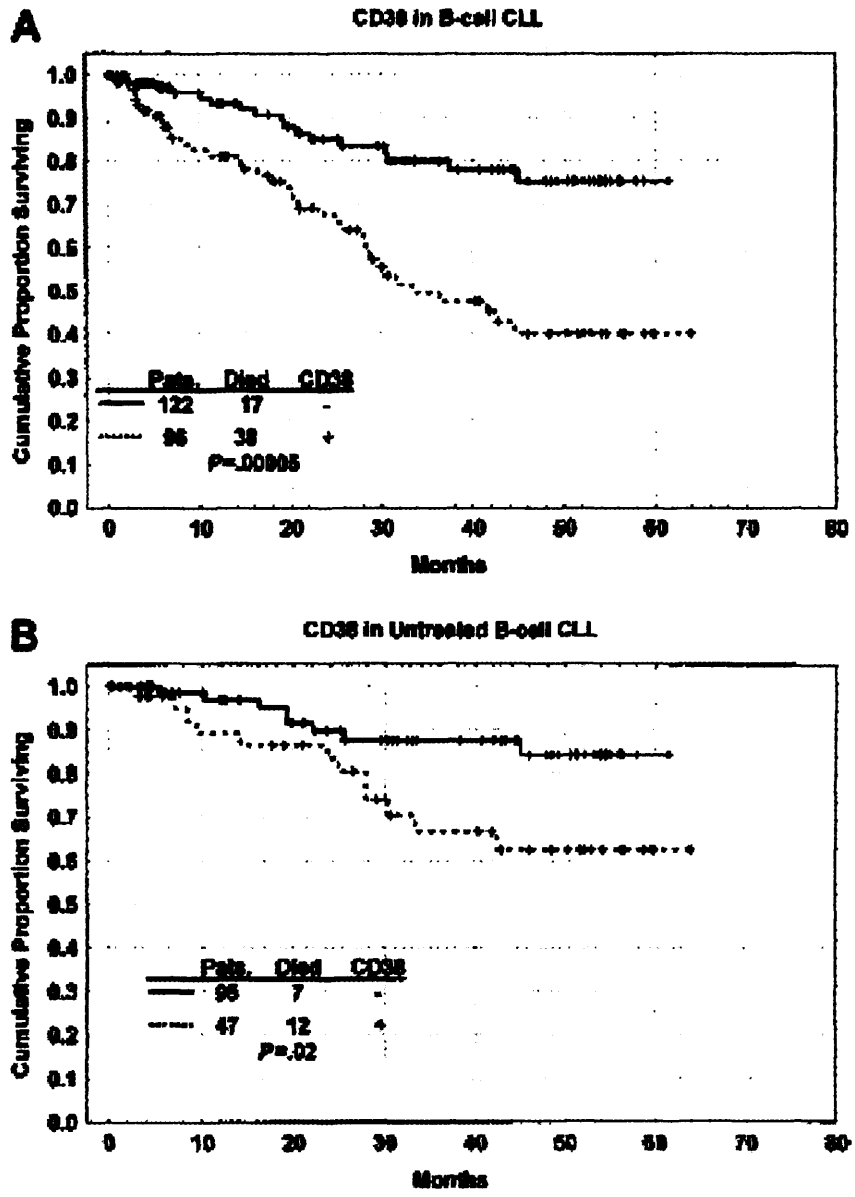

Figure 1.9 Survival and CD38 status (adapted from Ibrahim *et al.*, 2001)

1.2.6.7 ZAP-70 expression

ZAP-70 is part of signal transduction cascade in T-cells following T-cell receptor activation but is not usually expressed in normal B-cells (Qian *et al.*, 1997). Although its role in CLL is not yet clear, it has been shown to increase

the effectiveness of B-cell signalling (Chen *et al.*, 2002; 2005) but this would appear to be independent of its kinase activity (Chen et al., 2008). It also appears to enhance migratory and tumour survival responses and contribute to aggressive clinical behaviour (Richardson *et al.*, 2006; Zanotti *et al.*, 2007). Microarray analysis of CLL revealed that transcription of ZAP-70 was significantly higher in patients with unmutated IgV_H genes compared to patients with mutated IgV_H genes. It was therefore suggested that ZAP-70 could be a surrogate marker for unmutated patients (Rosenwald *et al.*, 2001). ZAP-70 was first described as a promising prognostic marker in CLL by Crespo *et al.*, in 2003 and was shown to possess a strong correlation with mutational status (Crespo *et al.*, 2003; Orchard *et al.*, 2004).

ZAP-70 can be assessed by reverse transcriptase polymerase chain reaction, Western blotting, immunohistochemistry and flow cytometric methods (Crespo *et al.*, 2003; Orchard *et al.*, 2004; Rassenti *et al.*, 2004). Although there are issues surrounding the optimal method for assessing ZAP-70 expression for clinical purposes, flow cytotometry-based methods are the most widely used (Letestu *et al.*, 2006, Gachard *et al.*, 2008). ZAP-70 expression by immunohistochemistry showed significant correlation with advance Binet stages B and C, diffuse bone marrow infiltration, β2M and LDT<12 (Zanotti *et al.*, 2007). Currently ZAP-70 is not a routine test in clinical practice and further clinical trials are needed to standardise the method of assessment and determine whether it should affect the management of patients with CLL.

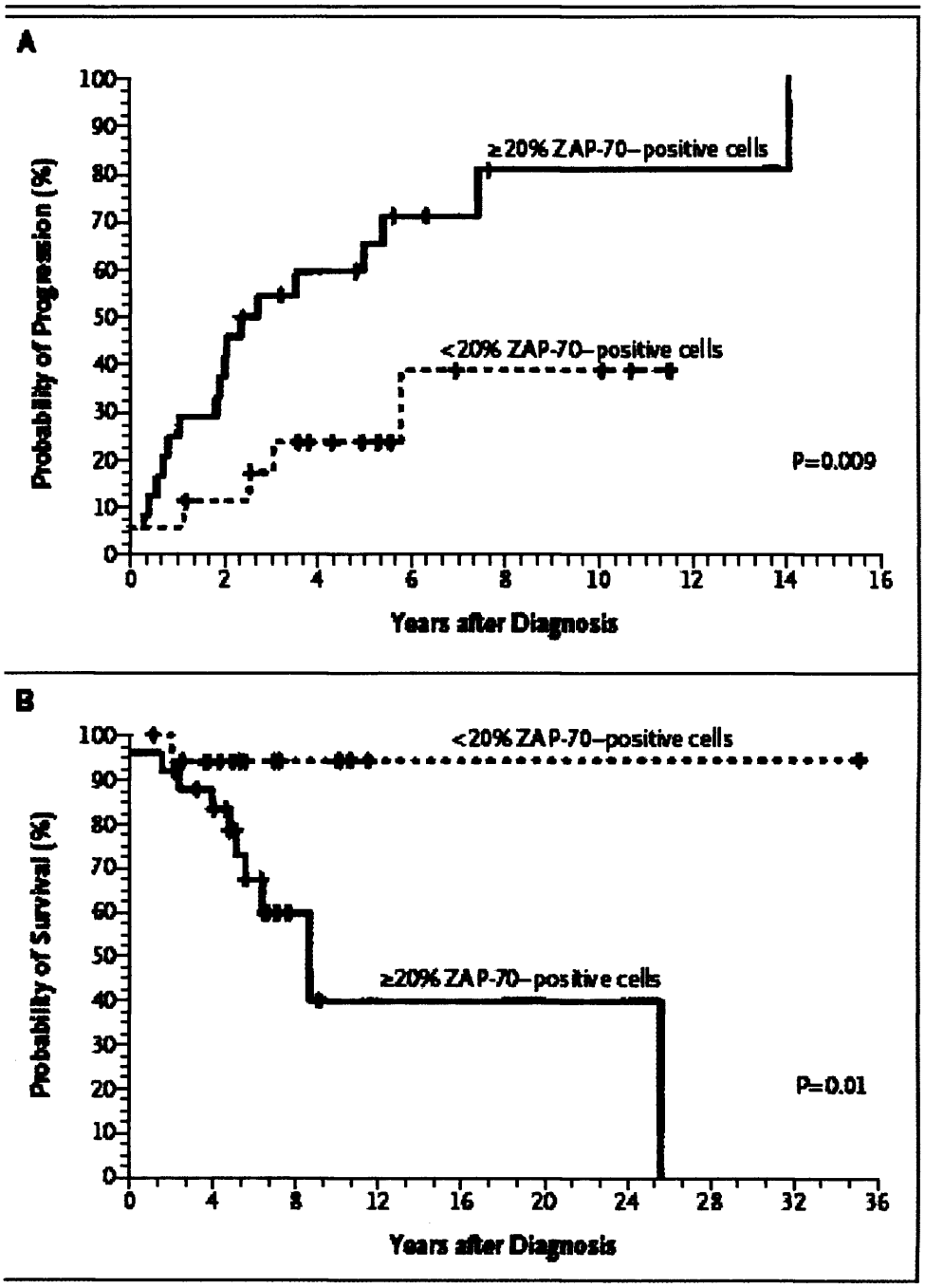

Figure 1.10 Survival and ZAP-70 expression in CLL (adapted from Crespo *et al.*, 2003)

1.2.6.8 Other prognostic markers

The list of other prognostic markers under investigation in CLL is long. These markers should only be used in clinical trials until their validity is proven. Several studies have found serum markers CD23, thymidine kinase and β2M may predict progression-free survival but how these markers can be factored into the clinical management of CLL is yet to be established (Reinisch *et al.*, 1994; Hallek *et al.*, 1999; Sarfati *et al.*, 1996; Magnac *et al.*, 2003; Wierda *et al.*, 2007).

1.2.7 Treatment of CLL

1.2.7.1 Active disease (disease progression)

Recent reports indicate that approximately 50% of patients with early-stage CLL experience rapidly progressive disease and require therapy. Furthermore, these patients have a median survival period that is significantly shorter than that suggested by the original publications of the Rai and Binet staging systems. Most patients diagnosed as having CLL, including the majority of patients with early-stage disease, die of CLL or its complications, despite aggressive treatment. Disease progression is defined according to the criteria described by the National Cancer Institute Working Group (NCI-WG) in 2008 (Hallek *et al.*, 2008). Patients are considered to have an active disease if there is:

1) Evidence of progressive marrow failure

2) Massive or progressive splenomegally

3) Massive or symptomatic lymphadenopathy

4) Progressive lymphocytosis with an increase of more than 50% over two month period or LDT <6 months
5) Autoimmune anaemia or thrombocytopenia poorly responsive to corticosteroids
6) Disease related symptoms like loss of weight

1.2.7.2 Indications for treatment in CLL

As there is no curative therapy for CLL and all drugs have side-effects, the decision to initiate therapy is an important consideration in the management of CLL. It has been shown that treatment of early stage disease does not prolong survival and about one third of patients do not progress and will never need any treatment (Shustik *et al.*, 1988; Richardson *et al.*, 2006). Well-established criteria have been developed by the NCI-WG for the treatment of patients with CLL and these include patients with Binet stage C disease as well as patients with stage A or B disease with features of disease progression (Hallek *et al.*, 2008). Treatment decisions also need to include consideration of the patient's own wishes, their age and performance status.

1.2.7.3 Treatment of CLL

Most patients do not receive treatment immediately after initial diagnosis unless they present with clear pathological symptoms. Indeed, it has been shown that treating CLL patients with indolent disease does not prolong survival (Shustic *et al.*, 1988; Montserrat *et al.*, 1991; Dighiero *et al.*, 1998). Treatment of patients with CLL have been improved over the last few years due to advances in our understanding of the biology of the disease. Response

rates to most conventional chemotherapeutic agents differ according to the clinical and biological characteristics of the disease. Furthermore, response rates to treatment and survival are better for women than men (Catovsky *et al.*, 1989) although the precise reasons for this remain obscure. Recent data suggests that treatment should be tailored to the genetic features of each patient as these can predict the outcome of fludarabine-based regimens (Van Bockstaele *et al.*, 2008). Pharmacological therapy may consist of chemotherapy or chemo-immunotherapy. Chemotherapy can be delivered as monotherapy or combination therapy involving glucocorticoids, alkylating agents and purine analogues. In addition, there are a large number of novel agents currently in clinical development that may be used on their own or in combination with conventional chemotherapy.

1.2.7.4 Single agent therapy

a) Alkylating agents

The alkylating agent chlorambucil has shown response rates between 47% and 71% and had been the mainstay of chemotherapy of CLL for many decades (Sawitsky *et al.*, 1977; Montserrat *et al.*, 1985; Hansen *et al.*, 1988; Catovsky *et al.*, 2007). Chlorambucil can be administered as either a continuous or an intermittent single agent regimen or in combination with other alkylator agents. However, chlorambucil either alone or in combination has never demonstrated any difference in progression-free survival (PFS) or overall survival (OS) in clinical trials (Montserrat *et al.*, 1985; Hansen *et al.*, 1988; Raphael, *et al.*, 1991).

b) Purine nucleoside analogues

Fludarabine, cladribine and pentostatin have been shown to be active in the treatment of CLL (Saven *et al.*, 1995; Robak *et al.*, 2000a; 2000b). Fludarabine treatment gives better overall responses (OR) and complete responses (CR) compared with chlorambucil though again no study has ever shown a survival benefit (Keating *et al.*, 1991; Johnson *et al.*, 1996; Byrd *et al.*, 2000; Wierda *et al.*, 2006; Catovski *et al.*, 2007).

c) Monoclonal antibodies

Rituximab (anti-CD20) and alemtuzumab (anti-CD52) are the most commonly used monoclonal antibodies at the moment. Although only possessing limited activity as a single agent, Rituximab is used in many combination chemotherapeutic regimens with successful results (Byrd *et al.*, 2001; Huhn *et al.*, 2001; O'Brien *et al.*, 2001a). Alemtuzumab is primarily used in relapsed / refractory patients with mainly bone marrow disease and patients with p53 deletions (Lundin *et al.*, 2002; Robak *et al.*, 2008).

1.2.7.5 Combination therapy

The unique mode of action of fludarabine, which affects DNA synthesis including DNA repair, opened up the possibility of potentiating its effects in combination with other drugs. Fludarabine inhibits excision repair of DNA inter-stand cross links induced by cyclophosphamide (Yamauchi *et al.*, 2001) and has been shown to synergise in laboratory experiments (Bellosillo *et at.*, 1999; Laurenti *et al.*, 2008). A research team from the MD Anderson Cancer

Centre in Houston, Texas (O'Brien *et al.*, 2001b) showed in a clinical trial that the combination of fludarabine (F) with cyclophosphamide (C) was effective in previously untreated patients with CLL. This combination was also assessed in a large scale clinical trial in UK (Catovsky *et al.*, 2007). According to this trial, there were better complete and overall response rates with fludarabine plus cyclophosphamide than fludarabine or chlorambucil alone but still there was no significant difference in overall survival.

Laboratory studies have shown fludarabine and rituximab can compliment each other. Rituximab sensitized leukaemic cells to fludarabine-induced apoptosis by down regulating the anti-apoptotic protein Bcl-2 (Alas *et al.*, 2001). In addition, fludarabine down-modulated the expression of complement resistance proteins CD46, CD55, CD59 on malignant B-cells and sensitized them to rituximab-induced complement dependent cytotoxicity (Di Gaetano *et al.*, 2001). This was translated in to improved patient responses as shown in clinical trials; fludarabine, cyclophosphamide in combination with rituximab was highly effective for the treatment of CLL (Keating *et al.*, 2005; Wierda *et al.*, 2005; Tam *et al.*, 2008). However, fludarabine plus cyclophosphamide (with or without Rituximab) cannot be recommended for poor-risk patients characterised by 17p (p53) deletion, since they are unlikely to respond well to this combination and show severe haematological toxicity (Kay *et al.*, 2007).

Alemtuzumab has been shown to be effective in combination with other chemotherapeutic agents (Elter *et al.*, 2005). It has been used as first line therapy in p53 deleted patients, for consolidation of fludarabine-based

treatment or subsequent therapy in refractory disease in high risk patients (Lundin *et al.*, 2002; Stilgenbauer *et al.*, 2002; Lozanski *et al.*, 2004).

1.2.7.6 Stem cell transplantation

Autologous or allogeneic stem cell transplantation is considered as another treatment option for patients with resistant / refractory disease (Paneesha *et al.*, 2005; Dreger *et al.*, 2007). In some instances, such as in young patients with progressive disease with 17p deletion /dysfunction, allogeneic transplantation may be considered upfront due to the lack of effective chemotherapeutic options. However, it is generally accepted that transplantation in CLL should be carried out in the context of a clinical trial. The consensus opinion is that allogeneic transplantation should be considered in younger patients with non-responsive or early relapse disease and patients with a p53 abnormality requiring treatment.

1.3 Transcription factor Nuclear Factor kappa B (NF-κB)

1.3.1 Transcription factors

The term "transcription factor" describes a class of biological molecules that has the capacity to alter gene transcription. Typically, these proteins contain a domain capable of binding DNA and a domain involved in protein-protein interaction to allow the transcription factors to increase gene expression. The mechanisms for increasing gene expression can include the recruitment of the basal transcription machinery, thus increasing RNA synthesis, and the recruitment of other enzymes that allow better physical access to DNA, for example, those involved in chromatin remodelling (Brennan *et al.*, 2008).

1.3.2 Nuclear Factor kappa B (NF-κB)

NF-κB is a collective name for a group of inducible homo- and heterodimeric transcription factors made up of members of the reticuloendotheliosis family (Rel family) of DNA binding proteins. In humans this family is comprised of Rel A (also known as p65), Rel B, c-Rel (also known as Rel), p50 (a processing product of p105, both of which are known as NF-κB1) and p52 (a processing product of p100, both of which are known as NF-κB2). All family members contain a highly conserved amino- terminal of approximately 300 amino acids, rel homology domain (RHD) which is important for DNA binding, dimerisation and nuclear localisation (known as nuclear localisation signal; NLS)(Perkins *et al.*,1999). But only c-Rel, Rel A and Rel B contain a transactivation domain in their carboxy terminal which contains a sequence that is required for

interaction with the transcriptional apparatus and for transcriptional activation (Perkins *et al.*, 1999; Schmid *et al.*, 2008). In their inactive state most transactivating NF-κB dimers are bound to inhibitory molecules (IκB family) (Ghosh *et al.*, 1998; Karin *et al.*, 2002). The IκB family consists of classical IκBs (IκBα, IκBβ, and IκBε), the NF-κB precursors (p100 and p105) and the unusual IκBs (BCL3, IκBξ and IκBNS) (Nolan *et al.*, 1991; Hayden *et al.*, 2004). These inhibitory molecules possess ankyrin domains containing five to seven ankyrin repeat sequences which function by binding and inhibiting RHDs through masking NLSs and hence preventing their nuclear translocation (Ghosh *et al.*, 1998). IκBα also blocks DNA binding and promotes nuclear export of DNA bound dimers (Tam *et al.*, 2000; Lee *et al.*, 2001; Schmid *et al.*, 2008).

Many genes are activated only by a subset of NF-κB proteins, while others are only expressed in certain cell types. In addition, NF-κB proteins interact with other DNA-binding proteins and associate with non DNA-binding coactivator proteins and components of the basal transcription complex. It is the differential expression of Rel proteins, their ability to heterodimerise with different family members and the interaction of these proteins with different components of the transcription apparatus that contribute to the diverse effects following activation of the NF-κB pathway (Perkins *et al.*, 1999).

NF-κB is activated by signalling through many receptors involving classical (IKKβ and IKKγ dependent activation of NF-κB or canonical pathway) and alternate (IKKα and NF-κB inducing kinase, NIK dependent or non-canonical pathway) pathways (Hoffman *et al.*, 2006a; 2006b). Also, there are other less

well identified pathways releasing limited amounts of NF-κB (tyrosine phosphorylation-induced dissociation of IKBs and casein-kinase-2-induced increased turnover of IκBs) (Hoffman *et al.*, 2006b). These receptors can be grouped in to two classes. The first, receptors that mainly activate the classical pathway which include the B-cell receptor (BCR), the T-cell receptor (TCR), tumour necrosis factor receptor (TNFR), interleukin-1 receptor (IL-1R) and members of toll-like receptor family (TLR). The second, receptors that activate both classical and alternate pathways which include lymphotoxin-β receptor (LT-βR), receptor activator of NF-κB (RANK), CD40 and B-cell activating factor receptor (BAFFR). After they have been released from their inhibitory partners, NF-κB dimers translocate to the nucleus, bind their DNA-binding sites and induce transcription of target genes (Ghosh *et al.*, 1998; Karin *et al.*, 2002). This in turn leads to the synthesis of proteins through alteration of the pattern of gene expression (Sen *et al.*, 1986). Figure 1.11 shows a simplified schematic diagram of the NF-κB activation pathway.

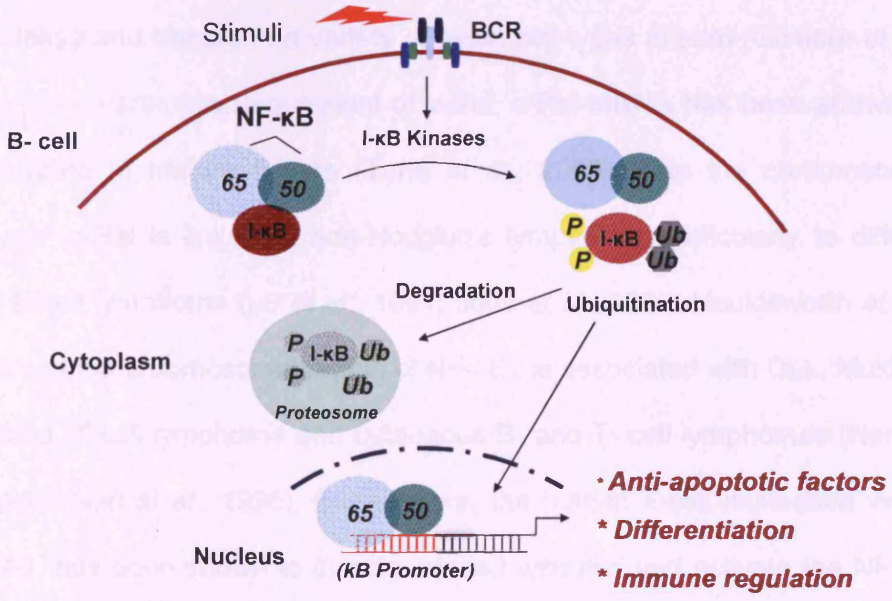

Figure 1.11 Schematic representation of NF-κB activation.

1.3.3 NF-κB and Cancer

Although the maintenance of appropriate levels of NF-κB activity is a critical factor in achieving normal cellular proliferation, constitutive NF-κB activation is likely involved in the enhanced growth properties seen in a variety of cancers (Karin *et al.*, 2006a; 2006b). Tumourogenesis requires self sufficiency in growth signals, insensitivity to growth inhibition, evasion of apoptosis, sustained angiogenesis, tissue invasion and metastasis (Hanahan *et al.*, 2000). NF-κB proteins regulate genes controlling most of these processes particularly apoptosis (Beg *et al.*, 1996), proliferation (Cao *et al.*, 2001), angiogenesis (Koch *et al.*, 1992) and metastasis (Wang *et al.*, 1999).

The initial evidence for the association between Rel proteins and cancer came from v-Rel, an oncoprotein belonging to the NF-κB transcription factor family produced by avian reticuloendotheliosis virus T (REV-T), oncovirus causing rapidly fatal lymphomas and leukaemia in chickens and its ability to immortalise and transform a variety of avian cell types *in vitro* (Gilmore *et al.*, 1999). The mammalian equivalent of v-Rel, c-Rel mRNA has been shown to be elevated in mature B-cells (Tarte *et al.*, 2003). Also the chromosomal region of c-Rel is linked to non-Hodgkin's lymphoma, particularly to diffuse large B-cell lymphoma (Lu *et al.*, 1991; Joos *et al.*, 1996; Houldsworth *et al.*, 1996) and the chromosomal region of NF-κB2 is associated with CLL, Multiple myeloma, T-cell lymphoma and cutaneous B- and T- cell lymphomas (Neri *et al.*, 1991; Neri *et al.*, 1995). Furthermore, the human T-cell leukaemia virus, HTLV-1, has been shown to directly interact with IKK and activate the NF-κB signalling pathway (Xiao *et al.*, 2001).

1.3.4 NF-κB and CLL

CLL cells have been reported to exhibit high constitutive NF-κB activation compared to normal B-lymphocytes (Furman *et al.*, 2000; Cuni *et al.*, 2004; Tracey *et al.*, 2005). Whilst the exact factors responsible for the constitutive expression of NF-κB are not fully resolved, many factors including Akt activation, BCR signalling, CD40 ligation, IL-4 and BAFF have been shown to increase NF-κB activity and enhance CLL cell survival with members of the Bcl-2 family being principal transcriptional targets (Dancescu *et al.*, 1992; Barragan *et al.*, 2002; Zaninoni *et al.*, 2003; Kern *et al.*, 2004). Furthermore, currently used chemotherapeutic agents can induce NF-κB activation as an unwanted side effect which confers apoptosis suppression and hence resistance to these drugs (Tergaonkar *et al.*, 2002; Nakanishi *et al.*, 2005).

1.3.5 Inhibition of NF-κB in haematological malignancies

Numerous inhibitors of NF-κB are under development, but because of the widespread importance of this factor to normal tissues it has been difficult to develop NF-κB inhibitors that act specifically on cancer cells (Yamamoto *et al.*, 2001; Braun *et al.*, 2006). Therefore, a better understanding of NF-κB regulation is crucial for therapeutic approaches to specifically target cancer cells. Intrinsic NF-κB activation as well as its upstream and downstream regulator subunits and NF-κB target genes could be targeted in the process of developing inhibitors to dampen NF-κB expression (Braun *et al.*, 2006).

A number of well established dietary chemo-preventative compounds have been shown to inhibit NF-κB (Yamamoto *et al.*, 2001). The most common of

these is red wine which has been shown to inhibit NF-κB activity and induce apoptosis in transformed cells (Holmes-McNary *et al.*, 2000). Also, a variety of drugs commonly used in clinical medicine have been shown to possess NF-κB suppressive potential; glucocorticoids, non-steroidal anti-inflammatory drugs, Cyclosporine A, Tacrolimus, Rituximab, Thalidomide and Proteosome inhibitors are just a few from the long list (Braun *et al.*, 2006).

There is a published list of NF-κB inhibitors from the literature divided in to 4 main categories (http://people.bu.edu/gilmore/nf-kb/index.html) (Gilmore *et al.*, 2006a; 2006b): anti-oxidants that have been shown to inhibit activation of NF-κB, proteasome and protease inhibitors that inhibit Rel/NF-κB, IkBα phosphorylation and/or degradation inhibitors and miscellaneous inhibitors of NF-κB. These distinct categories reflect diverse strategies and show the different research avenues available in the search for inhibitors of the NF-κB pathway.

Bortezomib (PS-341), Thalidomide and Rituximab are widely used for the treatment of haematological malignancies including CLL. PS-341, a proteosome inhibitor that can inhibit NF-κB activity, has shown consistent anti-tumour activity against chemosensitive and chemoresistant (high NF-κB activity) multiple myeloma cells (Berenson *et al.*, 2001) and promising results in clinical trials for the treatment of multiple myeloma, a disease characterised by constitutive NF-κB activation. In addition, PS-341 is cytotoxic to CLL cells *in vitro* and augments the cytotoxic effect of fludarabine (Duechler *et al.*, 2005). Thalidomide, which inhibits NF-κB activation via suppression of IKK activity, (Keifer *et al.*, 2001; Mitsiades *et al.*, 2002) has shown impressive

results in clinical trials for the treatment of multiple myeloma. The monoclonal antibody rituximab, which targets the surface molecule CD20 was also shown to decrease the phosphorylation of NIK and IκBα, diminish IKK and NF-κB DNA binding activity in NHL cell lines and lead to the sensitisation of these cells to chemotherapeutic drug induced apoptosis (Jazirehi *et al.*, 2005). However, these three molecules (Bortezomib, Thalidomide and Rituximab) exert other actions beyond NF-κB and highly specific inhibitors of NF-κB are still missing from clinical practice. There are potentially more specific NF-κB inhibitors under development which have been tested on cells *in vitro* and in animal models. These include PS-1145 (Lam *et al.*, 2005), BAY 11-7082 (Pickering *et al.*, 2005), Curcumin (Everret *et al.*, 2007), the sesquiterpene lactone parthenolide (Steele *et al.*, 2006) and its analogue DMAPT (Guzman *et al.*, 2007). PS-1145, an IKK inhibitor, has been shown to overcome the growth and survival advantage of B-cell malignancies like multiple myeloma cells (Hideshima *et al.*, 2002; Hideshima *et al.*, 2007) and was also found to be selectively toxic to subtypes of diffuse large B-cell lymphoma cells that are associated with NF-κB activation by down regulating NF-κB dependent genes (Lam *et al.*, 2005). It remains an attractive agent to be tested in other B-cell malignancies like CLL. BAY 11-7082 is a well characterised inhibitor of IKK (Pierce *et al.*, 1997) shown to induce apoptosis in CLL samples with relatively low toxicity to normal B-cells. Induction of apoptosis was associated with cytochrome c release from the mitochondria and caspase 9 activation (intrinsic pathway) suggesting a predominant role of NF-κB in preventing the activation of the intrinsic mitochondrial pathway in CLL (Pickering *et al.*, 2007). In the study by Pickering *et al.*, 2007, another NF-κB inhibitor, Kamebakaurin, a complex natural kaurane dierpene that directly inhibited DNA binding of p50

(Lee *et al.*, 2002) showed comparable results. Curcumin, a diferuloylmethane derived from turmeric, an inhibitor of IKK and NIK/IKK complex was shown to suppress the activation of NF-κB and expression of cyclin D1, Bcl-2 and Bcl-XL; all known NF-κB target genes (Everett *et al.*, 2007). It causes apoptosis in human B-cell malignancies, Hodgkin's lymphoma, multiple myeloma and activated B-cell neoplasms like DLBCL, CLL (Thomas *et al.*, 2005; Everett *et al.*, 2007) and mantle cell lymphoma cell lines (Shishodia *et al*, 2005a; 2005b). In addition, curcumin has been shown to augment the apoptotic effect of fludarabine, dexamethasone and vincristine (Everett *et al.*, 2007).

The sesquiterpene lactone parthenolide (PTL) has been shown to be cytotoxic to cells isolated from patients with CLL with minimal cytotoxicity to normal T-lymphocytes or CD34 (+) haematopoietic progenitor cells (Steele *et al.*, 2006). It was shown to decrease nuclear levels of NF-κB and diminish phosphorylation of its negative regulator IκB. The mechanism of cell killing appears to be via PTL-induced generation of reactive oxygen species, resulting in turn in a conformational change in the pro-apoptotic protein Bax, and the subsequent release of mitochondrial cytochrome c and caspase activation (Steele *et al.*, 2006). Some of the patients used in this study were resistant to the conventional chemotherapeutic drug chlorambucil indicating the importance of identifying new targets in treating this incurable leukaemia. Dimethyl amino parthenolide (DMAPT) was shown to induce rapid death of primary human leukaemic cells from myeloid leukaemia via inhibition of NF-κB, induction of oxidative stress responses and activation of p53 (Guzman *et al.*, 2007).

In summary, NF-κB is an extremely attractive transcription factor to target for the treatment of leukaemia and lymphomas either alone or in combination with other drugs. The development of new agents acting upstream of NF-κB or perhaps agents that target the molecule itself using the strategies described above may offer valuable new anti-cancer drugs in the future.

2.0 MATERIALS AND METHODS

2.1 Tissue culture

2.1.1 Sample collection

Peripheral blood samples and / or bone marrow samples from patients with CLL were obtained with the patients' written informed consent (LREC # 02/4806). CLL was defined by clinical criteria as well as cellular morphology and the co-expression of CD19 and CD5 in lymphocytes simultaneously displaying restriction of light-chain rearrangement. Clinical information including treatment histories was available for all patients and none of the previously treated patients had received chemotherapy within 3 months prior to sample collection.

2.1.2 Isolation of mononuclear cells

Reagents and instruments Used

Histopaque 1077 (Sigma-Aldrich, Dorset, UK)

Phosphate-buffered saline (PBS) pH 7.2 (Sigma-Aldrich, Dorset, UK)

Ammonium chloride 0.87M of (Sigma-Aldrich, Dorset, UK)

Vi-cell XR cell counter (Beckman-Coulter, High Wycombe, Buckinghamshire)

Isolation of mononuclear cells

Peripheral blood was layered onto Histopaque 1077 and centrifuged at 300xg for 30 min. After centrifugation, the opaque interface containing mononuclear

cells was aspirated and transferred into a clean sterile centrifuge tube (Figure 2.1). The mononuclear cells were then washed with PBS at 300xg for 5 min. After washing, the supernatant was aspirated and discarded. The pellet containing mononuclear cells were then washed with 0.87% (w/v) of ammonium chloride solution to lyse contaminating erythrocytes. Cells were then centrifuged at 300xg for 5 min. The supernatant was aspirated and discarded and the pellet was then washed twice with PBS followed by centrifugation at 300xg for 5 min after each washing step. After the final wash, the mononuclear cells were resuspended in PBS and counted using a Vi-cell XR counter. All mononuclear preparations contained at least 95% viable cells according to the Trypan Blue exclusion method.

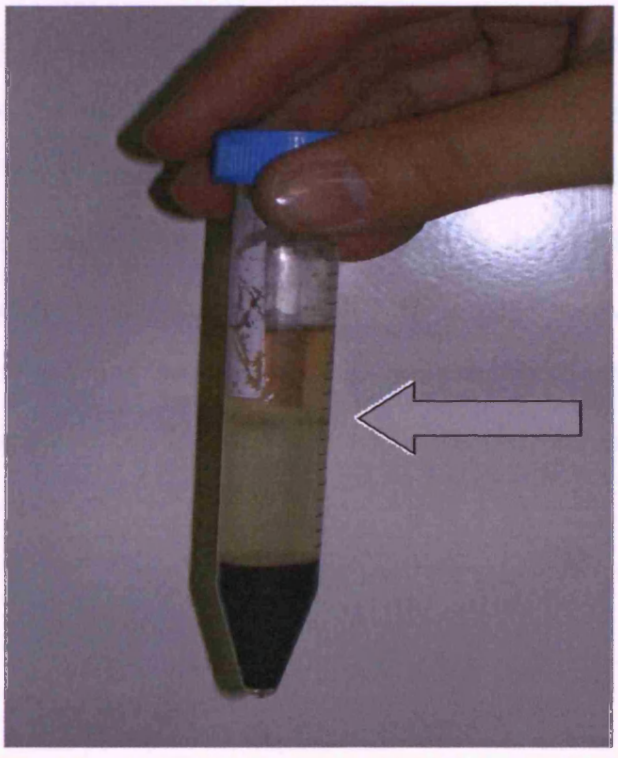

Figure 2.1 Illustration of MNC separations on Histopaque centrifugation

2.1.3 Separation of B- and T-lymphocytes

Reagents and instruments Used

Dynabeads CD19-labelled (Dynal, Invitrogen, Paisley, UK)

Magnetic particle concentrator (MPC, Invitrogen, Paisley, UK)

Separation of B and T-lymphocytes

A volume of 7.5 µl CD19-labelled Dynabeads was used for the isolation of 1×10^6 target cells. The required volume of Dynabeads was added to a 1.5 ml microtube together with 1ml of 2% foetal calf serum in PBS. The microtube was then placed in the magnetic particle concentrator (MPC) for 60 sec. After 60 sec, the fluid was aspirated and the washed Dynabeads were resuspended again in 1ml of 2% foetal calf serum in PBS. The washed Dynabeads were then added to the isolated mononuclear cells and incubated for 30 min at 2-4°C with gentle tilting and rotation. After incubation, the rosetted cells were isolated by placing the microtube in the MPC for 2 min. The supernatant was then aspirated and the microtube was removed from the MPC. The supernatant contained non B-cells while the beads were bound to the B-cell fraction (Figure 2.2).

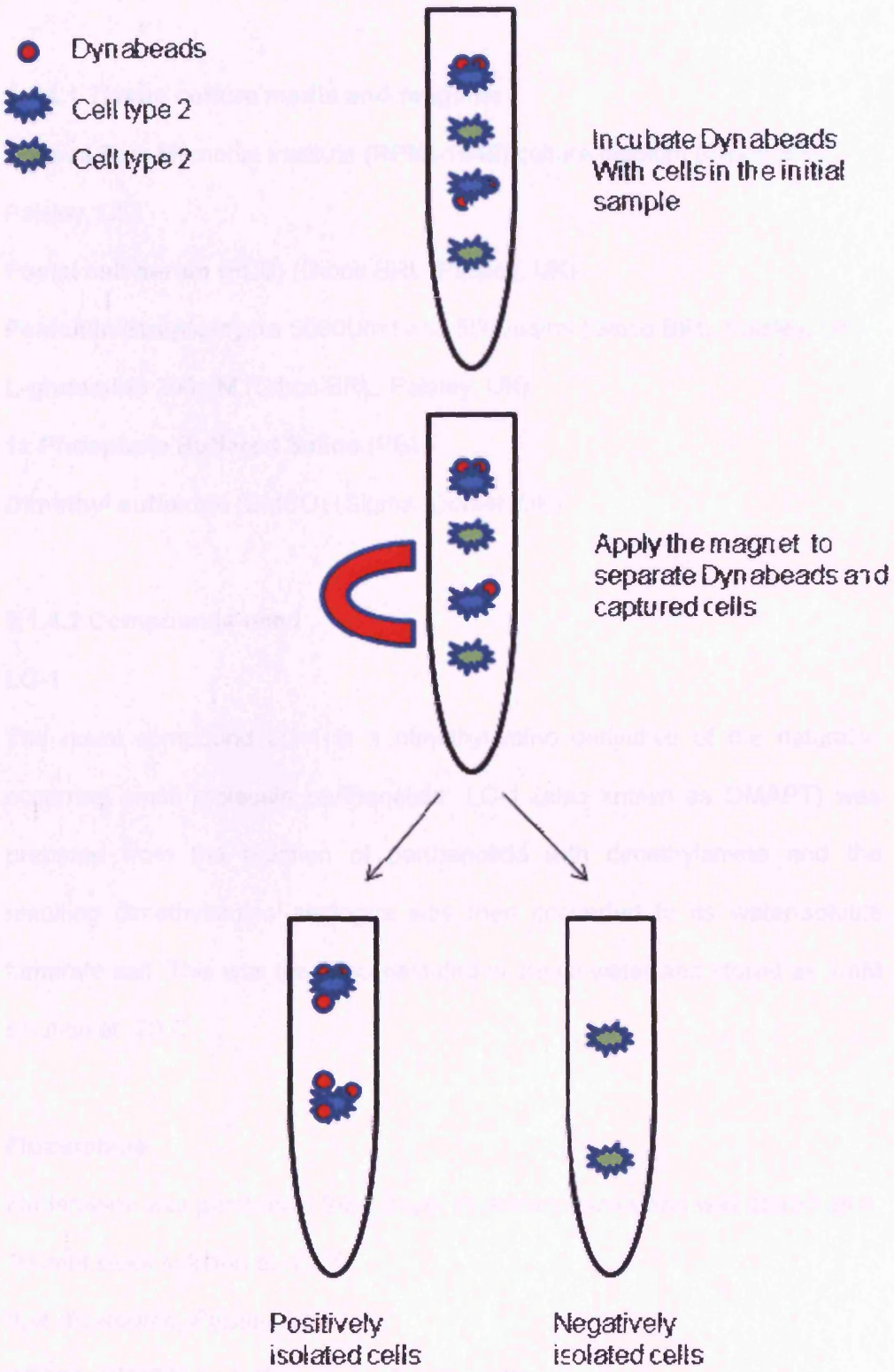

Figure 2.2 Schematic representation of B-cell separation

2.1.4 Tissue culture

2.1.4.1 Tissue culture media and reagents

Roswell **P**ark **M**emorial **I**nstitute **(RPMI-1640)** culture medium (Gibco BRL, Paisley, UK)

Foetal calf serum (FCS) (Gibco BRL, Paisley, UK)

Penicillin/Streptomycin 5000U/ml and 5000µg/ml (Gibco BRL, Paisley, UK)

L-glutamine 200mM (Gibco BRL, Paisley, UK)

1x Phosphate Buffered Saline (PBS)

Dimethyl sulfoxide (DMSO) (Sigma, Dorset, UK)

2.1.4.2 Compounds used

LC-1

The novel compound LC-1 is a dimethylamino derivative of the naturally-occurring small molecule parthenolide. LC-1 (also known as DMAPT) was prepared from the reaction of parthenolide with dimethylamine and the resulting dimethylamino analogue was then converted to its water-soluble fumarate salt. This was then reconstituted in sterile water and stored as 1mM solution at -20 C.

Fludarabine

Fludarabine was purchased from Bayer Pharmaceuticals and was stored as a 100mM stock solution at -20^0 C.

IL-4 (Biosource, Paisley, UK)

CD154 (CD40 ligand) (Biosource, Paisley, UK)

2.1.4.3 Preparation of 1x Phosphate Buffered Saline (PBS)

1 x PBS was made up by dissolving 50 PBS tablets (Sigma Aldrich) in 5 litres of distilled water. 500 ml aliquots were sterilised by autoclaving and stored at room temperature.

2.1.4.4 Preparation of media and cell culture

RPMI media was supplemented with L-glutamine (1%), FCS (10%), penicillin and streptomycin (2%) for all cell cultures. $1x10^6$-$5x10^6$ mononuclear cells were cultured alone or with relevant compounds at 37°C in a 5% CO_2 moist chamber for up to 48 hours. Cells were harvested by centrifugation and analysed by flow cytometry, processed for RNA extraction or sub-cellular fractionation.

2.2 Flow cytometry

Flow cytometry enables the discrimination of distinct cellular populations based on differential light scatter and fluorescence characteristics. The process of flow cytometry involves focusing cells into a unicellular stream of fluid and passing the stream through a laser beam. Specific antibodies against a particular antigen can be conjugated with fluorescent dye and then excited with a laser light. The subsequently emitted light differs in the wave length from the excitation light which enables separation using optical filters. This can be used for the quantification of antigens of interest.

2.2.1 ZAP-70 and CD38 expression

2.2.1.1 Reagents and instruments used

Fix and Perm kit (Reagent A-fixative, Reagent B- permeabilisation) (CALTAG laboratories, Buckingham, UK)

ZAP-70-Alexafluor 488 (CALTAG laboratories, Buckingham, UK)

CD19-Allophycocyanin (CD19-APC) (CALTAG laboratories, Buckingham, UK)

CD38-Phycoerythrin (CD38-PE) (CALTAG laboratories, Buckingham, UK)

Summit 4.3 analysis software was used to analyse data generated from this machine (DAKO, Cambridgeshire, UK)

2.2.1.2 Determination of ZAP-70 and CD38 expression

Lymphocytes were aliquoted ($1x10^6$ cells) in duplicate; control and test samples. For test samples, the cells were stained with the following monoclonal antibodies: CD19-APC (4µl) and CD38-PE (4µl). For control samples, cells were labelled with a dual negative control (4µl) containing IgG_1/ FITC and IgG_1 /RPE (Dako) as well as CD19-APC. Both test and control samples were incubated in a dark place for 10min at room temperature. After incubation, samples were washed with 3-4 ml of PBS and centrifuged at 300xg for 5 min. Then 50µl of Reagent A (fixative) was added and the cells were incubated for 10mins followed by a washing step in PBS and centrifugation at 300xg for 5 min. The supernatant was removed and 50µl of Reagent B (permeabilisation) and ZAP-70 Alexafluor 488 were added only to the test sample and both tubes were incubated for 10 min at room temperature in the dark. Then both samples were washed with PBS and

centrifuged at 300xg for 5 min. The supernatant was discarded and the pellet was resuspended in 500µl of 1% paraformaldehyde. Stained cells were analysed on a FACS Calibur flow cytometer (Becton Dickinson, USA). Data were analysed using Summit 4.3 software (Dako, Ely, UK). The cut-off point for both CD38 positivity and ZAP-70 positivity was ≥20% (Figure 2.3 and 2.4).

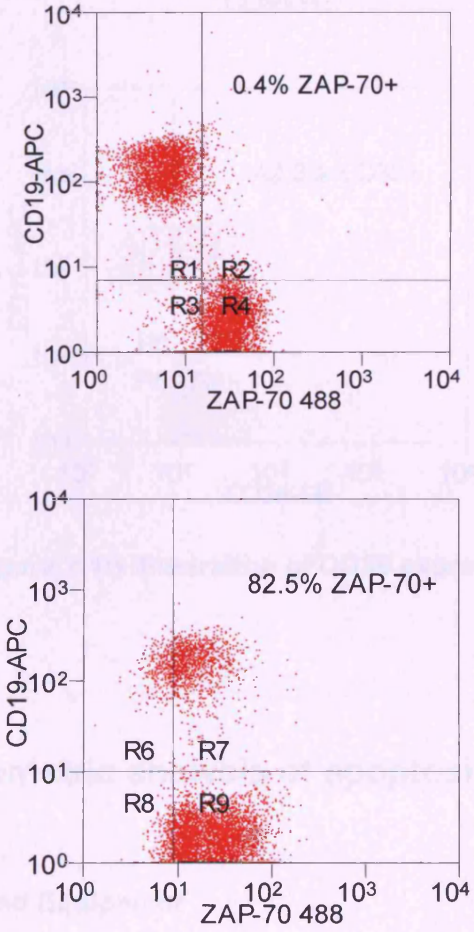

Figure 2.3A and Figure 2.3B illustration of ZAP-70 expression on CLL samples.

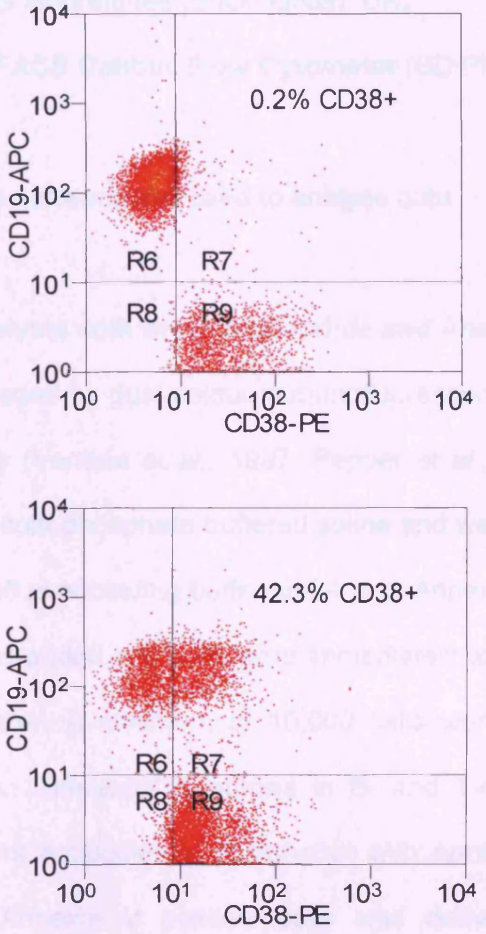

Figure 2.4A and Figure 2.4B illustration of CD38 expression on CLL samples.

2.2.2 Flow cytometric analysis of apoptosis

2.2.2.1 Reagents and Equipment

Propidium Iodide (PI) (CALTAG laboratories, Buckingham, UK)

Annexin V-FITC (Fluorescence isothiocyanate) (CALTAG laboratories, Buckingham, UK)

4 x binding buffer (CALTAG laboratories, Buckingham, UK)

CD3 PE (CALTAG laboratories, Buckingham, UK)

CD19 APC (CALTAG laboratories, Buckingham, UK)

Becton Dickinson FACS Calibur Flow Cytometer (BD Pharmingen, Oxford, UK)

Summit 4.3 analysis software was used to analyse data

2.2.2.2 Viability analysis with propidium iodide and Annexin V

Apoptosis was assessed by dual-colour immunofluorescent flow cytometry as described previously (Vermes *et al.*, 1997, Pepper *et al.*, 2003). $1x10^6$ cells were washed in ice-cold phosphate buffered saline and were incubated for 15 min in the dark in 196 µl of binding buffer and 4 µl of Annexin V-FITC. 10µg/ml propidium iodide was added and cells were immediately analysed on a FACS Calibur flow cytometer. A minimum of 10,000 cells were counted in each sample for analysis. Differential apoptosis in B- and T-cells was analysed using CD3 and CD19 antibodies in conjunction with Annexin V. For B-cells, the proportion of Annexin V positive cells was derived from the total population of $CD3^-/CD19^+$ cells. For T-cells, the proportion of Annexin V positive cells was derived from the total population of $CD3^+/CD19^-$ cells. A minimum of 10,000 events were acquired for each sample.

2.2.3 Flow cytometric analysis of caspase activation

2.2.3.1 Reagents and Equipment

PhiPhiLuxTM G1D2 substrate (Calbiochem, Nottingham, UK)

Caspase-8 inhibitor (ZIETD.fmk) (Calbiochem, Nottingham, UK)

Caspase-9 inhibitor (ZLEHD.fmk) (Calbiochem, Nottingham, UK)

Pan-caspase inhibitor (ZVAD.fmk) (Calbiochem, Nottingham, UK)

2.2.3.2 Analysis of caspase activation

CLL cells were incubated at 37°C in a humidified 5% carbon dioxide atmosphere in the presence of LC-1 (0.5 to 8μM) for up to 48h. Cells were then harvested by centrifugation and labelled with CD19-APC antibody. Subsequently the cells were incubated for 1h at 37°C in the presence of the PhiPhiLux^{TM} G_1D_2 substrate (Calbiochem, Nottingham, UK). The substrate contains two fluorophores separated by a quenching linker sequence that is cleaved by active caspase-3. Once cleaved, the resulting products fluoresce green and can be quantified using flow cytometry. The caspase activation cascade was further analyzed using a caspase-8 inhibitor (ZIETD.fmk, 50μM), a caspase-9 inhibitor (ZLEHD.fmk, 50 μM) and the pan-caspase inhibitor (ZVAD.fmk, 50μM).

2.3 Molecular biology

2.3.1 Preparation of cytosolic and nuclear extracts

2.3.1.1 Reagents and instruments used

Low salt detergent lysis buffer (10mM HEPES pH7.9, 1.5mM $MgCl_2$ and 10mM KCl)

High salt buffer (20mM HEPES pH7.9, 420mM NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA and 25% glycerol)

Storage buffer (10mM HEPES pH7.9, 25mM KCl, 0.1mM EDTA and 10% glycerol)

Phosphatase Inhibitor Cocktails I and II (Sigma, Dorset, UK)

Nonidet P40 (NP40)

Phenylmethosulfonylfluoride (PMSF)

2.3.1.2 Cytosolic and Nuclear extraction

Cytosolic and nuclear extracts were prepared using a method previously described (Brennan *et al.*, 1995). Cytosolic extracts were prepared by lysis of cells for 5 minutes on ice in 100 µl of low salt detergent lysis buffer supplemented with 1mM phenylmethylsulfonylfluoride (PMSF), 1:100 dilutions of Phosphatase Inhibitor Cocktails I and II (Sigma) and 0.1% NP40 detergent. These supplements were added immediately prior to use due to their short half-lives. Following centrifugation (Heraeus Biofuge) (16,600xg for 5 min at 4°C), the supernatant was retrieved representing the cytosolic extract. Nuclear extracts were prepared by incubating the remaining pellet for 15 minutes in 50 µl of high salt buffer supplemented with 1mM PMSF and 1:100 dilutions of Phosphatase inhibitor cocktails I and II immediately prior to use. Following centrifugation (16,600xg for 5 min at 4°C), the supernatant was collected representing the nuclear extract. For extracts prepared specifically for evaluation by EMSA, 50 µl storage buffer was added to the nuclear extract. All extracts were then stored at -80°C.

2.3.2 Quantification of protein concentration

2.3.2.1 Reagents and instruments used

Bovine Serum Albumin (BSA) (Sigma, Dorset, UK)

Protein assay reagent (500-0006) (Bio-Rad Hemel Hempstead, UK)

Microplate reader (170-6850) (Bio-Rad Hemel Hempstead, UK)

The NanoDrop® ND-1000 Spectrophotometer (Labtec international, Wilmington, USA)

2.3.2.2 Protein quantification using Bradford method

Protein concentration was determined using a method developed by Bradford (Bradford M, 1976). Protein concentration standards were generated using 1mg/ml BSA solution. Doubling dilutions of the 1mg/ml BSA solution were prepared in a flat-bottomed 96 well plate. For the generation of a protein standard curve, duplicate 10 µl aliquots of each BSA dilution were used. Two wells containing 10 µl distilled H_2O were included for the generation of the standard curve. A fixed volume (1 µl) of cytosolic or nuclear extract was pipetted into the 96 well plate. Protein assay reagent was diluted 1 in 5 with distilled H_2O and 200 µl was added to each standard and extract. A microplate reader was used to read the absorbance of each well at 570nm. The protein concentration for each extract was first determined by plotting a standard curve (Figure 2.5) using the optical density at 570nm (OD_{570nm}) of all the protein standards on a Microsoft Excel spreadsheet. The concentration of each sample extract was then calculated using the equation from the standard curve.

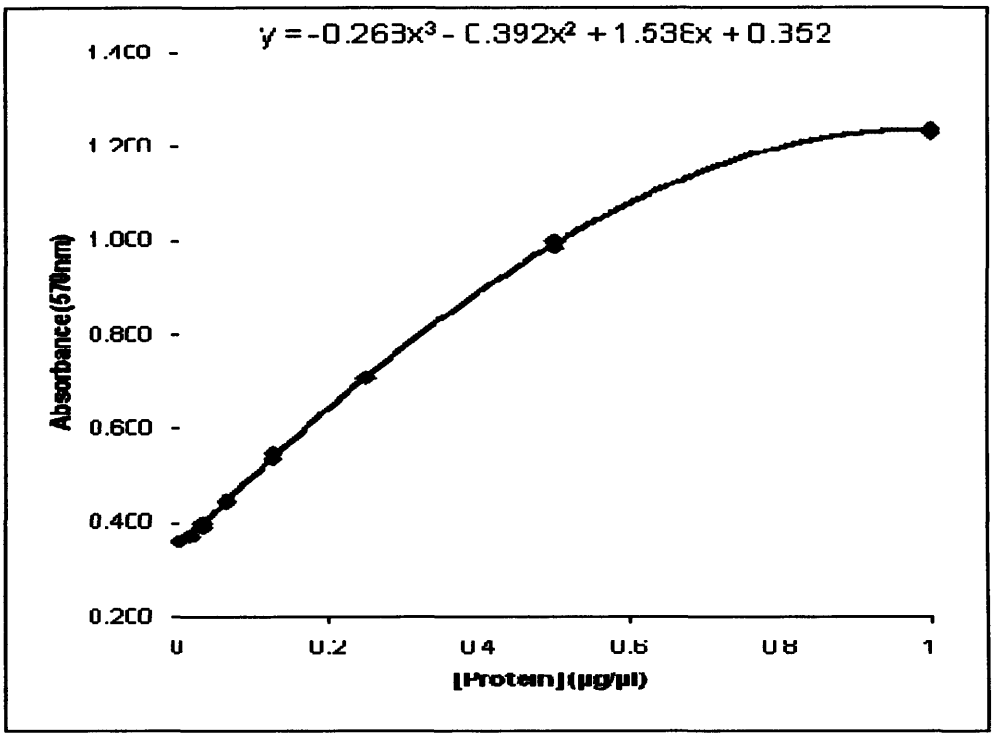

Figure 2.5 Standard curve generated for Bradford assay

2.3.2.3 Protein quantification using NanoDrop® ND-1000

1µl of each sample was pipetted onto the measurement pedestal of the apparatus (Figure 2.6).The protein concentration of sample was measured using absorbance at a wavelength of 280nm.

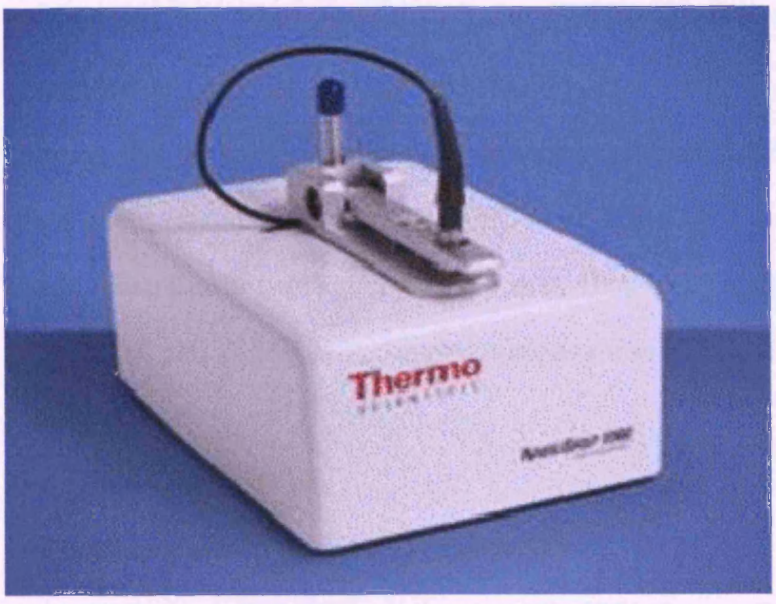

Figure 2.6 NanoDrop® ND-1000

2.3.3 Electrophoretic Mobility Shift Assay (EMSA)

2.3.3.1 Reagents and instruments used

Glycerol

Bovine Serum Albumin (BSA)

3M Whatman paper

Redivue adenosine 5'-[γ-^{32}P] triphosphate triethylammonium salt (Amersham (A0068) with relative radioactivity levels of 9.25MBq/250μCi)

10x T4 polynucleotide kinase buffer (Promega, Southampton, UK)

T4 polynucleotide kinase (100 units) (Promega, Southampton, UK)

Phenol: Chloroform: Isoamyl alcohol (25:24:1) (Sigma, Dorset, UK)

5x TBE (1L) containing 54g Tris HCl, 55g Boric acid and 7.4g EDTA and dH_2O

Poly (dI dC) (100 A260 units, Amersham, Buckinghamshire, UK) was re-suspended at 1µg/µl l in 5 ml distilled H_2O

Loading dye containing 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 30% (v/v) glycerol and 50mM EDTA and dH_2O

10x binding buffer containing 40% glycerol, 10mM EDTA, 50mM DTT, 100mM Tris pH7.5, 1M NaCl, 1mg/ml nuclease-free BSA and dH_2O up to 500µl

Acrylamide (40%) (BDH)

Ammonium persulphate (APS) (Sigma, Dorset, UK)

N N N'N'-Tetramethylethylenediamine (TEMED) (Sigma, Dorset, UK)

NF-κB binding oligonucleotide - sequence (5'-CAA CGG CAG GGG AAT CTC CCT CTC CTT-3') (Promega, Southampton, UK)

p50, p52, cRel, Rel B and Rel A antibodies (Kindly supplied by Nancy Rice, USA)

2.3.3.2 End-labelling of double stranded oligonucleotides with [γ-^{32}P]-ATP

Prior to the addition of Redivue adenosine 5'-[γ-^{32}P] triphosphate triethylammonium salt, a labelling mix was first formed in a 1.5 ml eppendorf tube. This included 4 µl NF-κB binding oligonucleotide,10 µl 10x T_4 polynucleotide kinase buffer, 2.5 µl T_4 polynucleotide kinase (25 units) and 78.5 µl added to the mixture and incubated for 2.5 hours at 37°C in a Perspex container. Post-incubation, the Perspex box was returned to the controlled radiation area. 2.5 µl of 0.5M EDTA pH8 and 100 µl of phenol: chloroform was added to the mix. The mixture was briefly vortexed and centrifuged at 1390xg for 5 min in a microcentrifuge. The upper aqueous layer was removed to a

fresh 1.5 ml eppendorf. 4 µl of 5M NaCl and 200 µl of absolute ethanol were then added to the aqueous layer. The mixture was incubated at -20°C for 60 min in a lead container. The mixture was centrifuged at 1390xg for 10 min in a microcentrifuge and the supernatant was removed. The pellet was allowed to air-dry and dissolved in 50 µl TE buffer.

2.3.3.3 Preparation of native 4% polyacrylamide gels

Gels were prepared according to the following recipe: 35 ml distilled H_2O, 5x TBE, 5 ml 40% acrylamide, 0.1g ammonium persulphate and 20µl TEMED. Acrylamide gel mix was then poured into gel plates and a 12-well forming comb was inserted and gels were allowed to set for 40 min. The comb was then removed and wells were rinsed with distilled H_20 and then filled with 0.5x TBE.

2.3.3.4 EMSA

Nuclear extracts were prepared as a reaction mix which included 1 µl 10x binding buffer, 2 µl poly (dI dC) and 2 µg nuclear extract and dH_2O to make a final volume of 10µl. For supershift or cold competitor experiments, 2 µg of antibody or 100ng unlabelled NF-κB binding oligonucleotide respectively was pre-incubated for 30 min at 4°C. 1µl of ^{32}P-labelled NF-κB binding oligonucleotide probe was added to the reaction mix and incubated at room temperature for 30 min. 1.2 µl of loading dye was then added to the mix. Samples were loaded under the 0.5x TBE in the wells using gel loading tips. The electrophoresis apparatus (Amersham) was assembled and 0.5x TBE was added to both the upper and lower reservoirs. The gels were then run at 200V for 90 min using a Bio-Rad power pack. Following this, gels were placed

on 3M Whatman paper was and dried under vacuum on a gel drier (Bio-Rad, model 583) set at 80°C for 120 min. Gels were then visualised by autoradiography. A typical result is schematically represented in diagram 2.7.

2.3.3.5 Supershift EMSA

For the super-shift assay, the binding mixture was pre-incubated with the indicated antibodies for 30 min before adding the labelled probe. I used p50, p52, cRel, Rel B and Rel A antibodies for these experiments. Due to the change in size of the complexed molecules the mobility is altered which enables the visualization of different components.

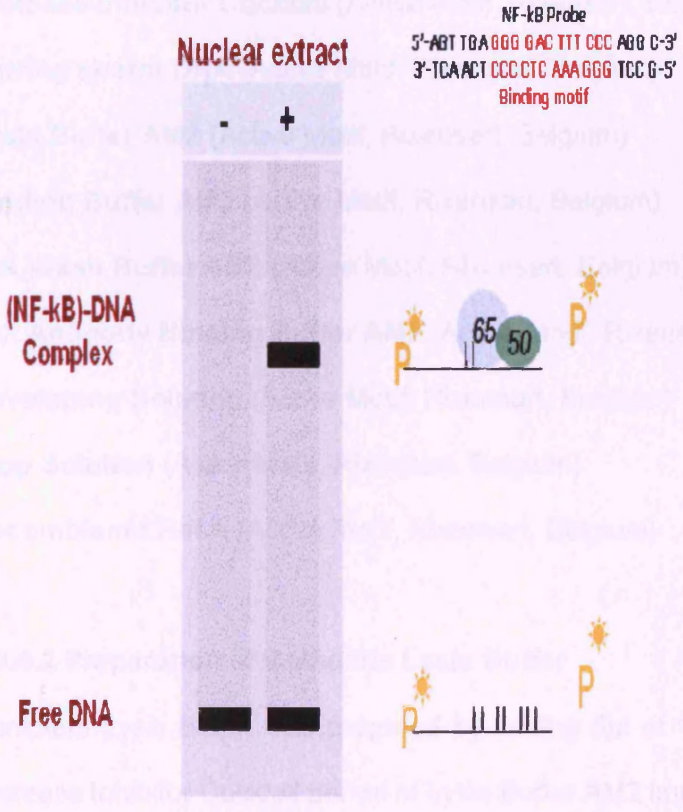

Figure 2.7 Simplified schematic of an EMSA result.

2.3.4 Enzyme Linked ImmunoSorbant Assay (ELISA)

(Trans AM™ NF-κB Rel A Transcription Factor Assay Kit, version E3)

2.3.4.1 Reagents and Equipment

Microplate reader (Bio-Rad, 170-6850)

NFκB p65 antibody (Active Motif, Rixensart, Belgium)

Anti-rabbit HRP-conjugated IgG (Active Motif, Rixensart, Belgium)

Wild-type oligonucleotide (Active Motif, Rixensart, Belgium)

Mutated oligonucleotide (Active Motif, Rixensart, Belgium)

Positive control nuclear extract (Active Motif, Rixensart, Belgium)

Dithiothreitol (DTT) (Active Motif, Rixensart, Belgium)

Protease Inhibitor Cocktail (Active Motif, Rixensart, Belgium)

Herring sperm DNA (Active Motif, Rixensart, Belgium)

Lysis Buffer AM2 (Active Motif, Rixensart, Belgium)

Binding Buffer AM3 (Active Motif, Rixensart, Belgium)

10X Wash Buffer AM2 (Active Motif, Rixensart, Belgium)

10X Antibody Binding Buffer AM2 (Active Motif, Rixensart, Belgium)

Developing Solution (Active Motif, Rixensart, Belgium)

Stop Solution (Active Motif, Rixensart, Belgium)

Recombinant Rel A (Active Motif, Rixensart, Belgium)

2.3.4.2 Preparation of Complete Lysis Buffer

Complete Lysis Buffer was prepared by adding 5µl of 1 M DTT and 10µl of Protease Inhibitor Cocktail per ml of Lysis Buffer AM2 immediately before use.

2.3.4.3 Preparation of Complete Binding Buffer

Complete Binding Buffer was prepared by adding 2µl of 1M DTT, and 10µl of 1µg/µl Herring sperm DNA per ml of Binding Buffer AM3 immediately before use.

2.3.4.4 Preparation of 1X Wash Buffer

Wash Buffer (1x) was prepared by diluting 1 to 10 dilution in distilled water and mixing it gently to avoid foaming. (10 ml 10x Wash Buffer AM2 with 90 ml distilled water).

2.3.4.5 Preparation of 1X Antibody Binding Buffer

10X Antibody Binding Buffer AM2 was warmed to room temperature and vortexed for 1 min prior to use. Antibody binding buffer (1x) was prepared by diluting 1 to 10 dilutions in distilled water and mixing gently to avoid foaming (Dilute 1 ml 10x antibody binding buffer AM2 with 9 ml distilled water). Both primary and secondary antibodies were diluted to 1:1000 with the 1xantibody binding buffer.

2.3.4.6 Preparation of standard curve

To quantify the amount of Rel A in the samples, I used recombinant Rel A protein to generate a standard curve. The following concentrations were used: 0.5, 0.25, 0.125, 0.0625, 0.0312, 0.0156, 0.008 and 0 ng/µl for the standard curve.

2.3.4.7 ELISA

For each sample well 30µl of complete binding buffer was added followed by 1µg of nuclear extracts diluted in 20µl of complete lysis buffer. For the positive control wells 30µl of complete binding buffer was added followed by 1µl of Jurkat nuclear extract diluted in 20µl of complete lysis buffer. For the competitive binding experiments 30µl of complete binding buffer containing 20pmol of wild-type or mutated consensus oligonucleotide was added followed by sample nuclear extracts diluted in 20µl complete lysis buffer. For the standard curve, 30µl of complete binding buffer followed by serial dilutions of recombinant Rel A diluted in 20µl of complete lysis buffer were used. Each plate was incubated for 1h at room temperature with mild agitation (100 rpm on a rocking platform). Each well was washed 3 times with 200µl 1x washing buffer followed by 100µl of pre-diluted Rel A antibody (1:1000 dilution in 1x antibody binding buffer) per well. After incubation for 1h at room temperature without agitation the plate was washed 3 times with 200µl 1x washing buffer followed by 100µl of diluted HRP antibody (1:1000 dilution in 1x antibody binding buffer) per well. Each plate was then incubated for 1h at room temperature without agitation and then washed 4 times with 200µl 1x washing buffer. Subsequently, 100µl of developing solution was added to all the wells followed by incubation for 5 min at room temperature protected from direct light. Finally, 100µl of stop solution was added and the absorbance was read using a spectrophotometer within 5 min at 450nm with a reference wavelength of 655 nm (Benchmark, microplate reader, Bio-Rad). The processing steps for ELISA are represented schematically in Figure 2.8. An example of standard curve generated using recombinant Rel A proten is shown in Figure 2.9.

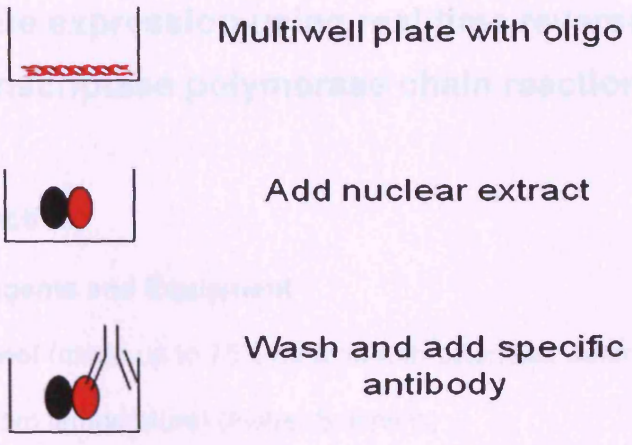

Figure 2.8 Illustration of steps involved in ELISA analysis

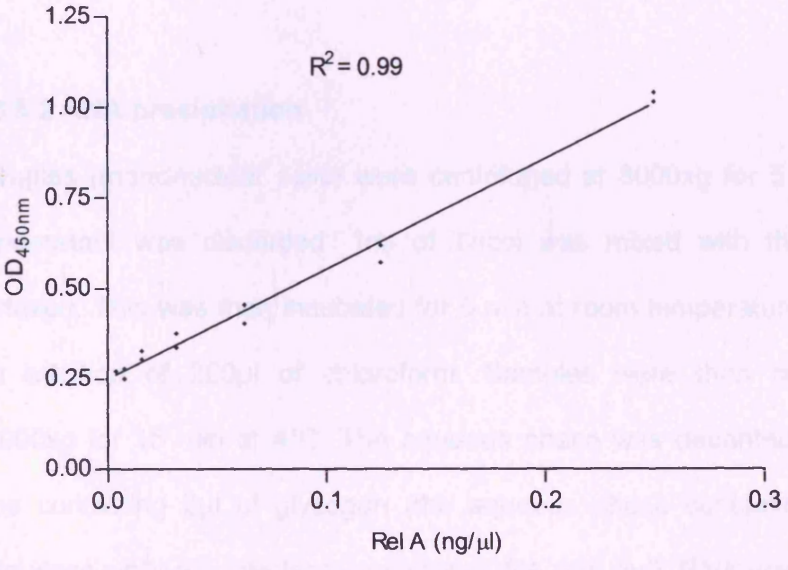

Figure 2.9 Rel A was quantified in the patient samples by using a Rel A standard curve generated using recombinant proteins.

2.3.5 Gene expression using real-time reverse transcriptase polymerase chain reaction (RT-PCR)

RNA extraction

2.3.5.1 Reagents and Equipment

100% Ethanol (made up to 75% ethanol with deionized water prior to use and stored at room temperature) (Fisher Scientific)

Trizol (Invitrogen) stored at 4°C

Chloroform (stored at 4°C) (Fisher Scientific)

Glycogen (5 mg/ml) (Applied Biosystems, stored at -20°C)

Isopropyl alcohol (Stored at room temperature) (Fisher Scientific)

RNase free water

2.3.5.2 RNA precipitation

Samples (mononuclear cells) were centrifuged at 8000xg for 5 min and the supernatant was discarded. 1ml of Trizol was mixed with the pellet and vortexed. This was then incubated for 5 min at room temperature followed by the addition of 200µl of chloroform. Samples were then centrifuged at 12000xg for 15 min at 4°C. The aqueous phase was decanted into a fresh tube containing 2µl of glycogen (the aqueous phase containing RNA was colourless while the interface containing DNA was red). RNA was precipitated by adding 500µl of isopropyl alcohol followed by gentle mixing. This was incubated for 10 min at room temperature and centrifuged at 12000xg for 15 min. The supernatant was removed and mixed with 1ml of 75% ethanol. Then it was centrifuged at 7500xg for 10 min at 4°C and the supernatant removed. This was then stored at -80°C after adding 1ml of 75% ethanol. Before use

this was centrifuged at 7500xg for 5mins. The supernatant was then removed and the pellet was air dry before re-dissolving in 13µl of RNA free water. RNA was quantified using the NanoDrop® ND-1000 Spectrophotometer (Labtec international).

2.3.5.3 Reverse transcription reaction

Reagents and Equipment

$MgCl_2$ (Applied Biosystems, Cheshire, UK)

10x PCR buffer II (Applied Biosystems, Cheshire, UK)

dGTP (Applied Biosystems, Cheshire, UK)

dATP (Applied Biosystems, Cheshire, UK)

dTTP (Applied Biosystems, Cheshire, UK)

dCTP (Applied Biosystems, Cheshire, UK)

RNAse inhibitor (Applied Biosystems, Cheshire, UK)

MuLV (Applied Biosystems, Cheshire, UK)

Random Hexamer primers (Applied Biosystems, Cheshire, UK)

For the master mix, 4µl of $MgCl_2$ was added in addition to 2µl of 10x PCR buffer II, 1µl RNAse inhibitor and 2µl of each of dGTP, dATP, dTTP and dCTP. Subsequently, 1µl MuLV and 1µl Random hexamer primers were added and mixed thoroughly. To this 3 µl of RNA extract was added to make a 20µl total reaction volume. Complementary cDNA was synthesized using a Thermal Cycler 480 (Applied Biosytems). The operating conditions were as follows: 10 min at 25°C followed by 30 min at 42°C followed by 5 min at 95°C. The resulting cDNA was stored at -20°C until use.

2.3.5.4 Real-time quantitative polymerase chain reaction (qRT-PCR) (Light Cycler)

Reagents and Equipment

LightCycler System (Roche Diagnostics, Meylan, France)

Primers for target genes (Eurogentec S.A, Belgium)

CFLAR	Forward 5′-AGA-GTG-AGG-CGA-TTT-GAC-CTG-3′
	Reverse 5′-AAG-GTG-AGG-GTT-CCT-GAG-CA-3′
BIRC5	Forward 5′-TGTTGGGAATCTGGAGATGA-3
	Reverse 5′-CGGATGAACTCCTGTCCTTT-3′
BCL2	Forward 5′-GGTCATGTGTGTGGAGAGCG-3′
	Reverse 5′- GGTGCCGGTTCAGGTACTCA-3′

Primers for endogenous control

RPS14	Forward 5′-GGCAGACCGAGATGAACTCT-3′
	Reverse 5′-CCAGGTCCAGGGGTCTTGGT-3′

$MgCl_2$ (25μM stock)

Forward Primer (10μM stock)

Reverse Primer (10μM stock)

Syber Green (10x) 1A and 1B

RNase free H_2O

2.3.5.5 Preparation of Syber green for use

Syber green was supplied by Roche in two separate tubes 1A and 1B. Immediately before use a mixture was prepared by adding 10μl of 1A to 1B and labelled as 1(According to manufacturer's instructions).

2.3.5.6 Master mix for each study gene and endogenous control

The master mix for the PCR reaction was prepared by adding 1.6µl 5mM $MgCl_2$, 0.5µl of 10µM forward primer, 0.5µl of 10µM reverse primer, 1µl of 10x Syber Green and 5.4µl of RNase free water (making a total master mix for each reaction of 9µl). Subsequently 1µl of cDNA was added to each capillary to make a final volume of 10µl.

2.3.5.7 Real-time quantitative PCR

9µl of master was mixed with 1µl of cDNA in a capillary tube. Capillary tubes were loaded onto the light cycler machine and the reaction was carried out under the following conditions:

Denaturation: 95°C for 10 min

Amplification: 95°C for 3 sec

Annealing Temp 63°C 5 sec

Final extension: 72°C 10 min

The results of the real-time RT-PCR were expressed as normalized target gene values, e.g. the ratio between CFLAR and RPS14 transcripts calculated from the crossing points of each gene. All experiments were performed in duplicate.

2.3.6 IgV_H gene mutation analysis

2.3.6.1 Reagents and instruments used

Qiagen RNeasy mini kit (Qiagen, Crawley, UK)

Reverse-iT kit (ABgene, Epsom, UK)

RNA was extracted from the patient samples using Qiagen RNeasy mini kits (Qiagen). Complementary DNA was synthesized using the Reverse-iT kit (ABgene). The IgV_H gene mutation status of the CLL patients was analyzed according to the method previously described (Stankovic *et al.*, 2002) and the resulting PCR products were sequenced using the BIG dye terminator sequencing kit version 3 (Applied Biosytsems). The sequences were subsequently analyzed using the following public databases: Immunoglobulin BLAST (http://www.ncbi.nlm.nih.gov/igblast/) and IMGT (http://imgt.cines.fr/IMGT_vquest/vquest?livret=0&Option=humanIg). The sequences with a germ line homology of 98% or higher were regarded as unmutated and less than 98% as mutated.

2.4 Clinical data collection

Comprehensive clinical information, including treatment histories, was available for all patients either through their case notes or direct interviews with the patients. The characteristics of the patients used for each experiment are given in the relevant experimental section.

Definitions of clinical characteristics gathered for the study

Date of diagnosis:	The date the patient was diagnosed with CLL
White blood cell count (WBC):	Total white blood cell count at the time of taking the sample for NF-κB analysis
Lymphocyte count:	Lymphocyte count at the time of taking the sample for NF-κB analysis
Lymphocyte doubling time (LDT):	Time taken for the lymphocyte count to double (in months)
Time to first treatment (TTFT):	Time from diagnosis to the treatment for the first time

Time to subsequent treatment (TTST):	Time from enrolment in the study to the treatment.
Overall survival (OS):	Time to death from date of diagnosis
Overall survival from entry into study:	Time to death from date of entry into the study

2.5 Statistical analysis

Software packages used

Graphpad Prism 4.0 software (Graphpad Software Inc., CA, USA)

Calcusyn software (Biosoft, Cambridge, UK)

SAS statistical software (SAS Institute)

2.5.1 Assessment of dose of the drug required to kill 50% of the cells (LD_{50})

This was performed using Graphpad Prism 4.0 software (Graphpad Software Inc., San Diego, CA, USA). Drug sensitivity for each individual drug and drug combination was evaluated using non-linear regression and line of best fit dose-response curves.

2.5.2 Assessment of synergy

Data was analysed using the median effect method to determine the degree of synergy (Chou *et al.*, 1984) between the compounds tested. Combination

Indices (CI) and Dose reduction Indices (DRI) were calculated using Calcusyn software (Biosoft, Cambridge, UK).

2.5.3 Statistical analysis of prognostic parameters

The relationship between Rel A and known prognostic factors as well as time to first treatment and overall survival was explored through Wilcoxon rank sum tests for the categorical variables Binet stage, CD38, ZAP-70 and IgV_H gene mutation status. Survival curves were constructed using the method of Kaplan and Meier and the Log-Rank test was used to assess any differences between patient and tumour characteristics. Cox regression analysis determined important independent prognostic factors for time to first treatment and overall survival. Statistical analysis was carried out using GraphPad Prism software 4.0 (Graphpad, CA) and SAS statistical software (SAS Institute).

3.0 QUALITATIVE AND QUANTITATIVE ASSESSMENT OF NF-κB IN CLL

3.1 Introduction

The factors that contribute to the pathogenesis and progression of CLL are poorly understood but decreased susceptibility to apoptosis (Kern *et al.*, 2004; Romano *et al.*, 2005; Greaney *et al.*, 2006) and deregulated proliferation have been implicated (Messmer *et al.*, 2005). NF-κB is known to contribute to the regulation of both apoptosis and proliferation and CLL cells have been reported to exhibit high constitutive NF-κB activation compared to normal B-lymphocytes (Furman *et al.*, 2000; Cuni *et al.*, 2004; Tracey *et al.*, 2005). Therefore, I set out to investigate NF-κB activity in a cohort of primary CLL patient samples in order to determine its biological relevance in CLL and to evaluate its potential as a prognostic marker.

3.2 Measurement of NF-κB activity

In its inactive state NF-κB is bound to Inhibitor of κB (IκB) in the cytoplasm. Following cellular stimulation, IκB is phophorylated and degraded via proteosomal mechanisms allowing NF-κB proteins to be translocated in to the nucleus (Sun *et al.*, 1994). NF-κB can be measured at the protein level and mRNA level or alternatively IκB levels can be quantified as an indirect method of NF-κB activity. An overview of most commonly used methods for the measurement of NF-κB is given below.

3.2.1 Electrophoretic Mobility Shift Assay (EMSA)

EMSA (Schreck *et al.*, 1990) is the most widely used method for measurement of NF-κB and is a direct measure of NF-κB DNA binding. In this method, nuclear extracts are incubated with radioactive double-stranded oligonucleotide containing a consensus sequence for NFκB binding. Active NF-κB in the nuclear extracts will bind to its consensus sequence and samples are then resolved by native polyacrylamide gel electrophoresis followed by autoradiography. A retarded band corresponding to the NF-κB DNA complex can be detected as well as a band that migrates much further in unit time corresponding to free DNA. When NF-κB is in its inactive form, bound to IκB, it is unable to bind to DNA. A further adaptation of this method involves the incubation of cellular extracts with antibodies against specific NF-κB subcomponents prior to mixing with the radioactive oligonucleotide. The resulting protein-oligonucleotide complex can show decreased mobility thereby distinguishing the individual NF-κB subunits (super-shift Assay). This gel retardation method is sensitive but is not suitable for high throughput screening of large numbers of patient samples.

3.2.2 Enzyme Linked ImmunoSorbant Assay (ELISA)

This DNA binding assay is based on the use of multi-well plates coated with an oligonucleotide containing the consensus binding site for NF- κB. This is different to a normal ELISA as the protein of interest is captured by a double-stranded oligonucleotide containing the consensus binding sequence for NF-κB rather than an antibody. This technique only captures activated transcription factor which is then detected by primary antibody followed by a

secondary antibody conjugated to horseradish peroxidase. Finally, the reaction is quantified by a colourimetric or fluorometric reaction. This assay allows large scale screening than EMSA.

3.3 Programmed cell death

Programmed cell-death (PCD) is death of a cell in any form mediated by an intracellular programme and is often termed physiological cell death or apoptosis (Engelberg-Kulka *et al.*, 2006). In contrast, necrosis is a form of cell death that results from acute tissue injury and provokes an inflammatory response (pathological cell death). There are different pathways consisting of many steps leading to the DNA fragmentation in apoptosis (Elsasser *et al.*, 2000). There are several completely unresolved issues in apoptosis. These include the time course of activation of the death signal to DNA fragmentation, point the apoptotic process is irreversible and the best way of quantification of apoptosis (Rodriguez *et al.*, 2004). The first measurable phenomena are loss of mitochondrial transmembrane potential and activation of caspase 3. This is followed by transferred phosphatidyl serine (PS) to the outer cytoplasmic membrane. Chromatin condensation and DNA fragmentation are considered to be late features of apoptotic cell death. The reversibility of the apoptotic pathway may be cell type dependent. It has been shown that PS expression is reversible in some cell types while DNA single strand breaks are reversible in others (Chang *et al.*, 2003; Lamarche *et al.*, 2003).

Apoptosis is involved in several normal biological processes including foetal development, tissue renewal in the skin, gut and bone marrow and elimination of abnormal cells. The loss of effects as a controller may allow malignant cells

to arise (Evan *et al.*, 1997). In CLL, it has long been believed the accumulation of malignant cells is due to an inherent defect in apoptotic machinery. The diagram below illustrates the various pathways leading to apoptosis (Figure 3.1).

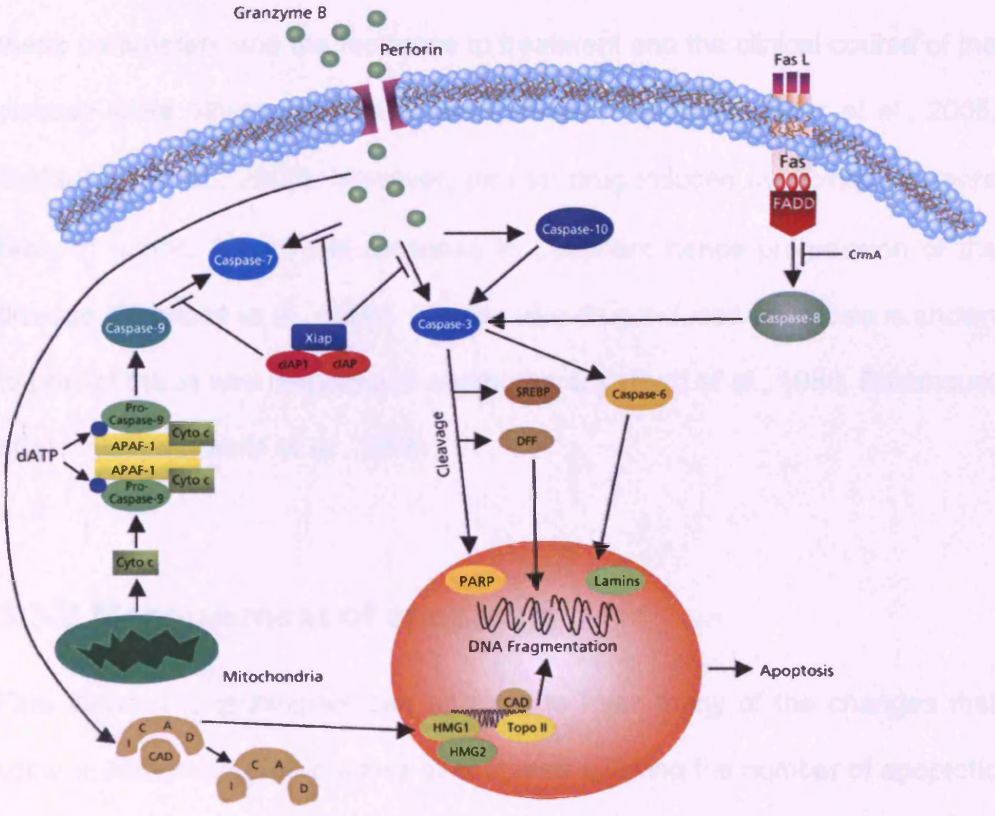

Figure 3.1 Apoptotic pathways

3.3.1 Apoptosis and CLL

CLL cell accumulation in peripheral blood and bone marrow is believed to result from failed apoptosis, alterations in cell cycle regulation and from cell proliferation (Kern *et al.*, 2004; Messemer *et al.*, 2005; Obermann *et al*, 2007). This leads to the progressive increase in malignant lymphocytes. Various studies have tried to identify the prognostic significance of both spontaneous and drug induced *ex vivo* apoptosis in CLL cells. However, results have not been conclusive with some authors describing a clear correlation between these parameters and the response to treatment and the clinical course of the disease while others attribute no such relationship (Jahrsdorfer *et al.*, 2005; Sieklucka *et al.*, 2008). However, *ex vivo* drug-induced cytotoxicity is more likely to predict the clinical response to treatment hence progression of the disease (Morabito *et al.*, 1998). Also *ex vivo* drug induced apoptosis is shown to predict the *in vivo* response to chemotherapy (Byrd *et al.*, 1988; Bosanquet *et al.*, 1997; Morabito *et al.*, 1998).

3.3.2 Measurement of apoptosis

Flow cytometric techniques can be used to track many of the changes that occur in cells during the process of apoptosis allowing the number of apoptotic cells in a sample to be accurately quantified (Ormerod *et al.*, 2000). During apoptosis there is a loss of plasma membrane phospholipid asymmetry, with phosphatidyl serine residues flipping from the inside to the outside of the plasma membrane. Annexin V is a calcium-dependent phospholipid binding protein that has a high affinity for PS and can be used flow cytometrically with non-fixed cells as a sensitive probe for PS exposure (Koopman *et al.*, 1994;

Vermes *et al.*, 1995). When using the Annexin V binding method to quantify apoptotic cells, it is customary to add a cationic dye such as propidium iodide to distinguish between apoptotic cells with an intact plasma membrane and those cells that have lost membrane integrity and are undergoing secondary necrosis.

3.4 Results

Patient characteristics used for this part of the study are described in Table 3.1 and Table 3.2.

Table 3.1 Patient characteristics

Patient ID	Gender	Stage	CD38	ZAP-70	IgV_H status
1	M	A	2	0	M
2	M	A	14	0	M
3	F	A	1	1	M
4	F	C	2	1	M
5	M	B	46	2	M
6	M	A	79	3	M
7	F	A	1	5	M
8	M	A	3	8	M
9	M	A	7	10	M
10	F	B	34	10	M
11	F	A	1	20	M
12	M	A	46	20	M
13	F	A	9	22	M
14	M	C	2	28	M
15	F	A	3	29	M
16	M	C	18	31	M
17	M	A	35	32	M
18	M	A	23	36	M
19	F	C	68	56	M
20	F	A	100	61	M
21	M	C	5	0	U
22	M	A	35	1	U
23	M	C	3	20	U
24	M	A	54	24	U
25	F	A	47	25	U

Patient ID	Gender	Stage	CD38	ZAP-70	IgV_H status
26	M	C	3	29	U
27	F	A	70	33	U
28	M	C	70	33	U
29	M	B	39	37	U
30	M	B	98	39	U
31	M	B	42	40	U
32	M	A	39	48	U
33	M	B	81	1	ND
34	F	A	3	1	ND
35	F	A	4	2	ND
36	F	A	87	4	ND
37	F	A	29	22	ND
38	F	A	47	33	ND
39	F	C	87	88	ND
40	M	A	16	ND	ND
41	F	A	21	ND	ND
42	M	A	ND	ND	ND
43	F	A	ND	ND	ND

Table 3.2 Patient characteristics

Patient ID	Gender	Stage	CD38	ZAP-70	IgV_H status
1	M	A	8	11	M
2	F	A	7	3	U
3	F	A	9	13	M
4	M	B	4	0.2	M
5	M	A	5	70	M
6	M	A	2	0.3	M
7	M	A	13	24	M
8	F	A	0.7	5.4	M
9	M	A	10	0.2	M
10	M	B	42	14	ND
11	F	A	99	63	M
12	M	A	9.8	10.4	M
13	M	A	8	3	M
14	M	A	84	13	M
15	M	B	36	34	U
16	M	A	99	90	ND
17	M	B	99	40	U
18	M	A	35	32	M
19	M	A	26	7	M
20	F	A	2.2	6.5	M
21	F	A	3.2	0.8	M
22	M	A	4.6	10.3	M
23	M	A	10	4	M
24	M	A	ND	ND	ND
25	M	A	0.4	0.6	M
26	M	A	13	85	M
27	M	A	ND	ND	ND
28	M	A	85	99	ND
29	M	A	4.2	11	ND
30	F	A	5	3	ND

3.4.1 Total NF-κB binding is heterogenous in CLL patient samples

CLL cells have been shown to exhibit high levels of nuclear NF-κB when compared with non malignant B-cells (Furman *et al.*, 2000; Endo *et al.*, 2006). However, a detailed analysis of the variation in levels of NF-κB across multiple patients or a dissection of the constituent subunits in primary CLL cells has not been previously described. Therefore, in this study, the first objective was to determine the range of constitutive DNA binding of NF-κB within our patient cohort.

NF-κB DNA binding was investigated in 43 unselected CLL patient samples using electrophoretic mobility shift assay (EMSA). Two cell lines were chosen as reference for the assay: BL41, a Burkitt's lymphoma line with relatively low NF-κB activity, and IARC-171, an EBV immortalised line derived from the same patient. EBV activates NF-κB during B-cell immortalization so IARC-171 cells represent a positive control for a B-cell high in NF-κB. Figure 3.2 shows a representative sample of nuclear extracts derived from 12 patients demonstrating the differential expression of NF-κB DNA binding. DNA binding varied from almost undetectable (patients 2, 4, 7and 9) to levels comparable or higher than those found in the EBV immortalized cells (patients 3, 8 and 11) with other samples showing intermediate NF-κB DNA binding. The specificity of the NF-κB binding was confirmed by an EMSA cold competitor assay (Figure 3.3). A non-radioactively labelled NF-κB DNA oligonucleotide eliminated binding while an oligonucleotide for another transcription factor, AP1, left DNA binding unaffected.

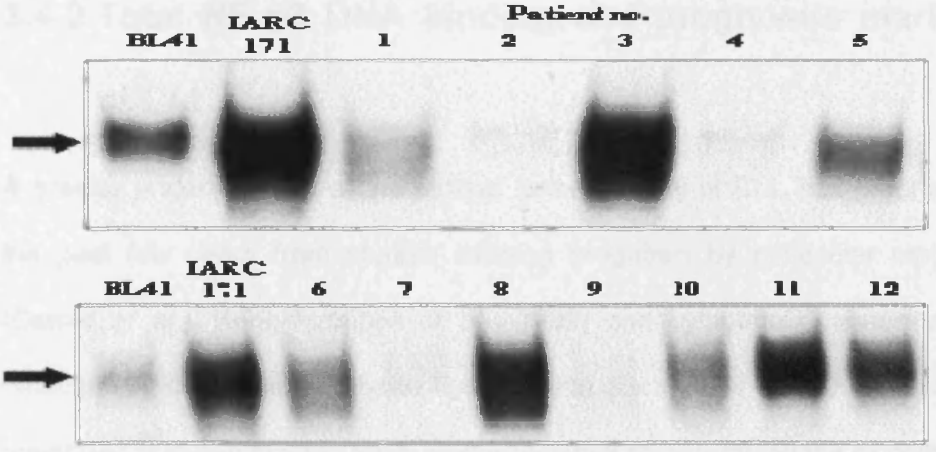

Figure 3.2 CLL patients show heterogeneity of NF-κB DNA binding. NF-κB DNA binding activity in cell nuclear extracts was measured using electrophoretic mobility shift assays. An oligonucleotide corresponding to the consensus sequence to NF-κB was radiolabeled and incubated with 2 μg nuclear extract. The DNA-protein complex was restored by electrophoresis in a 4% native polyacrylamide gel in 0.5X TBE buffer. The gels were dried and protein binding was visualized by autoradiography.

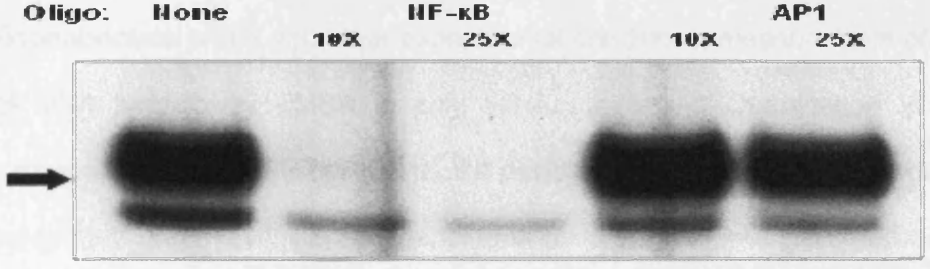

Figure 3.3 The specificity of the NF-κB binding was confirmed by an EMSA cold competitor assay. To 2 μg of nuclear extracts of CLL patient 11, a cold, non-radiolabeled NF-κB and a non-specific oligonucleotide, AP1 was added at 10X and 25X the concentration of radiolabeled NF-κB and incubated for 30 minutes, prior to addition of the radiolabeled NF-κB consensus oligonucleotide. The DNA-protein complex was resolved by electrophoresis in a 4% native polyacrylamide gel in 0.5X TBE buffer. The gels were dried and protein binding was visualized by autoradiography.

3.4.2 Total NF-κB DNA binding and prognostic markers of CLL

A greater understanding of the clinical heterogeneity of CLL has emerged in the past few years from studies defining prognosis by molecular methods (Damle *et al.*, 1999; Hamblin *et al.*, 1999) and cytogenetic abnormalities detected by fluorescence in-situ hybridisation (Dohner *et al.*, 2000). Specific molecular features of CLL, such as the absence of IgV_H mutations or deletions in chromosomes 11 or 17, identify patients who are likely to progress and have a high probability of dying as a result of their disease (Dohner *et al.*, 2000; Crespo *et al.*, 2003; Rassenti *et al.*, 2004). Although major advances have been made in identifying subsets of patients with different prognoses, none of the currently available markers are able to predict the clinical course of every patient. Also there is a significant heterogeneity among availabe prognostic markers. I compared NF-κB DNA binding with other widely available prognostic markers in CLL. Due to variation in the radioactivity of the oligonucleotide probe and other experimental conditions, measurement of NF-κB DNA binding by EMSA is only semi-quantitative. Quantitation in this context utilised the IARC cell line as the positive control in each gel retardation experiment and the NF-κB DNA binding of each sample was standardised by generating the ratio between sample value and value for IARC. These values (relative NF-κB DNA binding) were then compared with other currently used prognostic markers in CLL. Relative NF-κB DNA binding was not significantly different in different prognostic subsets. P values as tested by non-parametric *t*-test for CD38+ ≥20% , ZAP-70+ ≥20% or IgV_H status were 0.53, 0.15 and 0.66 respectively (Figure 3.4 A, B and C). These findings illustrate that there

was no corelation of total NF- κB DNA binding with ZAP-70, CD38 expresssion or IgV_H mutational status.

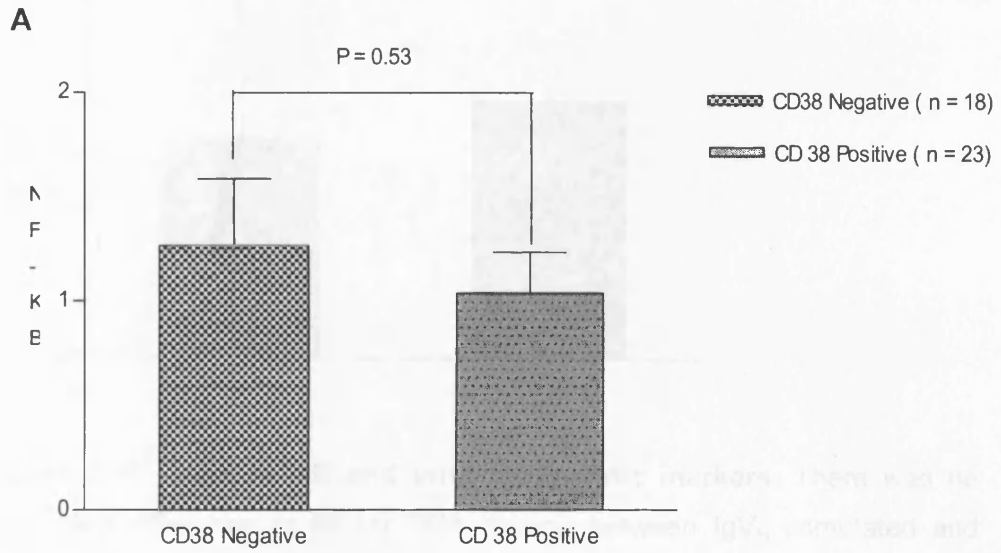

Figure 3.4A Total NF-κB and other prognostic markers. There was no significant difference in NF-κB DNA binding between CD38 positive and CD38 negative samples.

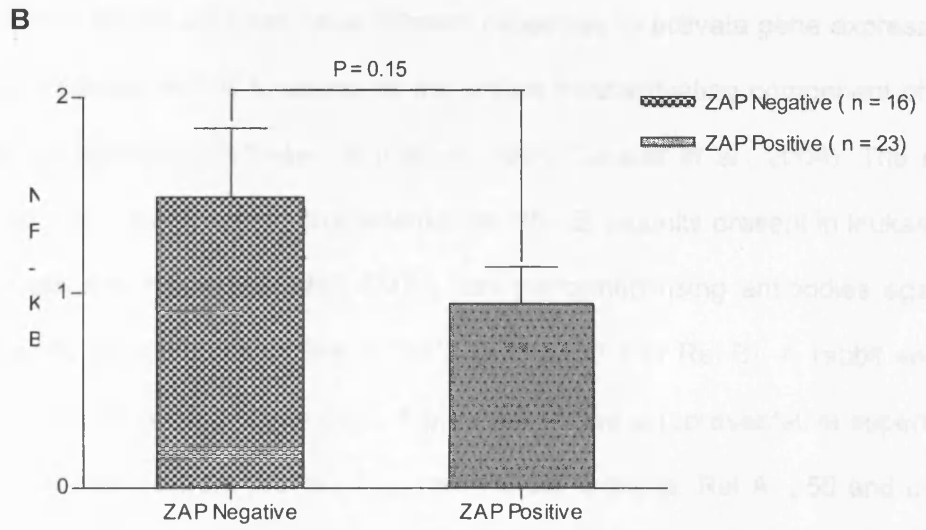

Figure 3.4B Total NF-κB and other prognostic markers. There was no significant difference in NF-κB DNA binding between ZAP-70 positive and negative samples.

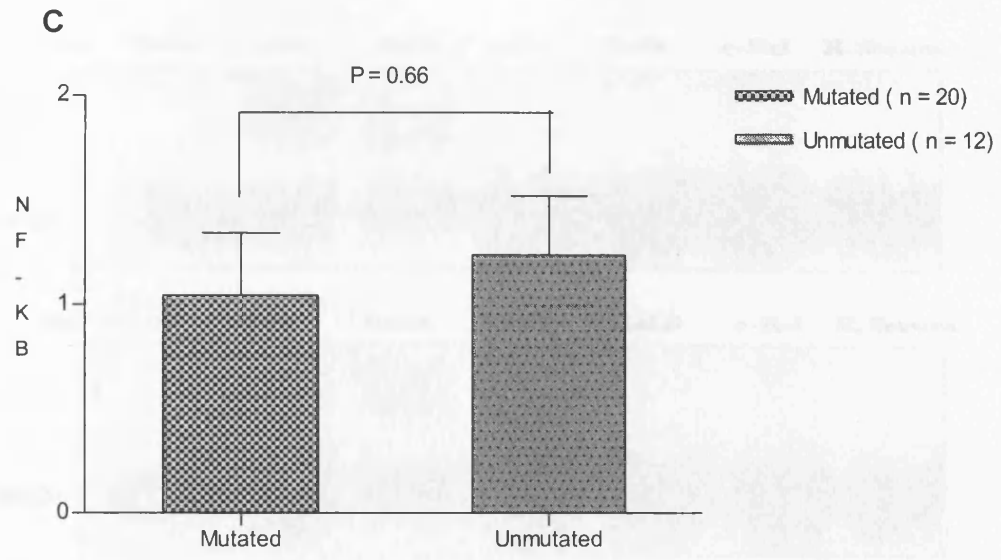

Figure 3.4C Total NF-κB and other prognostic markers. There was no significant difference in NF-κB DNA binding between IgV_H unmutated and mutated samples.

3.4.3 Qualitative analysis of NF-κB subunits

Specific NF-κB subunits have different capacities to activate gene expression. For instance, Rel A functions as the critical transactivating component of the NF-κB signalling pathway (Sun *et al.*, 1994; Takada *et al.*, 2004). The next step in this study was to characterise the NF-κB subunits present in leukaemic B-cells. For this, super-shift EMSA was performed using antibodies against specific NF-κB proteins (Rel A, p50, p52, c-Rel and Rel-B). A rabbit serum control was used in each case. Figure 3.5 shows a representative supershift EMSA derived from primary CLL cell nuclear extracts. Rel A, p50 and c-Rel subunits were detected in all the samples tested, as evidenced by shifts in their respective bands following antibody binding. However, there was marked variation in the amounts of each in the different patient samples.

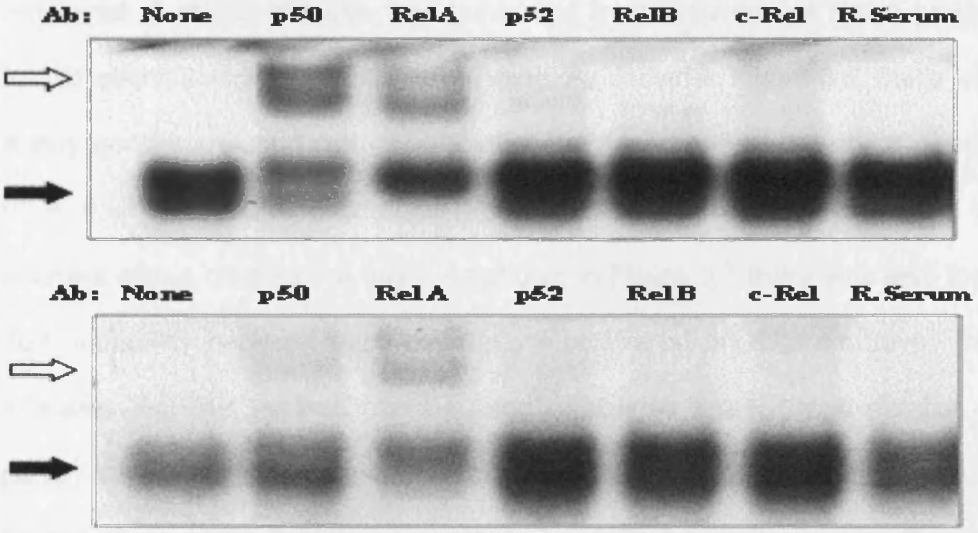

Figure 3.5 Rel A, p50 and c-Rel are the predominant NF-κB subunits in CLL patient samples. For qualitative analysis of NF-κB subunits, supershift analysis was performed on NF-κB using p50, Rel A, p52, Rel B, c-Rel antibodies and normal rabbit sera. 2 µg nuclear extracts from CLL nuclear extracts were used for these experiments. The different lanes marked, none, p50, Rel A, p52, Rel B, c-Rel and Rab ser (Rabbit sera), represent incubation with different antibodies. They were then incubated with a radiolabelled oligonucleotide corresponding to the consensus sequence of NF-κB for 30 minutes. Ab: represents the different antibodies used. "None" indicates that no antibody was incubated. White arrows indicate the antibody-protein-DNA complexes, while the black arrows indicate the protein-DNA complexes.

3.4.4 Quantitative analysis of NF-κB

NF-κB subunits Rel A, Rel B and c-Rel are shown to have transactivation domains (Schmid *et al.*, 2008) and this analysis shows that, of the three, Rel A is the most abundant subunit therein CLL cells. Furthermore constitutively activated Rel A has been shown to have oncogenic potential in cell lines (Beauparlant *et al.*, 1994). Quantification of Rel A was the next step of this study. In order to accurately quantify NF-κB in the CLL samples a colourimetric enzyme linked immunosorbant assay (ELISA) technique was

employed. A standard curve was generated from recombinant Rel A protein for the quantification of Rel A DNA binding. As shown in Figure 3.6, there was a very good correlation with recombinant Rel A and optical density at 450nm (r^2 = 0.99). In addition, samples from the same patients were tested on different plates on different days. As shown in Figure 3.7 there was less than 10% variability between each experiment performed on different days. This indicates that this method can be used reliably to screen large number of patient samples and suggests that results derived from different centres could be directly compared with one another. Rel A DNA binding measured using ELISA and total NF-κB DNA binding as measured by EMSA was compared. There was a correlation between these two values (r^2 = 0.2) (Figure 3.8).

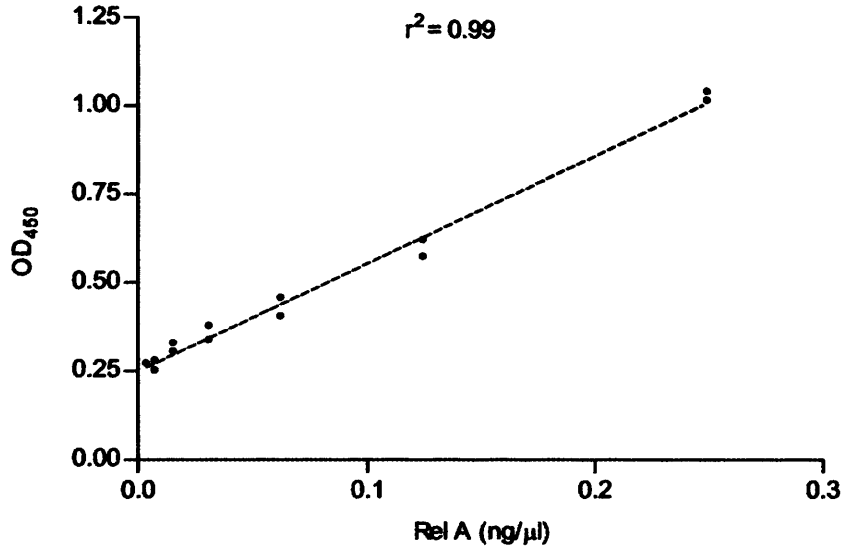

Figure 3.6 Standard curve was generated using recombinant Rel A proteins. ELISA was performed using various concentrations of (doubling dilutions) recombinant Rel A. There was a linear relationship with the concentration of recombinant protein and optical density.

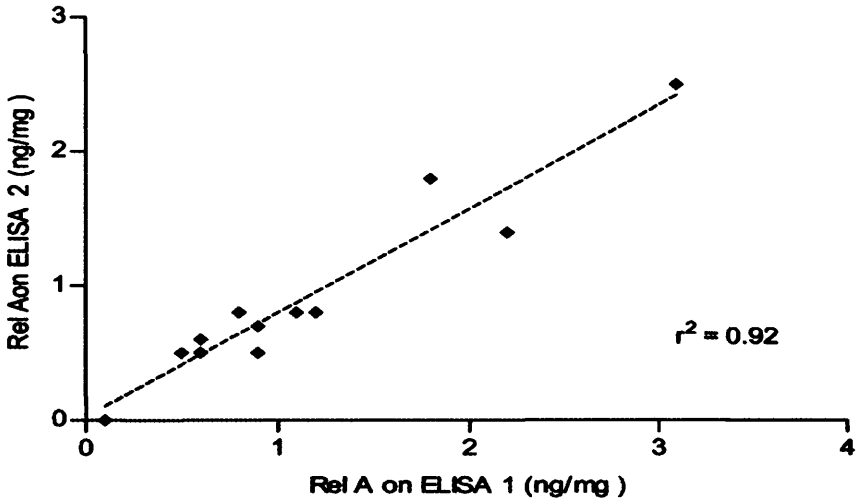

Figure 3.7 Inter assay variability of Rel A DNA binding was less than 10%. To assess the inter assay variability, Rel A DNA binding was measured from the same samples on two different ELISA plates. There was a strong correlation between two results generated on different days (r^2 = 0.92).

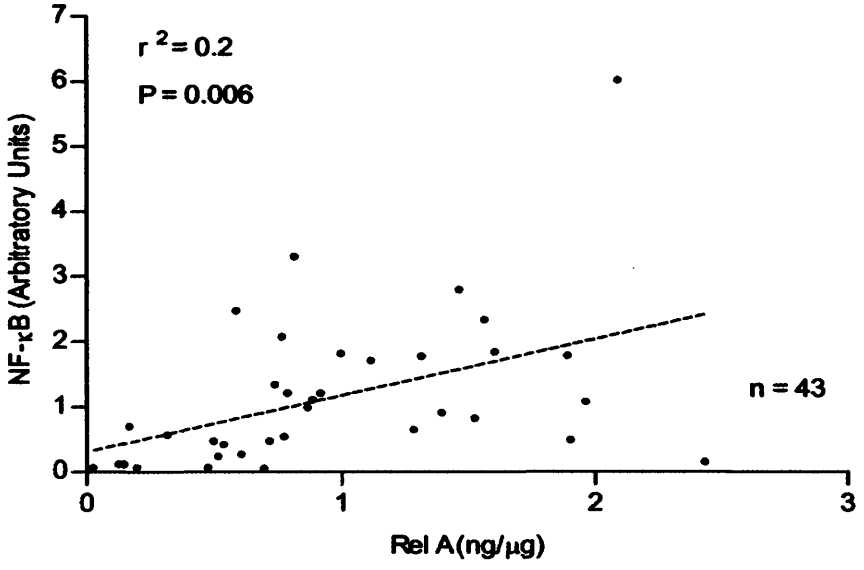

Figure 3.8 Rel A DNA binding correlates with total NF-κB DNA binding. NF-κB DNA binding was measured by EMSA and were compared with Rel A DNA binding measured by ELISA. There was a correlation between total NF-κB and its subunit Rel A measured by DNA binding methods (r^2 = 0.20)

After initial experiments to assess the reliability of quantifying Rel A using DNA binding ELISA, this technique was used for subsequent experiments. Figure 3.9 demonstrates Rel A DNA binding in B-cells, T-cells and monocytes from a normal donor. Samples derived from normal B- and T-lymphocytes had levels of Rel A below the threshold of detection. As shown in this figure there was a higher Rel A DNA binding among CLL samples when compared with normal B- and T-cells. Furthermore, CLL cells show wide variation in Rel A DNA binding. In the next stage of the study Rel A DNA binding was quantified in 30 CLL patient samples (Figure 3.10). There was considerable variability in Rel A: from undetectable levels up to 1.909 ng/μg of nuclear extract.

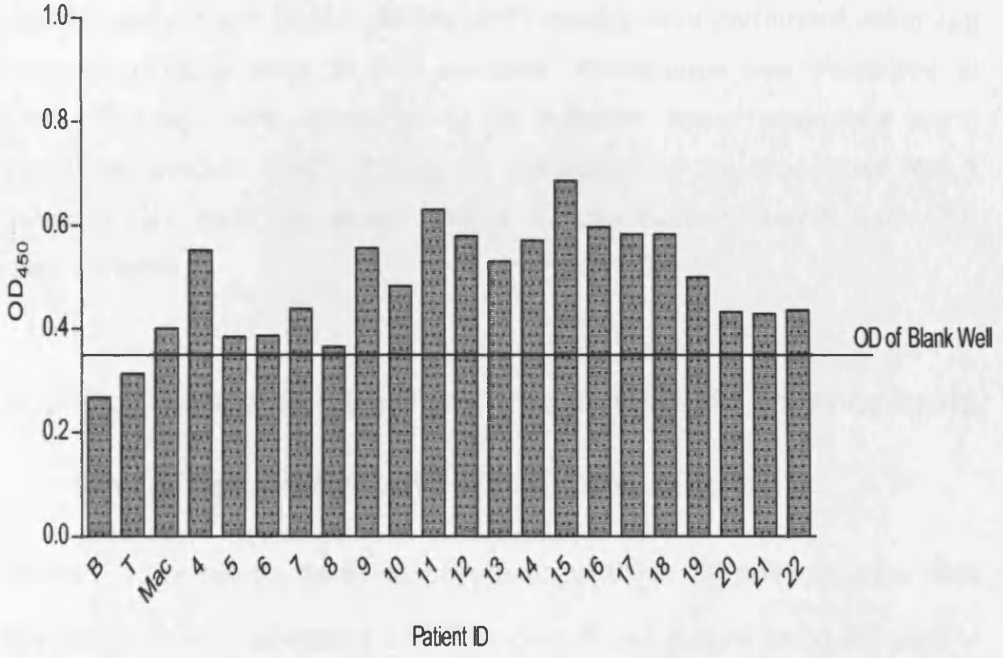

Figure 3.9 Rel A DNA binding was variable among CLL B-cells but higher than normal B- and T-cells. Subunit-specific Rel A ELISA (Active Motif) assays were performed using 2μg of nuclear extracts from B-cells, T-cells and macrophages from normal donors and 19 CLL samples. Absorbance was measured at 450nm.

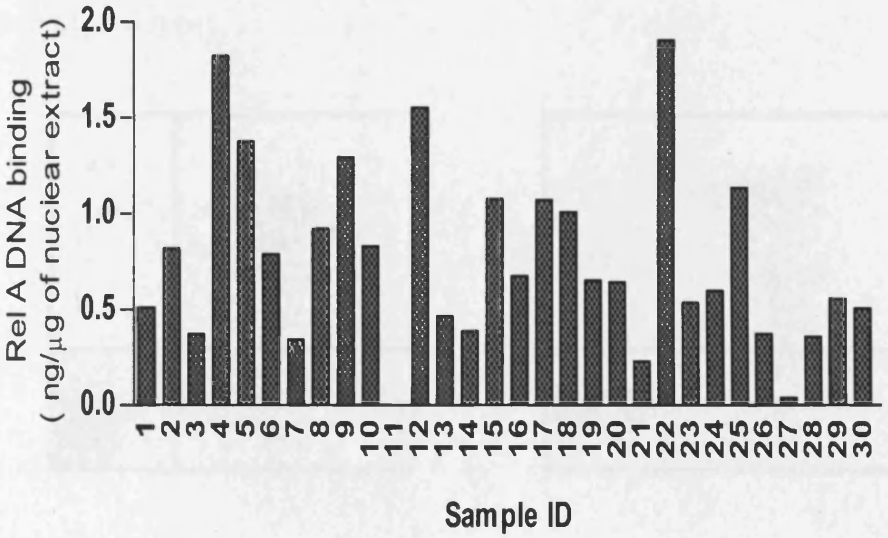

Figure 3.10 Quantitative analysis of Rel A DNA binding in CLL B-cells. Subunit-specific Rel A ELISA (Active Motif) assays were performed using 2μg of nuclear extracts from 30 CLL samples. Absorbance was measured at 450nm. Results were compared to a standard curve generated using recombinant protein which allowed the calculation of the amount of Rel A present in CLL cells expressed relative to total nuclear protein from CLL patient samples.

3.4.5 CLL samples show marked variability in apoptosis which correlates with Rel A DNA binding

In parallel experiments, samples from each of these 30 patients were also analysed for *in vitro* apoptosis after 48 hours in cell culture using Annexin V and propidium iodide labelling (Figure 3.11). Samples showed a wide variation ranging from 9% to 80% (Figure 3.12). This variation in spontaneous apoptosis was then correlated with the ex vivo Rel A DNA binding activity of each sample. Figure 3.13 shows that there was a significant inverse

correlation between CLL cell *in vitro* apoptosis and Rel A DNA binding activity ($r^2 = 0.21$, P = 0.01).

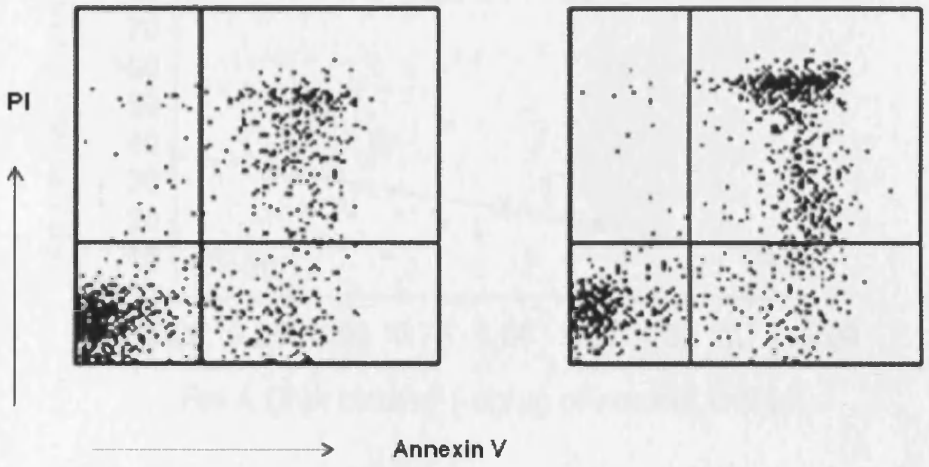

Figure 3.11 Apoptosis was assessed using Annexin V and PI staining. CLL cells were cultured for up to 48h and apoptosis was asseessed using Annexin V and PI staining.

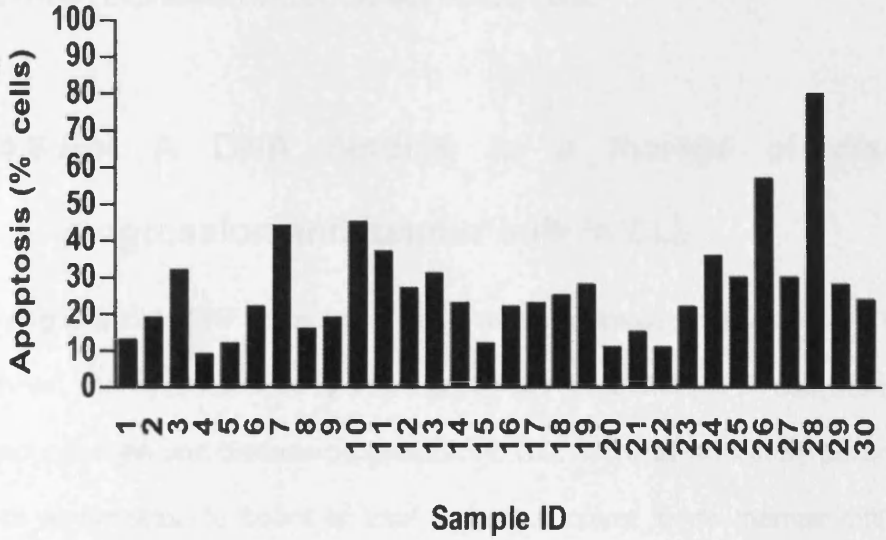

Figure 3.12 CLL samples show marked variability in spontaneous apoptosis. The *in vitro* spontaneous apoptosis of CLL cells was measured using Annexin V and Propodium Iodide (PI) staining (Top Panel). Percentage apoptosis was defined as the total Annexin V+ cells (PI+ and PI-) after 48 hours in culture derived from the same 30 CLL patients (Bottom Panel).

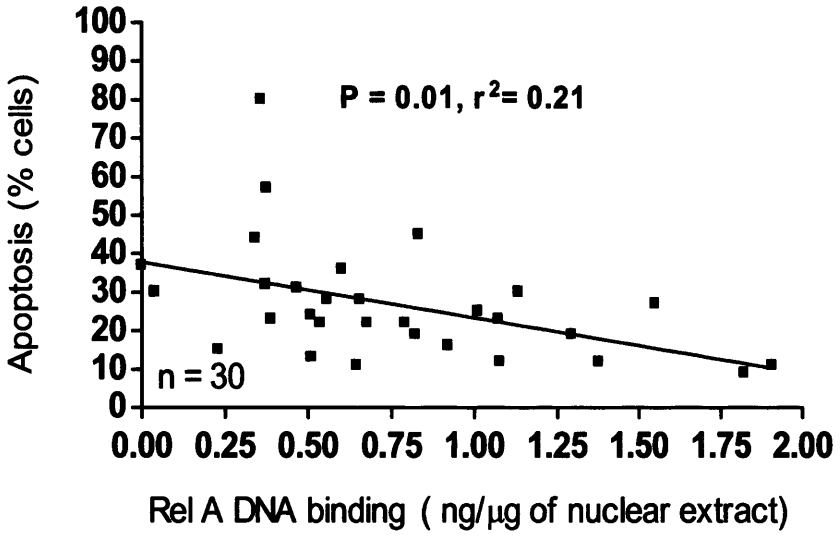

Figure 3.13 Correlation between Rel A DNA binding and apoptosis in CLL cells. Rel A DNA binding was measured using subunit specific ELISA and was correlated with spontaneous apoptosis measured by Annexin V and PI staining. The level of apoptosis showed a significant negative correlation with Rel A expression in the nucleus of CLL cells.

3.4.6 Rel A DNA binding is a marker of disease progression and tumour bulk in CLL

Having implicated NF-κB subunit Rel A as being important for *in vitro* CLL cell survival, the next step was to investigate Rel A in relation to clinical markers of tumour burden and disease progression in CLL. Clinical laboratory parameters such as lymphocyte count or total white cell count, bone marrow infiltration pattern and lymphocyte doubling time (LDT) reflect the tumour burden and/or disease activity in CLL (Montillo *et al.*, 2005). Rel A DNA binding, using the subunit-specific ELISA was compared with lymphocyte doubling time (LDT) and total white cell count on the date of sample collection. Rel A was

significantly higher in patients with an LDT less than 12 months (P=0.01; Figure 3.14). There was also a significant correlation (r^2=0.2, P=0.01) between Rel A DNA binding and total white cell count (Figure 3.15). Taken together these data suggest that Rel A contributes to tumour burden and disease activity in CLL.

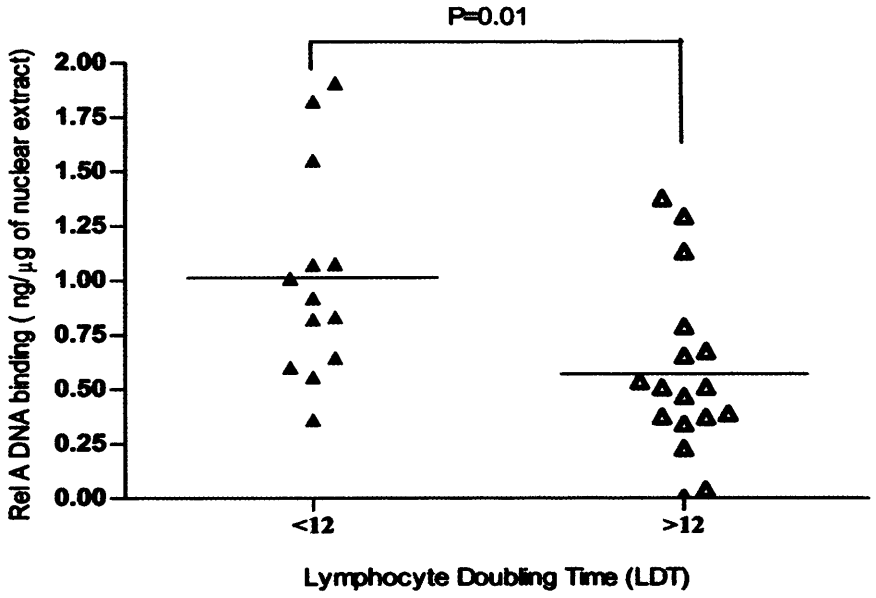

Figure 3.14 Rel A DNA binding is higher in samples from patients with shorter lymphocyte doubling time. Rel A levels were measured by DNA binding ELISA (Active Motif) and were compared in patients with a lymphocyte doubling time above or below 12 months. Patients with lymphocyte doubling times of less than 12 months showed significantly higher levels of Rel A (P=0.01).

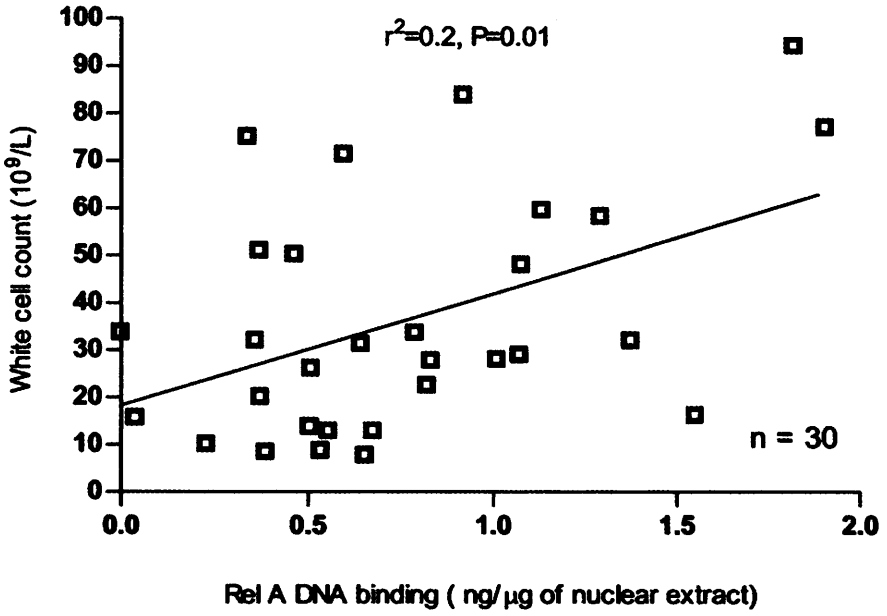

Figure 3.15 Correlation between Rel A DNA binding and total white cell count. Rel A levels were measured by DNA binding ELISA (Active Motif) and were compared in patients with WBC. There was a significant positive correlation between Rel A DNA binding activity in cells from patients and their total white cell count (r^2=0.2, p= 0.01).

3.5 Discussion

One of the key achievements of this part of the study was to move beyond the relatively simplistic concept that NF-κB is elevated in CLL patient samples towards a more detailed qualitative and quantitative study of this important family of transcription factors. CLL cells show wide variation in total NF-κB expression but typically express p50, Rel A and c-Rel but in varying amounts. In agreement with previous reports (Furman *et al.*, 2000; Cuni *et al.*, 2004), even the CLL samples that had low NF-κB expression had higher levels than primary B-cells in which no NF-κB could be detected by ELISA.

This present study used more than one method to assess NF-κB activity in CLL samples. EMSA is regarded as the "gold standard" of measuring NF-κB and ELISA is a new development within the last few years as a mean of quantifying NF-κB subunits. A comparison of total NF-κB and Rel A measurement in CLL patient samples showed that the correlation between Rel A DNA binding and total NF-κB DNA binding was only 20%. This further highlights the results of the supershift EMSA experiments that showed that p50 was the commonest subunit in CLL cells.

NF-κB has been implicated in tumourogenesis and survival of a growing list of leukaemias and lymphomas, including Hodgkin lymphoma, diffuse large B-cell lymphoma, multiple myeloma, acute lymphoblastic leukaemia and chronic myeloid leukaemia (Karin *et al.*, 2002). However, no previous study has quantified NF-κB subunits in primary patient samples. Furthermore, this is the first study to relate this quantification to *in vitro* cell survival, tumour burden and disease activity (LDT) thus demonstrating that an individual component of the NF-κB complex can contribute to the regulation of a human disease.

This study builds directly on others that have shown NF-κB provides pro-survival signals to a number of different cell types. Both primary cancer tissues and cell culture models of these cancers exhibit constitutive activated NF-κB and inhibition of this activity by over expression of IκB or pharmacological agents induces *in vitro* apoptosis (Romano *et al.*, 2000; Habens *et al.*, 2005; Pickering *et al.*, 2006). Our data correlating Rel A with spontaneous *in vitro* apoptosis revealed that the variation in Rel A can explain 21% ($r^2 = 0.21$) of the variation in apoptosis of CLL cells.

Surprisingly, there was no significant correlation between Rel A and IgV_H gene mutation status, CD38 expression or ZAP-70 expression. In contrast, there was a clear association between elevated Rel A, short LDT and high WBC. LDT and WBC are markers of disease progression and tumour burden and have been shown to correlate with overall survival in a number of studies and reinforce the importance of this transcription factor in CLL (Montserrat *et al.*, 1986; Vinolas *et al.*, 1987; Wierda *et al.*, 2007). I hypothesize that the lack of correlation between basal Rel A DNA binding and IgV_H gene status, CD38 and ZAP-70 is because these factors contribute to the ability of CLL cells to induce NF-κB following stimulation rather than constitutive expression. This notion is currently being tested experimentally in our laboratory.

In summary, in this part of the study I analysed both total and NF-κB subunit Rel A in relation to other prognostic parameters. The data highlight the importance of NF-κB subunit Rel A in relation to *in vitro* survival and possibly proliferation of CLL cells. Importantly, this work suggests that NF-κB has the potential to be a target for therapy in CLL and the potential to be a valuable biomarker for disease. These two issues will be addressed in the next two chapters of this thesis.

4.0 NF-κB INHIBITION IN CLL

4.1 INTRODUCTION

Chemotherapeutic drugs, such as fludarabine, chlorambucil, prednisolone and certain monoclonal antibodies induce CLL cell apoptosis *in vivo*, although complete remission is difficult to attain and most patients eventually relapse (Schriever *et al.*, 2003). The results of the UK CLL4 clinical trial clearly showed improved responses to fludarabine plus cyclophosphamide (FC) when compared to fludarabine or chlorambucil monotherapies. Therefore FC has now become the standard chemotherapy in the UK. However, this increase in response rates is at the expense of elevated off-target toxicities e.g. in the FC arm 56% of the patients developed neutropenia and 38% needed hospital admission (Catovsky *et al.*, 2007).

Most side effects are due to the non-selectivity of drugs toward target malignant cells. Development of new treatment options based on the biology of CLL cells is needed to improve prognosis, limit side effects and induce cures. Treatment strategies to target molecules that support the maintenance and growth of the tumour cells have been pursued (Griffin *et al.*, 2001); these approaches have the ability to alter the nature of clinical cancer treatment because they can distinguish cancer cells from their normal counter-parts (Kurzrock *et al.*, 2007). Agents like Imatinib mesylate, a Bcr-Abl and Kit kinase inhibitor in chronic myeloid leukaemia, and all-trans retinoid acid (ATRA) in acute promyelocytic leukaemia have revolutionised the treatment of these tumours by inducing remarkable responses with minimal host toxicities.

Several survival signalling mechanisms are implicated *in vivo* and *ex vivo* survival of CLL cells (Cuni *et al.*, 2004; Hajdu *et al.*, 2005; Perez-Chacon *et al.*, 2007; Natani *et al.*, 2007; Pablo *et al.*, 2007). It is now believed the relative resistance to apoptosis in CLL cells is caused by a combination of micro-environmental signals and inherent defects in cell cycle and apoptosis regulatory machinery in these cells (Endo *et al.*, 2006). The contribution of the micro-environment is believed to be mediated by humoral factors and cell to cell interactions (Ghia *et al.*, 2005). Bone marrow stromal cells and nurse like cells are prime candidates for cells 'feeding' survival signals to CLL cells (Ghia *et al.*, 2005; Endo *et al.*, 2006). IL-4 and IL-1β are among the humoral factors known to contribute to CLL cell survival. Also it has been shown that CLL cells can regulate their survival by secreting growth factors in an autocrine manner (Kern *et al.*, 2004; Novonty *et al.*, 2008). BCL-2 family proteins (Bcl-2, Bcl-XL, and Mcl-1) and IAP family proteins (IAP1, IAP2, XIAP, Survivin) are major candidate pro-survival proteins considered to be important in CLL (Pepper *et al.*, 2003; Schliep *et al.*, 2004; Grzybowska-Izydorczyk *et al.*, 2008; de Graaf *et al.*, 2008). Several cell signalling pathways, including the NF-κB pathway, are involved in regulation of these anti-apoptotic proteins; implicating a role of NF-κB in modulation of these anti-apoptotic factors and hence in the regulation of CLL cell survival (Munzert *et al*, 2002).

4.2 NF-κB inhibition in CLL

The NF-κB cell signalling pathway is an attractive therapeutic target as NF-κB proteins are implicated in the regulation of genes that control apoptosis, proliferation, angiogenesis and metastasis (Koch *et al.*, 1992; Beg *et al.*, 1996; Wang *et al.*, 1999; Cao *et al.*, 2001) in various cancers. Furthermore, CLL

cells have been reported to exhibit high constitutive NF-κB activation compared to normal B-lymphocytes (Furman *et al.*, 2000; Cuni *et al.*, 2004; Tracey *et al.*, 2005; see Chapter 3) and numerous NF-κB inhibitors have been shown to have *in vitro* efficacy in CLL cells (Escobar-Diaz *et al.*, 2005; Horie *et al.*, 2006; Steele *et al.*, 2006; Everett *et al.*, 2007; Geeraerts *et al.*, 2007; Pickering *et al*,. 2007). The sesquiterpene lactone parthenolide (PTL) and its analogue, dimethylamino-parthenlide (DMAPT), are probably the most widely studied NF-κB inhibitors to date. PTL has been shown to be cytotoxic to cells isolated from patients with CLL with minimal cytotoxicity to normal T-lymphocytes or CD34 (+) haematopoietic progenitor cells (Steele *et al.*, 2006).

4.3 Cytotoxicity (apoptosis) assessment

A number of different techniques can be used to quantify the cytotoxic effect of drugs on leukaemia cells. These include,

1) Trypan blue exclusion method.

2) ^{3}H-thymidine incorporation assay.

3) Colourimetric proliferation assays using compounds such as MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) or MTS (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulphophenyl] -2H- tetrazolium salt)

4) Apoptosis assays such as the Annexin V binding assay or the detection of poly (ADP)-ribose polymerase (PARP) cleavage.

5) Activation of various members of caspase cascade can be used as a measure of cell death.

(Martin *et al.*, 2001; Muppidi *et al.*, 2004; Rodriguez *et al.*, 2005)

4.4 Drug combination studies

Confusion surrounds the use of the terms synergism to describe the effects of drug combinations. Also terms like augmentation, potentiation, super-additivism and additivism are used in place of synergism in the literature of pharmacological studies (Berenbaum *et al.*, 1977). Several different terms are used to describe the same phenomenon and the same term means different things to different authors (Goldin *et al.*, 1957). The introduction of the combination index method by Chou & Talalay has achieved a greater degree of scientific consensus over fractional product method by Webb (Webb *et al.*, 1961; Chou *et al.*, 1984). The Chou & Talalay method is based on the median effect principle; which is a single generalised method that can be applied to analyse dose-effect relationships in enzymatic, cellular and whole animal systems. It is derived from the law of mass-action: the basic law in nature that forms the basis of chemical equilibrium dynamics, biological receptor theory and enzyme kinetics. The Chou & Talalay method is distincts from all other methods by the fact that it not only takes in to account the 'potency' but also the 'shape' of dose-effect curves of each drug and their combinations. The median effect equation correlates the 'dose' and 'effect' according to the relationship:

$$f_a/f_u = (D / D_m)^m$$

Where: D is the drug dose

D_m is the 'median-effect dose' – equivalent to the IC_{50}

f_a is the fraction affected by the dose

f_u is the fraction unaffected ($f_u=1-f_a$)

m is a coefficient signifying the sigmoidicity of the dose effect curve

The median effect plot is a plot of log (D) on the x-axis against log (f_a/f_u) on the y-axis. This plot has the form of a straight line, with the x-intercept equating to D_m: the median effect dose, and the slope of the line equating to m: the coefficient signifying the sigmoidicity of the dose-effect curve. The Chou and Talalay method designates a combination index (CI) for each drug combination studied. The combination index equation is derived from the median effect equation and describes the interaction between the two drugs. CI<1 indicate synergism, CI=1 indicate an additive effect while CI>1 indicate antagonism. In addition to calculating CI, newer computer software programmes also gives the dose reduction index (DRI). This determines how many fold of dose reduction is allowed for each drug in synergistic combinations. Reduction of dose (Dose reduction in combination drug experiments) leads to reduce toxicity while mainatining the desired efficacy.

In the current study, the software package Calcusyn (Biosoft) was used to construct median effect plots and calculate combination indices for each individual patient for each drug combination. Also we used this software to calculate the DRI for fludarabine in combination with LC-1. Non-mutual exclusivity was assumed for combinations of LC-1 with fludarabine. These assumptions were generally borne out in the layouts of the resulting median-effect plots. Combination indices differ according to the level of effect. If a drug is meant to stay in the body as an active agent for a long duration of time (drugs used for chronic disorders) it is important to demonstrate synergism at a low affect level. But some other drugs are meant to exert their effect more quickly and be inactivated or metabolised. In these circumstances it is more

important to be synergistic at a higher effect level. We used CI at 50% cell killing for interpretation of data.

4.5 RESULTS

Table 4.1 Patient characteristics

Patient ID	Gender	Stage	CD38	ZAP-70	IgV_H Status
1	M	A	32	12	M
2	M	A	20	7	M
3	F	A	16	22	M
4	M	B	45	75	U
5	M	A	2	6	M
6	M	A	5	2	M
7	M	A	37	9	U
8	M	A	3	36	M
9	F	A	62	78	M
10	F	A	56	34	U
11	F	A	97	79	U
12	M	A	8	18	M
13	M	B	62	27	U
14	M	A	3	9	M
15	M	C	3	29	U
16	M	A	7	3	M
17	F	B	4	3	M
18	M	A	4	4	M
19	M	A	36	87	M
20	F	C	99	100	M
21	F	A	47	76	U
22	M	A	24	37	M
23	M	A	31	23	U
24	M	A	3	0	M
25	F	A	4	ND	M
26	M	A	1	0	M
27	F	A	5	6	U
28	M	A	3	6	M
29	M	A	5	7	M

Patient ID	Gender	Stage	CD38	ZAP-70	IgV$_H$ Status
30	M	A	0	6	M
31	M	A	100	63	M
32	M	A	32	8	M
33	M	C	68	17	U
34	M	B	34	1	U
35	M	A	0	38	M
36	M	A	85	94	U
37	M	A	18	22	U
38	F	A	70	33	U
39	F	A	2	14	M
40	M	C	4	43	U
41	F	A	79	3	M
42	F	A	2	1	M
43	M	A	73	1	U
44	F	A	0	10	M
45	M	A	5	12	M
46	M	A	100	8	M
47	M	C	65	68	U
48	F	A	7	23	M
49	F	B	41	51	U
50	M	A	31	39	U
51	F	A	5	ND	U
52	M	B	2	67	U
53	M	A	94	7	U
54	F	B	6	64	U
55	F	A	97	43	ND
56	F	C	7.4	3	U
57	F	A	0.6	0.7	M
58	M	C	4.6	0.2	M
59	M	B	2.4	2	M
60	M	A	35	10.6	M
61	M	A	84	1	U

Patient ID	Gender	Stage	CD38	ZAP-70	IgV_H Status
62	M	A	0.6	17	ND
63	M	A	2	1.3	M
64	F	A	0.7	5.4	M
65	M	A	6.6	10	M
66	M	A	35	0.7	U
67	M	A	3.7	10	M
68	M	A	8.9	4.6	ND
69	M	A	ND	ND	ND
70	M	A	50.4	15.4	ND
71	F	A	6	3.6	ND
72	M	A	4.6	10.3	M
73	M	A	81	85	ND
74	M	C	38	65	ND
75	M	A	100	87	ND
76	F	A	5.4	3	ND
77	F	A	0.6	0.2	ND
78	M	A	2.3	2	ND
79	M	A	1	6.5	M
80	M	A	1.3	1.1	M
81	M	A	93.5	8.9	M
82	M	A	1.5	0.1	M
83	M	A	9.8	10.4	M
84	M	A	46	1.7	M
85	F	A	16	12.7	M
86	F	A	100	66	ND
87	F	A	100	94.5	M
88	F	A	0.6	1	ND
89	M	A	2	4.3	ND
90	M	A	0.34	6	M
91	F	A	1.1	20.4	M
92	F	A	12	20	ND
93	M	A	47	98	U

Patient ID	Gender	Stage	CD38	ZAP-70	IgV_H Status
94	M	A	85	99	ND
95	F	A	5	27	ND
96	M	A	0.4	3.9	M

4.5.1 LC-1 induces apoptosis of CLL cells

The *in vitro* effects of the NF-κB inhibitor, LC-1 were investigated in freshly isolated CLL patient samples. LC-1 is a derivative of the sesquiterpene lactone parthenolide which has been shown to selectively kill CLL cells *in vitro* (Steele *et al.*, 2006) and targets the transcription factor NF-κB. Parthenolide has poor solubility and unfavorable pharmacokinetics (Sweeney *et al.*, 2005). In contrast, LC-1 has superior pharmacokinetic properties and over 1000-fold greater solubility than the parent compound and therefore represents an excellent candidate drug. CLL cells were incubated with a range of concentrations of LC-1 (0.5-8 μM) for up to 48h and apoptosis was quantified using Annexin V and propidium iodide labelling. LC-1 induced time- and dose-dependent apoptosis in primary CLL cells (Figure 4.1). The drug concentration necessary to cause 50% cell death (LD_{50} values) were calculated for each patient sample using GraphPad prism software. An example from a representative patient sample is shown in Figure 4.2. The mean LD_{50} value (±SD) for LC-1 in the 96 CLL patient samples tested was 2.9 μM (± 1.4 μM) at 24h and 1.8μM ± 1.0 at 48h (Figure 4.3).

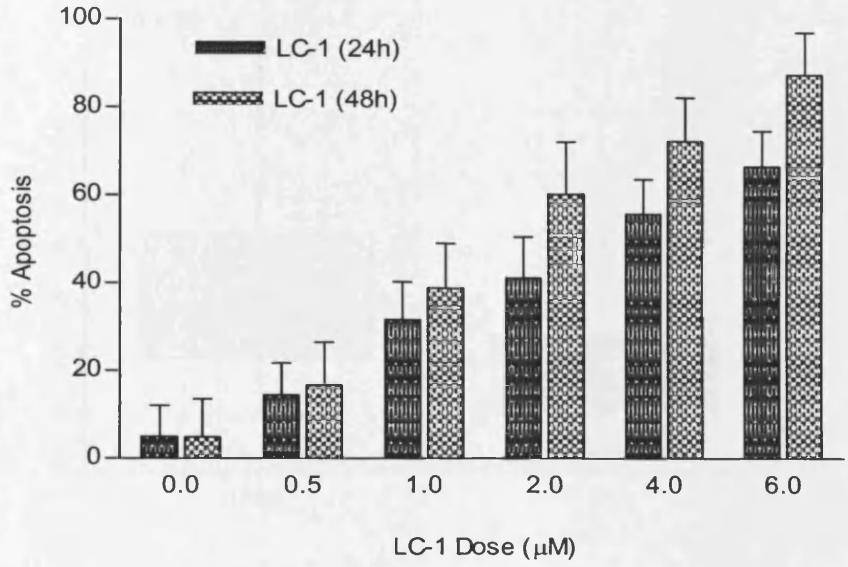

Figure 4.1 LC-1 induced time and concentration-dependent apoptosis in primary CLL cells. CLL cells were treated with LC-1 (0-8μM) for 24h and 48h and were then labelled with Annexin V and propidium iodide. The percentage of apoptotic cells was calculated as the sum of Annexin V+ / propidium iodide+ and Annexin V+ / propidium iodide-. A concentration-dependent and time-dependent increase in Annexin V positive cells was observed.

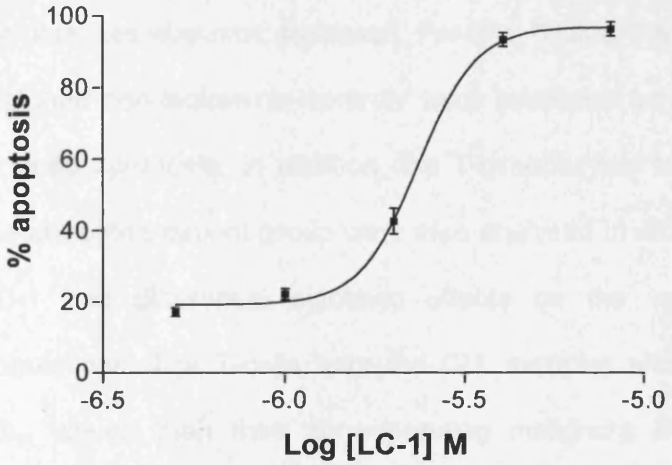

Figure 4.2 Representative example of a sigmoid dose-response curve generated from CLL cells cultured with LC-1 for 24h.

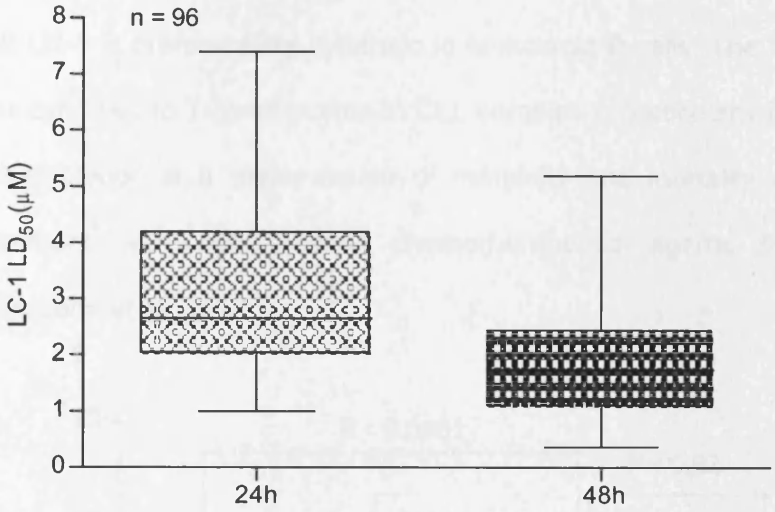

Figure 4.3 Mean LD_{50} values (± SD) for LC-1 were 2.9μM (± 1.4 μM) at 24h and 1.8μM (±1.0 μM) at 48h. CLL cells incubated with LC-1 (0-8μM) and apoptosis assayed using Annexin V and PI staining. LD_{50} values were calculated using Graphpad Prism software using best fit sigmoid curves.

4.5.2 LC-1 is preferentially cytotoxic to CLL cells

The relative cytotoxicity of LC-1 in CLL cells and normal B- and T-lymphocytes was next assessed. For this, B- and T-lymphocytes from 10 age-matched non-leukaemic controls were assessed for their sensitivity to LC-1-induced apoptosis. In addition, the T-lymphocytes from 6 CLL patients from the untreated patient group were also analysed in order to determine whether LC-1 had differential cytotoxic effects on the various lymphocyte sub-populations. The T-cells from the CLL samples showed consistently higher LD_{50} values than their corresponding malignant B-cell clones ($P<0.0001$; paired *t*-test). In addition, the normal age-matched control B-lymphocytes and T-lymphocytes demonstrated higher LD_{50} values than the CLL cells ($P<0.0001$). The relative sensitivities of the various lymphocyte populations to

LC-1 are illustrated in Figure 4.4. The conclusion from these experiments is that LC-1 is preferentially cytotoxic to leukaemic B-cells. The fact that LC-1 is less cytotoxic to T-lymphocytes in CLL samples is particularly interesting as T-cell depletion is a major cause of morbidity and mortality associated with treatment with conventional chemotherapeutic agents like fludarabine (Blijlevens *et al.*, 2005).

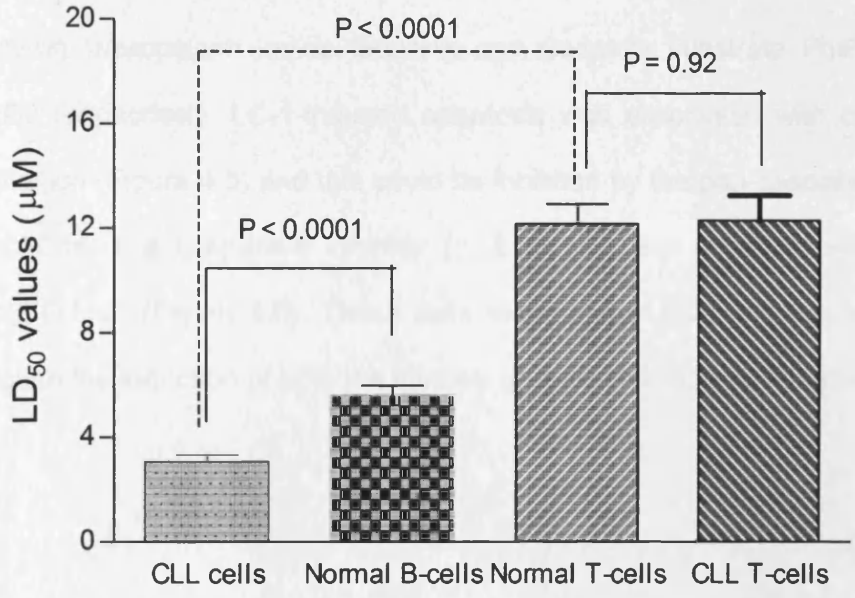

Figure 4.4 LC-1 is preferentially cytotoxic to leukaemic B-cells. B-cells and T-cells from non-leukaemic, age-matched, donors and T-cells from CLL patients were incubated with LC-1. Apoptosis was assessed using Annexin V and propidium iodide. The percentage of apoptotic cells was calculated as the sum of Annexin V+ / propidium iodide+ and Annexin V+ / propidium iodide-. LD_{50} Values calculated using Graphpad Prism software. Normal B-lymphocytes were more than 2-fold less sensitive to the apoptotic effects of LC-1 than CLL cells ($P<0.0001$).

4.5.3 LC-1 induces both the intrinsic and extrinsic apoptotic pathways

As the next step, the kinetics of LC-1-induced cell killing and the specific mechanism(s) of apoptosis induction in CLL patient samples were studied. CLL cells were incubated with a range of concentrations of LC-1 (0.5-8μM) for up to 48h and apoptosis and caspase-3 activation was quantified using Annexin V/propidium iodide labelling and Caspase substrate PhiPhiLuxTM G1D2 respectively. LC-1-induced apoptosis was associated with caspase-3 activation (Figure 4.5) and this could be inhibited by the pan-caspase inhibitor (z.VAD.fmk), a caspase-9 inhibitor (z.LEDH.fmk) and a caspase-8 inhibitor (z.IETD.fmk) (Figure 4.6). These data indicate that LC-1 induces apoptosis through the induction of both the intrinsic and extrinsic apoptotic pathways.

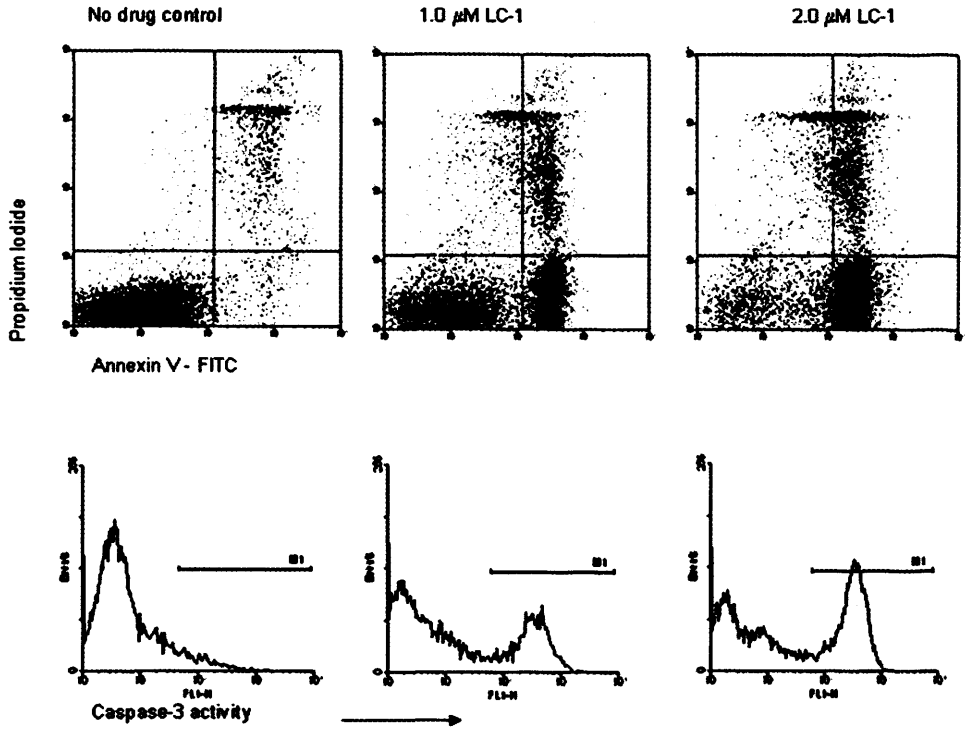

Figure 4.5 LC-1 induced apoptosis was associated with caspase-3 activation. (A) CLL cells were treated with LC-1 (0-8μM) for 24h and 48h and were then labelled with Annexin V and propidium iodide. A concentration-dependent increase in Annexin V positive cells was observed. (B) Under the same conditions, caspase-3 activity was determined by using a fluorogenic substrate molecule that once cleaved by active caspase-3, fluoresced green and was detected by flow cytometry. The increase in caspase-3 activation was concentration dependent and was correlated with an increase in apoptotic cell death.

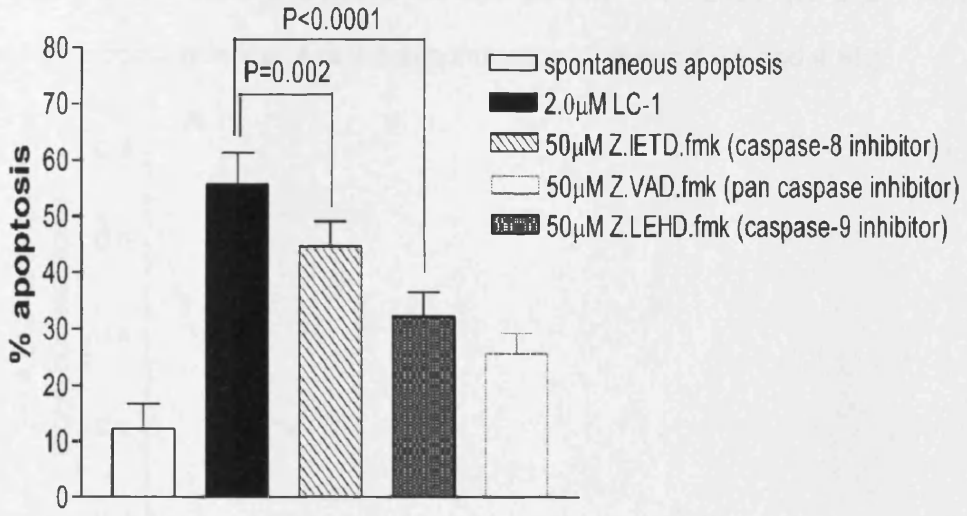

Figure 4.6 LC-1 induces both the intrinsic and extrinsic apoptotic pathways. CLL cells were cultured with a caspase-8 inhibitor (50 μM), a caspase-9 inhibitor (50 μM) or a pan caspase inhibitor (50 μM) with or without LC-1. Apoptosis was measured using Annexin V and PI staining. LC-1 induced apoptosis was partially abrogated by all three caspase inhibitors.

4.5.4 LC-1 inhibits Rel A DNA binding

To investigate the relationship between LC-1-induced apoptosis and inhibition of NF-κB DNA binding in CLL cells the following experiments were performed. CLL cells were incubated with LC-1 (0-8μM) for 4 hours before harvesting cells for apoptosis assessment or for protein extraction. Protein extracts were used for Rel A DNA binding assay by ELISA. Figure 4.7A demonstrates that LC-1 inhibits Rel A DNA binding in a dose-dependent manner. Also, CLL cells were incubated with 2μM of LC-1, and apoptosis was assessed and protein extracted hourly for up to 6h. Figure 4.7B demonstrates that LC-1 induced a time-dependent decrease in Rel A DNA binding. The inhibition is detectable at

4 hours and precedes any loss of cell viability and hence the effect is not merely a consequence of cell death induction (Figures 4.8A and 4.8B).

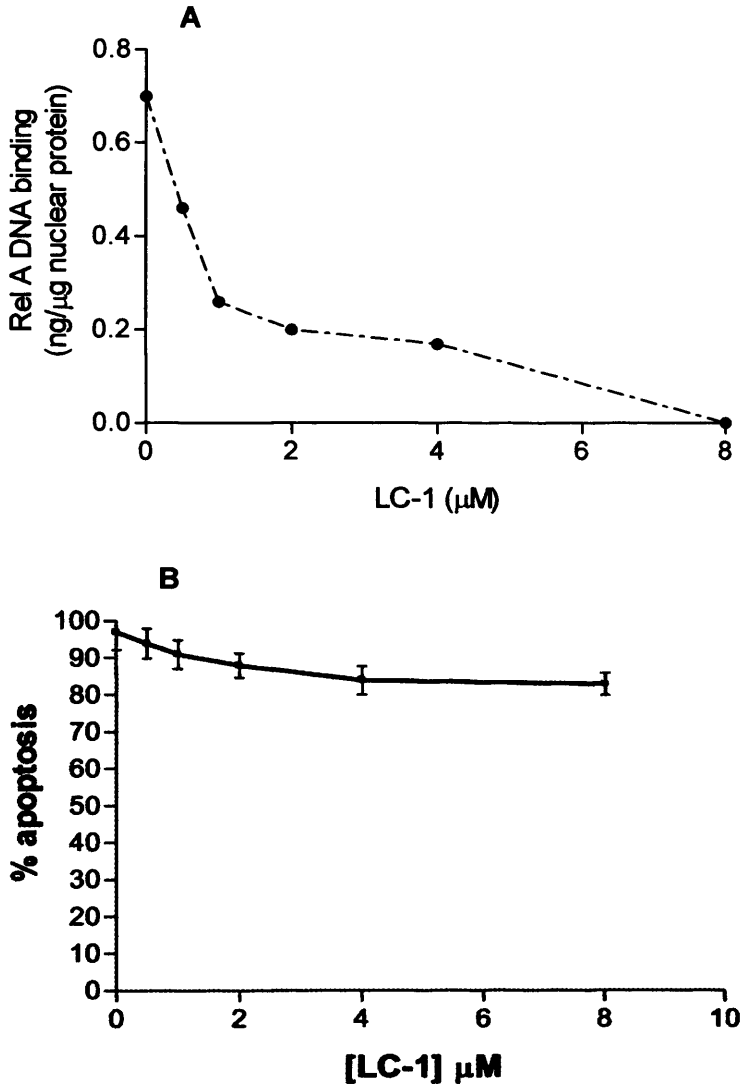

Figure 4.7 LC-1 causes a dose-dependent reduction in Rel-A DNA binding that precedes apoptosis induction in CLL samples. (A) CLL cells were treated with 2μM of LC-1 for 1h, 2h, 4h and 6h prior to apoptosis assay and nuclear protein extraction. ELISA analysis revealed a time-dependent decrease in Rel A DNA binding (B) Paired samples were analysed for apoptosis over the same time course with little evidence of elevated cell death.

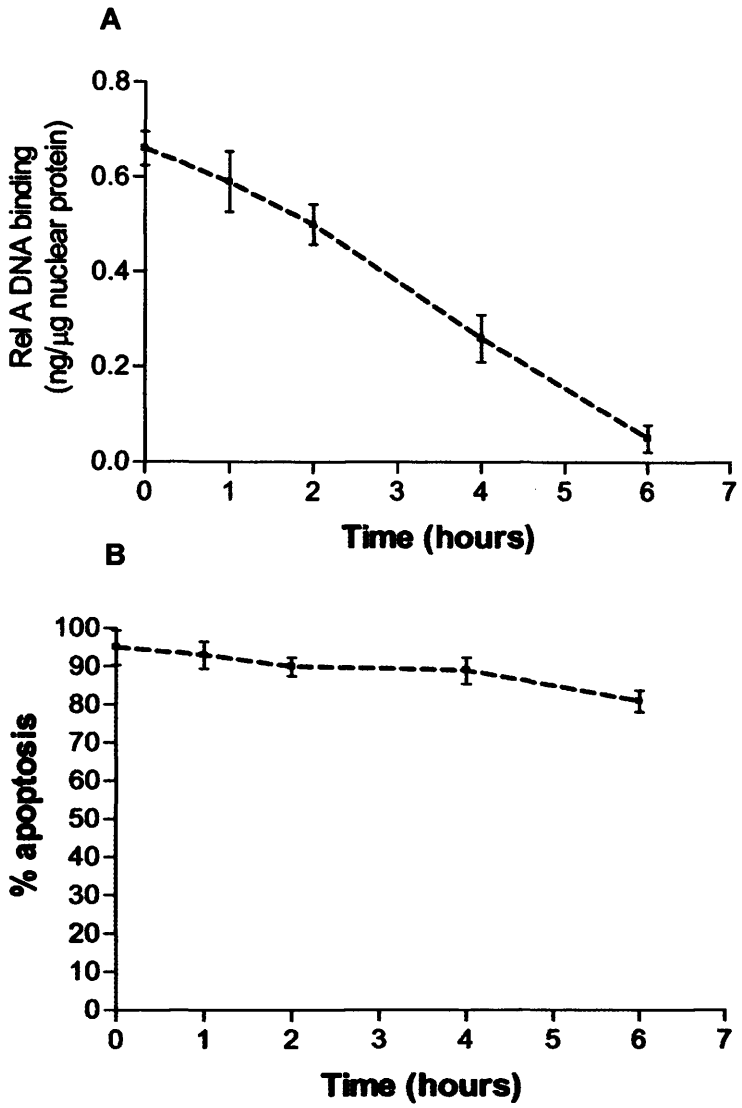

Figure 4.8 LC-1 induced a time-dependent reduction in Rel A DNA binding. (A) CLL cells were treated with 2μM of LC-1 for 1h, 2h, 4h and 6h prior to apoptosis assay and nuclear protein extraction. ELISA analysis revealed a time-dependent decrease in Rel A DNA binding. (B) Up to the 6h time point there was little evidence of apoptosis induction as measured by flow cytometry using the Annexin V / propidium iodide assay.

4.5.5 LC-1-induced suppression of NF-κB target gene transcription

In the next part of this study, I examined whether LC-1-mediated inhibition of Rel A had an effect on downstream gene transcription using real time RT-PCR. Three NF-κB-regulated genes (CFLAR, BIRC5 and BCL2) were selected for evaluation due to their known importance in CLL cell survival (Granziero *et al.*, 2002; Aron *et al.*, 2003; Tracey *et al.*, 2005). Gene transcription was compared in paired samples from individual patients (n = 6) with and without the addition of 2μM LC-1 for 4h. Figure 4.9 demonstrates significant reductions in the transcription of all 3 NF-κB-regulated genes following exposure to LC-1 (CFLAR by 2.7 fold, BIRC5 by 5.3 fold and BCL2 by 3 fold).

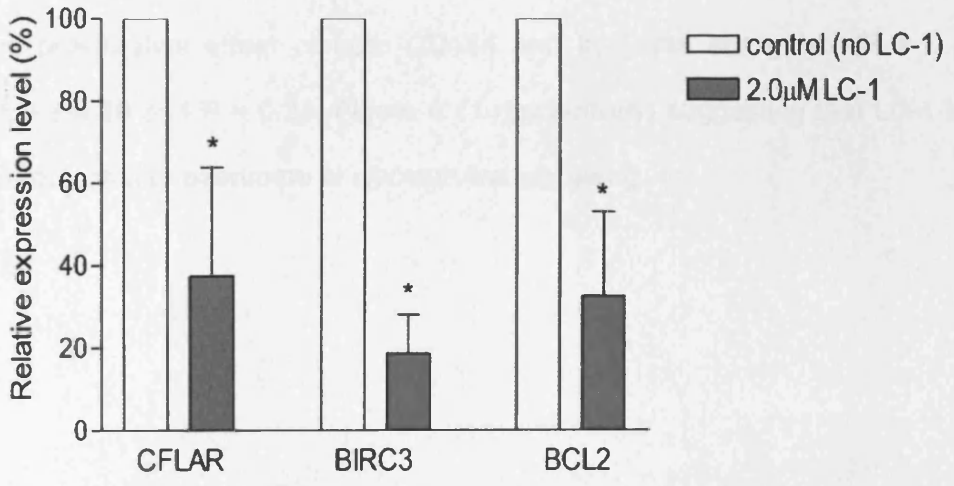

Figure 4.9 LC-1-induced suppression of NF-κB target gene transcription. $5x10^6$ CLL cells incubated with LC-1 (2μM) for 4h prior to RNA extraction. The expression of CFLAR, BIRC5 and BCL2 were quantified using real-time PCR. For each case, experiments were carried out in duplicate.

4.5.6 LC-1 overcomes the cytoprotective effects of CD40 ligand and Interleukin-4

There is growing evidence that micro-environmental factors play a major role in *in vivo* survival of CLL cells (Ghia *et al.*, 2000; Caligaris-Cappio *et al.*, 2000; Ghia *et al.*, 2005). A number of these factors, including CD40 ligand (CD154) and interleukin-4 (IL-4), cause their pro-survival effects, at least in part, via the induction of NF-κB (Furman *et al.*, 2000; Barragan *et al.*, 2002; Zaninoni *et al.*, 2003). As these signals appear crucial to CLL cells *in vivo*, it was important to assess the cytotoxic effects of LC-1 in the presence of these survival signals *in vitro*. Recombinant CD154 (1μM) or recombinant IL-4 (5nM) was added to the *in vitro* cytotxocity assays. As expected, the addition of CD154 and IL-4 significantly reduced spontaneous apoptosis in the untreated control cultures ($P = 0.0006$ and $P = 0.004$ respectively). However, in the presence of LC-1 the pro-survival effect of both CD154 and IL-4 was abrogated ($P = 0.49$, Figure 4.10 and $P = 0.33$, Figure 4.11 respectively) suggesting that LC-1 has the potential to overcome *in vivo* survival signalling.

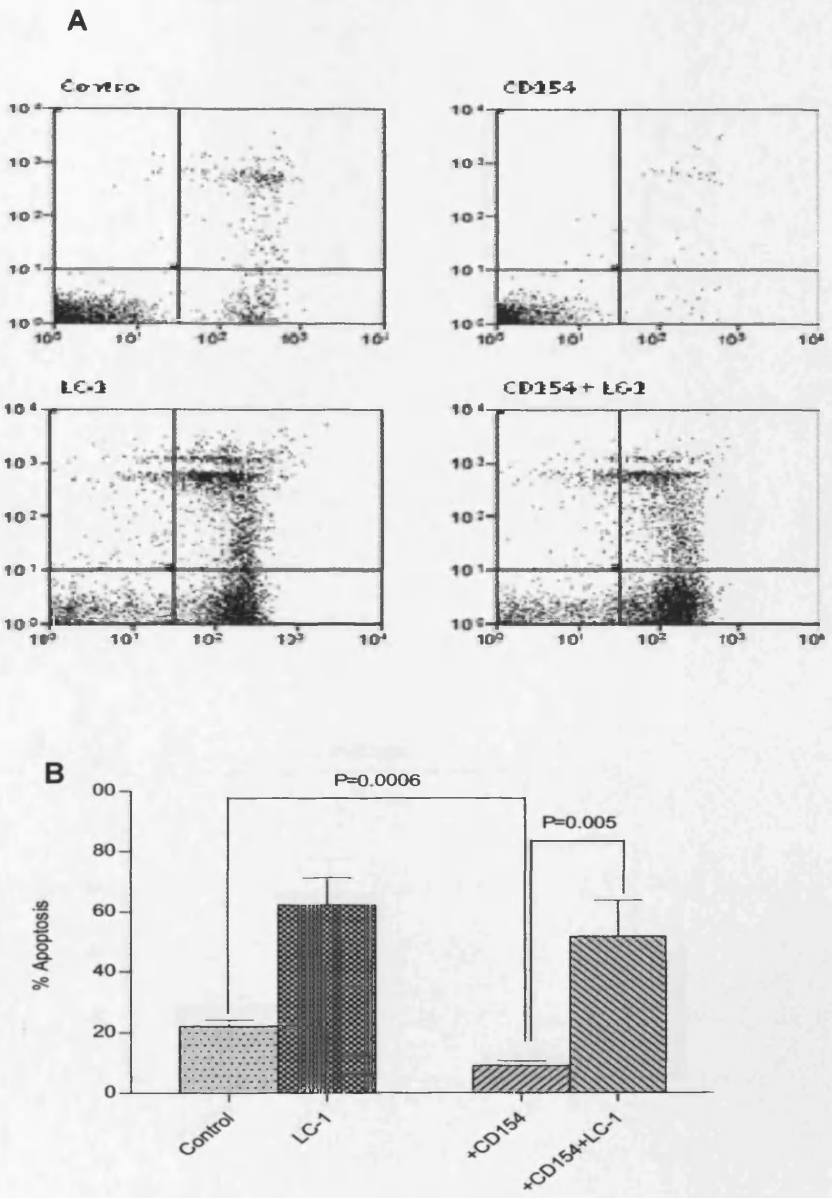

Figure 4.10 LC-1 over comes the cytoprotective effect induced by CD40 ligation. (A) CLL cells from 7 patient samples were cultured with or without CD154 (1μM) for up to 48h. (B) There was a significant cytoprotective effect by CD154 when compared with control cultures (P = 0.0006). Addition of LC-1 (2μM), overcome the pro-survival effect of CD154 (P = 0.49).

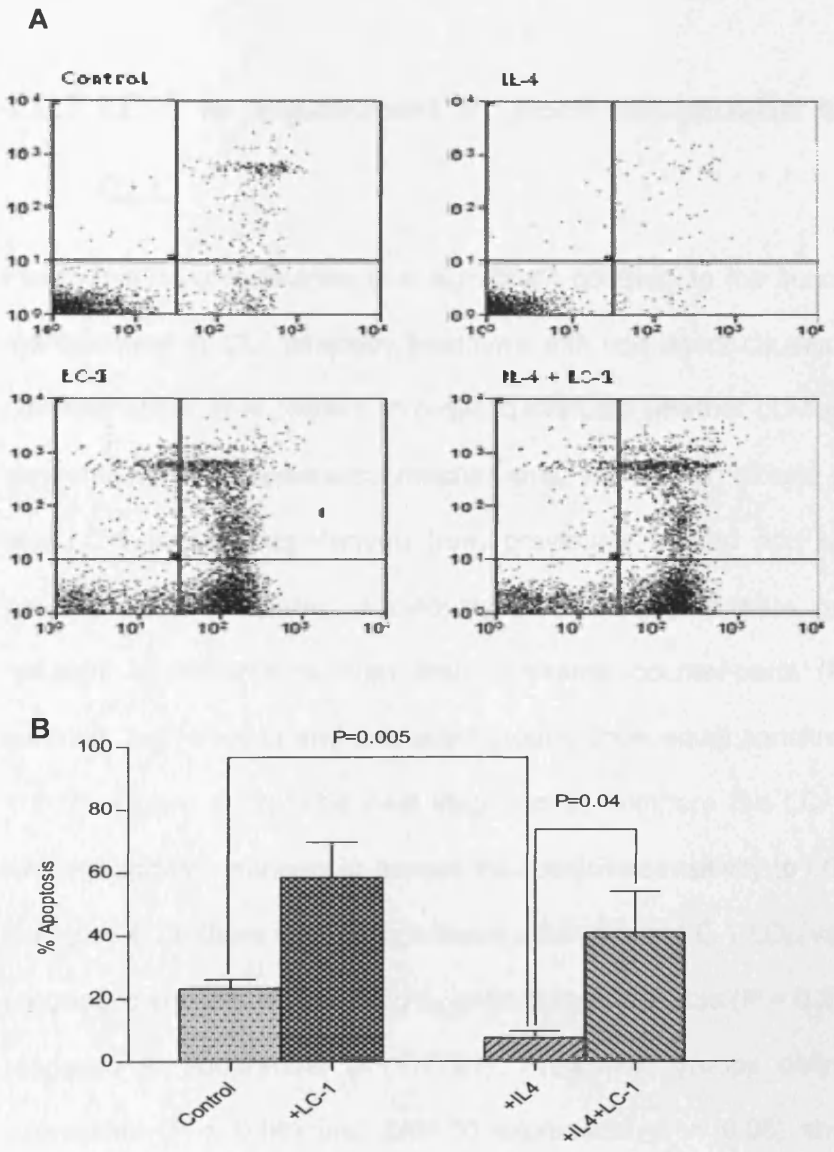

Figure 4.11 LC-1 over comes the cytoprotective effect induced by IL-4. (A) Mononuclear cells from 5 patient samples were cultured with or without IL-4 (5nM) for 24h. (B) There was a significant cytoprotective effect by IL-4 when compared with control cultures (P = 0.004). Addition of LC-1 (2μM), overcome the pro-survival effect of IL-4 (P = 0.33).

4.5.7 LC-1 is equipotent in poor prognostic subsets of CLL

Pleiotropic drug resistance is a significant obstacle to the successful clinical management of CLL whereby treatment with one agent causes resistance to others (Pepper *et al.*, 1999). In order to evaluate whether LC-1 was subject to conventional drug resistance mechanisms, the *in vitro* effects of fludarabine and LC-1 in samples derived from previously treated and untreated CLL patients were compared. Previously treated patients were relatively more resistant to fludarabine than their untreated counter-parts ($P<0.0001$). In contrast, both treated and untreated groups show equal sensitivity to LC-1 ($P = 0.53$, Figure 4.12). The next step was to compare the LC-1 LD_{50} values within prognostic subsets to assess their relative sensitivity to LC-1. As shown in Figure 4.13, there was no significant difference in LC-1 LD_{50} values between prognostic subsets define by IgV_H gene mutation status ($P = 0.21$) and *in vitro* response to fludarabine ($P = 0.21$). Prognostic groups defined by CD38 expression ($P = 0.06$) and ZAP-70 expression ($P = 0.05$) showed a trend towards increased sensitivity to LC-1 in the poor prognostic subsets. In addition, patients with 17p and 11q abnormalities also showed similar sensitivity to the apoptotic induction by LC-1. Taken together, these data show the potential value of inhibiting NF-κB as a therapeutic strategy in CLL particularly in resistant and relapsed disease.

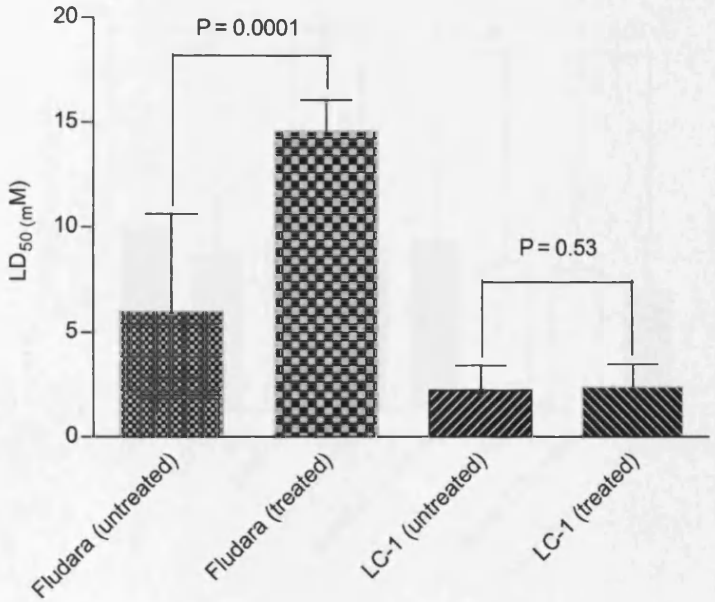

Figure 4.12 LC-1 is effective in patients who were treated previously. Fludarabine LD_{50} and LC-1 LD_{50} values were compared between treated and untreated patient groups. Previously treated patients showed significant *ex vivo* resistance to fludarabine ($P<0.0001$) but treated and untreated patient groups were equally sensitive to LC-1 ($P=0.53$).

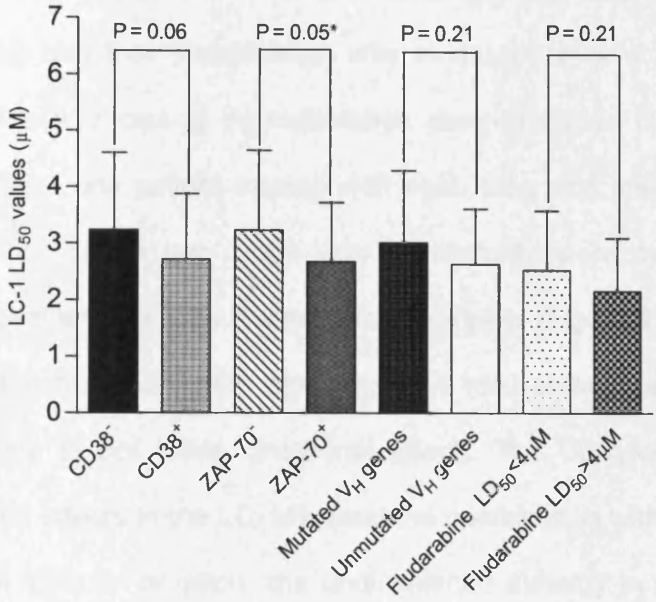

Figure 4.13 LC-1 is equipotent in poor prognostic subsets of CLL. LC-1 LD_{50} values were compared between prognostic subsets of patients. CLL cells derived from patients with CD38 expression ≥20% (n = 40) and <20% (n = 50), ZAP-70 expression ≥20% (n = 37) and <20% (n = 53), unmutated IgV_H genes (n = 23) and mutated IgV_H genes (n = 50), and *ex vivo* fludarabine resistance (n = 16) and fludarabine sensitive (n = 44) showed no significant difference in sensitivity to LC-1 (P = 0.06 , P = 0.05, P = 0.21 and P = 0.21 respectively).

4.5.8 LC-1 shows strong synergy with fludarabine in primary CLL cells

Having demonstrated the cytotoxic profile of LC-1 in poor prognostic subsets of CLL and its ability to overcome *in vitro* resistance to fludarabine, the next was to investigate potential synergy between LC-1 with fludarabine. The optimal molar ratio (2:1, LC-1: fludarabine) for the combination of the two drugs was experimentally determined by comparing the combination indices

derived from different molar combinations. Subsequently, a cytotoxic effect of each drug and their combination was measured over a 24h culture period. Figure 4.14A shows a representative dose-response curve for CLL cells derived from one patient treated with each drug and their combination. The combination of the two drugs was significantly more cytotoxic than either single agent as shown by the median effect plots (Figure 4.14B). Furthermore, the combination index (CI) plot (Figure 4.15A) showed synergism across a wide range of cell killing (fractional effect). The CI values revealed strong synergistic effects in the LC-1/fludarabine combination with a mean CI of 0.26 (Figure 4.15B). In addition, the undiminished synergy in samples from poor prognosis subsets was demonstrated (Figure 4.16). This finding that the LC-1/fludarabine combination exerts synergistic cytotoxicity in fludarabine resistant cells raises the possibility that the combination of NF-κB inhibitors and conventional chemotherapy may be clinically useful in poor prognosis groups of CLL.

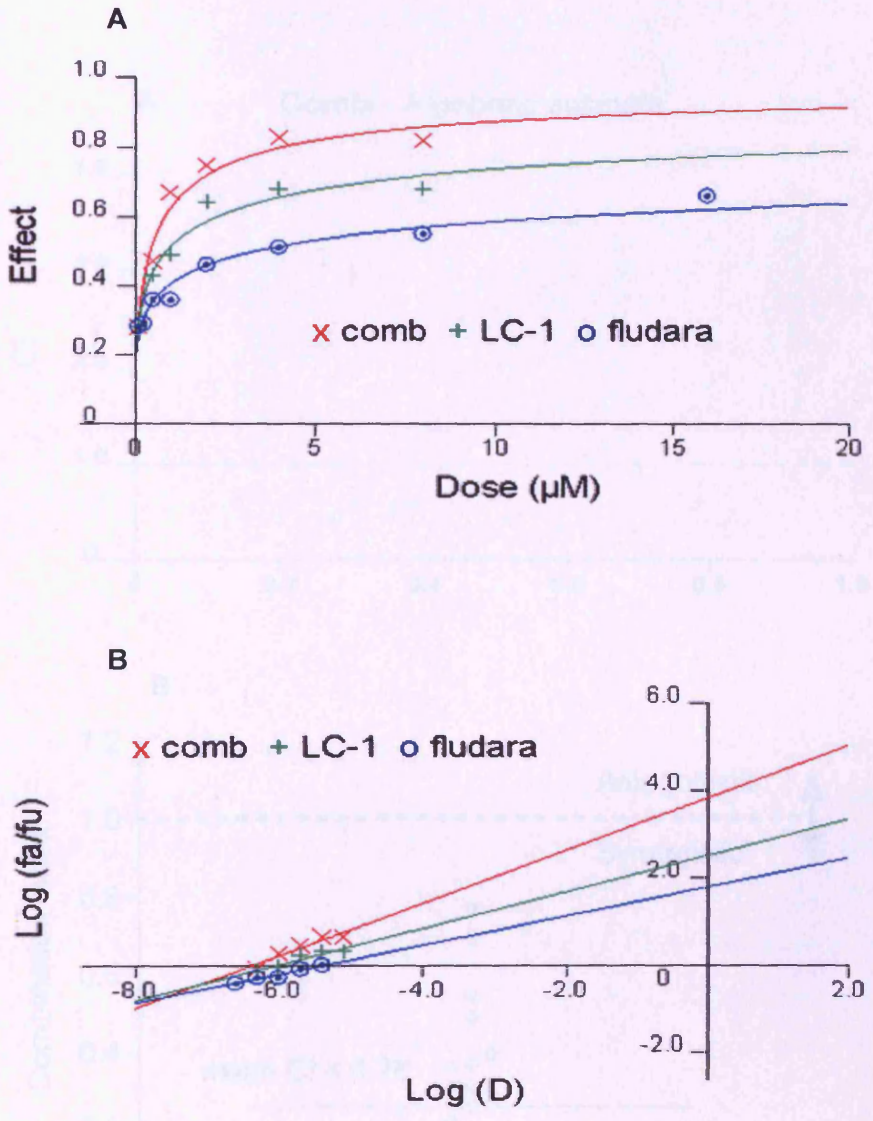

Figure 4.14 Synergistic effect of LC-1 with fludarabine in primary CLL cells. CLL cells were treated for up to 48h with LC-1 (0.5 to 8μM) and / or fludarabine (0.25 to 4μM) at a fixed molar ratio of 2:1. Cytotoxicity was quantified using an Annexin V/ propidium iodide assay. The median-effect plot was constructed using Calcusyn software where Fa = fraction affected and Fu = fraction unaffected. (A) Dose-response curve for CLL cells treated with LC-1 and/or with fludarabine. (B) Median-effect plot for CLL cells treated with LC-1 and/or with fludarabine.

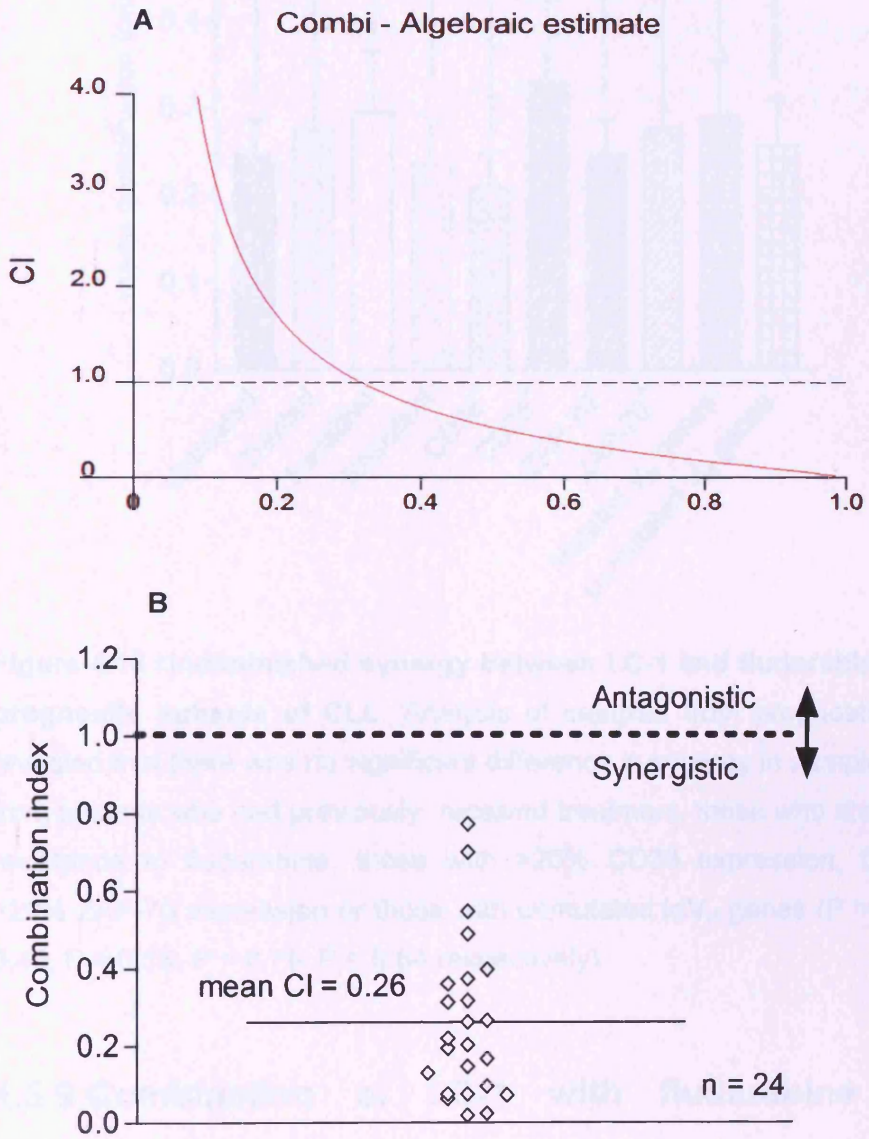

Figure 4.15 Synergistic effect of LC-1 with fludarabine in primary CLL cells. (A) The combination Index (CI) plot was constructed by computer analysis of the data. (B) Distribution of CI among 24 patients tested with mean value of 0.26 ± 0.20.

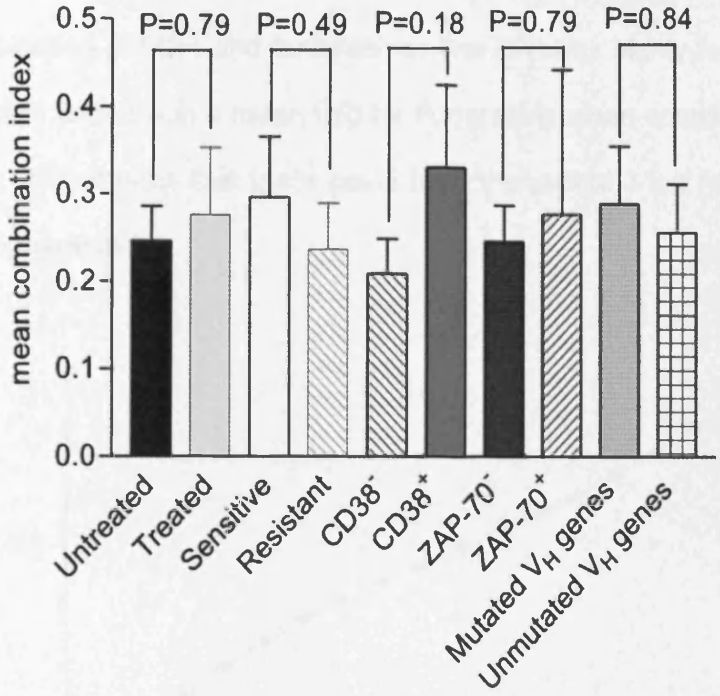

Figure 4.16 Undiminished synergy between LC-1 and fludarabine in poor prognostic subsets of CLL. Analysis of samples from prognostic subsets revealed that there was no significant difference in synergy in samples derived from patients who had previously received treatment, those who show *ex vivo* resistance to fludarabine, those with >20% CD38 expression, those with >20% ZAP-70 expression or those with unmutated IgV_H genes (P = 0.79, P = 0.49, P = 0.18, P = 0.79, P = 0.84 respectively).

4.5.9 Combination of LC-1 with fludarabine would facilitate a significant dose reduction of fludarabine

One of the major benefits of using combinations of drugs that have synergistic effects is that doses of individual drugs can be reduced to limit dose-dependent side effects, whilst maintaining efficacy. In this study the dose reduction index (DRI) for fludarabine in each patient sample tested was calculated (n = 24). Figure 4.17 shows a representative DRI for fludarabine

(DRI/ fractional effect curve) in CLL cells derived from one patient treated with the combination of LC-1 and fludarabine. The DRI was highly favourable in all the samples tested with a mean DRI for fludarabine when combined with LC-1 of >1000. This means that there could be a theoretical 3 log reduction in the dose of fludarabine.

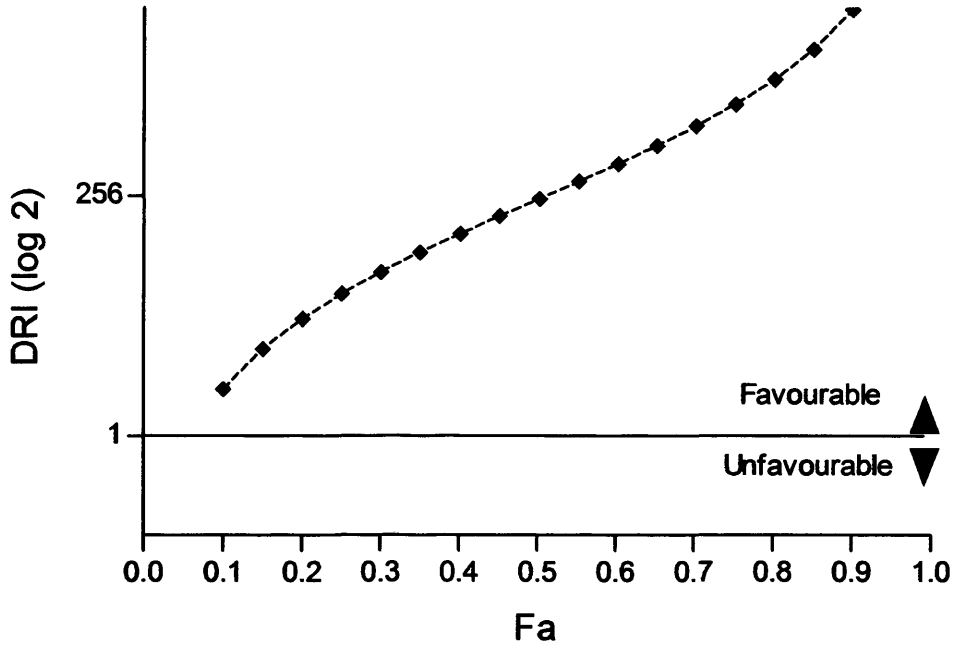

Figure 4.17 Combination of LC-1 with fludarabine would facilitate a significant dose reduction of fludarabine. CLL cells were treated for 48 h with LC-1 (0.5 to 8μM) and / or fludarabine (0.25 to 4μM) at a fixed molar ratio of 2:1.The cytotoxicity was quantified using an Annexin V/ propidium iodide assay. Dose reduction index- Fraction affected plots were calculated using CalcuSyn software. DRI values of more than 1 for fludarabine were observed over a wide range of inhibition levels when combined with LC-1.

4.6 DISCUSSION

In the previous chapter, I demonstrated that the NF-κB subunit Rel A is a biomarker of disease progression in CLL. This part of the study shows that inhibition of NF-κB by the novel parthenolide analogue LC-1 resulted in effective and preferential CLL cell killing. All of the samples tested were sensitive to LC-1 with a mean LD_{50} at 24h of 2.9μM. In addition, there was no significant difference in sensitivity to LC-1 within prognostic subsets. Indeed, LC-1 showed a trend towards increased efficacy in poor prognostic groups even in cells that showed marked *in vitro* resistance to fludarabine. This raises the possibility of using this agent as salvage treatment in poor prognostic subsets of CLL.

In addition, I investigated the specific mechanism(s) of LC-1-induced cell killing. It was previously shown that parthenolide-induced cell killing is mediated via intrinsic pathways of apoptosis pathway (Steele *et al.*, 2007). Unusually, LC-1-mediated apoptosis resulted in the activation of both the intrinsic and extrinsic pathways and this provides one possible explanation for the efficacy of this agent in samples with drug resistance to conventional therapy.

Importantly, I showed that LC-1 specifically inhibited the DNA binding of Rel A in a dose- and time-dependent manner. This inhibition preceded the induction of apoptosis indicating that it may be a critical regulator of LC-1-mediated cell killing. I then explored the downstream consequences of Rel A inhibition by quantifying the transcription of three NF-κB-regulated genes CFLAR, BCL2

and BIRC5 which have been implicated in CLL cell survival and resistance to chemotherapy (Pepper *et al.*, 1997; Munzert *et al.*, 2002; Morales *et al.*, 2005; Willimott *et al.*, 2007). Transcription of all three genes was significantly suppressed by LC-1 suggesting that NF-κB plays a role in preventing the activation of both the intrinsic and extrinsic apoptotic pathways in CLL. This is substantiated by the fact that CFLAR is involved in the inhibition of the extrinsic pathway and BCL2 inhibits the intrinsic pathway (Kroemer *et al.*, 2005)

Despite their prolonged survival *in vivo*, most CLL cells undergo spontaneous apoptosis when cultured *in vitro* (Collins *et al.*, 1989) suggesting that humoral factors and cellular interactions provide critical survival signals *in vivo*. There is growing evidence that the lymphoid organs are the sites of delivery of many of these pro-survival signals as it is here where CLL cells come into direct contact with stromal cells, T-cells and follicular dendritic cells (Jurlander *et al.*, 1998; Lagneaux *et al.*, 1998; Burger *et al.*, 1999). Two of the key survival stimuli appear to be derived from CD40 ligation and IL-4 and both of these interactions have the ability to induce NF-κB in CLL cells (Romano *et al.*, 1998; Dancescu *et al.*, 1992). I therefore included recombinant CD40 ligand and IL-4 into the drug sensitivity assay to determine whether LC-1 could overcome their cytoprotective effects. In accordance with previously published data CD40 ligation and IL-4 prevented CLL cells from apoptosis but the addition of LC-1 abrogated this pro-survival effect. Taken together, these data clearly show that NF-κB inhibition can overcome the survival signals induced by micro-environmental factors and this may be crucial in ensuring the clinical efficacy of these agents.

The ability to induce apoptosis is an essential property of most chemotherapeutic drugs (Thompson *et al.*, 1995). Perversely this may result in the activation of survival pathways, including the NF-κB pathway, as an unwanted side effect (Beg *et al.*, 1996; Wang *et al.*, 1996; Webster *et al.*, 1999; Nakanishi *et al.*, 2005). In keeping with this notion, samples from previously treated patients showed relative resistance to fludarabine in our study together with elevated Rel A DNA binding. However, I have demonstrated the equal potency of LC-1 in all samples tested irrespective of their previous treatment or other prognostic parameters. This highlights the potential value of NF-κB inhibitors for the treatment of CLL particularly in the context of resistant and relapsed disease.

One of the major benefits of using combinations of drugs that have synergistic effects is that doses of individual drugs can be reduced to limit dose-dependent side effects, whilst maintaining efficacy. The concept of the dose reduction index (DRI) was proposed by Chou (Chou *et al.*, 1994). DRI is a measure of how the dose of an individual drug in a synergistic combination can be reduced whilst retaining the same cytotoxic effect. A DRI >1 is beneficial and the greater the DRI value the greater the dose reduction for a given therapeutic effect. Remarkably, the mean DRI for fludarabine was >1000 indicating that the dose of fludarabine could theoretically be reduced by more than 3 logs thereby significantly reducing its immunosuppressive side effects while retaining the cytotoxic effects on CLL cells.

This part of the work provides a number of important new insights. It elucidated the mechanism of action of the novel NF-κB inhibitor LC-1 and also

demonstrates the efficacy of LC-1 in poor prognostic subsets of CLL including samples with fludarabine resistance. This data also provide a compelling argument for the use of LC-1 in combination with fludarabine in the clinical setting.

5.0 Rel A IS AN INDEPENDENT PROGNOSTIC MARKER OF CLINICAL OUTCOME IN CLL

5.1 INTRODUCTION

The clinical course of individual patients is highly variable and reliable predictors of prognosis are lacking in the clinical workup of this condition. This has stimulated the search for prognostic markers that can predict patient outcome in CLL. The need for these biomarkers is particularly important because of the introduction of more effective therapies that might be optimally employed in the early phase of the disease before the problems associated with high tumour burden and drug resistance are encountered. Clinical studies have consistently shown that unmutated IgV_H genes, high ZAP-70 expression, high CD38 expression and cytogenetic abnormalities (especially deletions of 11q and 17p) are all associated with inferior outcome (Damle *et al.*, 1999; Hamblin *et al.*, 1999; Dohner *et al.*, 2000; Ibrahim *et al.*, 2001; Rassenti *et al.*, 2004). However, none of these markers are able to accurately predict the clinical course of individual patients or their likely response to therapy. This limits their use in clinical management particularly in deciding if and when to treat. Therefore, the search for better markers is both timely and relevant.

The association of NF-κB subunit Rel A with *in vitro* survival and clinical disease progression in CLL was shown in chapter 3. I therefore hypothesized that Rel A could be a prognostic marker in this disease. In order to test this

hypothesis, Rel A DNA binding was analysed in 131 patients and the results were correlated with the established markers of prognosis and clinical outcome.

5.2 Results

5.2.1 The patient characteristics of the CLL cohort used for this part of the study are described in Table 5.1.

Patient ID	Gender	Stage	% CD38	% ZAP-70	IgV_H Status
1	F	B	6	4	ND
2	M	A	96	80	98%
3	M	B	11	5	ND
4	M	A	73	48	100%
5	M	A	3	2	87%
6	F	A	5	3	87%
7	F	A	88	82	94%
8	F	A	1	19	ND
9	M	A	3	0	ND
10	M	A	40	48	100%
11	M	A	22	2	92%
12	F	A	100	62	96%
13	M	C	2	29	95%
14	F	A	3	30	93%
15	F	A	100	92	82%
16	M	A	1	31	91%
17	M	A	100	14	89%
18	M	A	62	78	83%
19	M	A	10	13	93%
20	M	C	22	11	90%
21	F	C	47	86	98%
22	F	A	2	7	92%
23	M	A	13	24	94%

Patient ID	Gender	Stage	% CD38	% ZAP-70	IgV_H Status
24	M	A	84	1	100%
25	F	A	4	13	95%
26	M	B	47	2	96%
27	M	A	30	9	ND
28	M	A	0	4	93%
29	M	A	1	1	91%
30	M	A	42	14	75%
31	M	A	3	8	85%
32	M	A	35	11	94%
33	F	A	7	2	94%
34	F	A	13	11	96%
35	M	A	5	53	ND
36	M	A	35	32	96%
37	M	A	0	70	ND
38	F	C	5	14	98%
39	M	A	14	20	97%
40	M	A	1	17	ND
41	M	A	2	0	83%
42	M	B	99	39	100%
43	F	A	1	1	ND
44	M	A	0	1	90%
45	F	A	1	5	95%
46	M	C	6	4	ND
47	M	C	18	31	96%
48	F	A	87	4	ND
49	M	C	3	29	99%
50	F	A	46	31	95%
51	F	A	39	18	ND
52	M	A	6	37	ND
53	M	A	9	5	96%
54	M	A	5	8	90%
55	M	B	99	99	93%

Patient ID	Gender	Stage	% CD38	% ZAP-70	IgV_H Status
56	M	A	72	3	ND
57	M	B	43	40	100%
58	M	A	11	27	94%
59	F	B	4	3	85%
60	M	A	50	5	92%
61	F	A	6	34	ND
62	M	A	6	2	91%
63	F	A	16	13	83%
64	M	A	16	81	100%
65	M	A	3	81	ND
66	M	A	16	1	91%
67	F	C	7	3	100%
68	M	A	4	10	96%
69	F	C	69	57	91%
70	F	C	99	100	96%
71	M	A	80	1	100%
72	F	A	5	3	ND
73	M	A	8	23	93%
74	M	A	4	2	ND
75	F	A	23	2	90%
76	F	A	47	26	98%
77	M	A	36	1	100%
78	M	A	3	1	ND
79	M	A	9	3	ND
80	M	A	24	37	92%
81	M	A	27	25	92%
82	M	A	30	11	83%
83	M	A	50	0	96%
84	F	A	1	0	ND
85	M	A	1	10	ND
86	M	A	8	11	95%
87	F	A	3	54	ND

Patient ID	Gender	Stage	% CD38	% ZAP-70	IgV_H Status
88	M	A	98	47	99%
89	M	A	1	63	89%
90	M	A	5	10	94%
91	M	A	85	99	98%
92	M	A	34	1	100%
93	M	B	5	0	96%
94	M	A	2	2	ND
95	M	B	40	37	99%
96	F	A	70	33	99%
97	M	A	13	85	89%
98	M	A	7	10	91%
99	F	A	13	7	91%
100	M	C	47	21	97%
101	M	B	2	20	91%
102	M	C	4	43	100%
103	F	A	79	3	89%
104	M	A	73	1	100%
105	F	A	4	32	88%
106	M	C	3	21	100%
107	F	A	10	1	97%
108	M	A	2	4	ND
109	F	A	1	5	94%
110	M	A	10	4	93%
111	F	A	2	2	90%
112	M	A	26	7	94%
113	M	A	9	9	90%
114	F	A	100	7	91%
115	F	A	100	95	93%
116	M	A	3	7	97%
117	F	A	7	23	96%
118	F	A	3	22	94%
119	M	A	2	0	86%

Patient ID	Gender	Stage	% CD38	% ZAP-70	IgV_H Status
120	M	A	100	87	88%
121	M	A	8	58	92%
122	M	A	0	1	89%
123	M	B	3	18	90%
124	M	A	8	3	93%
125	M	A	47	10	94%
126	M	A	85	13	92%
127	M	A	0	6	91%
128	F	A	4	121	ND
129	M	A	36	34	98%
130	F	C	2	1	89%
131	F	A	7	20	92%

5.2.2 Measurement of Rel A DNA binding in CLL patient samples

Rel A was measured in nuclear extracts derived from 131 CLL patient samples using the quantitative DNA binding ELISA-based method described in chapter 3. Data from all 131 patients is shown in Figure 5.1; Rel A DNA binding ranged from undetectable to 2.44 ng/μg of nuclear extract with a median value of 0.44ng/μg (95% CI 0.48-0.66).

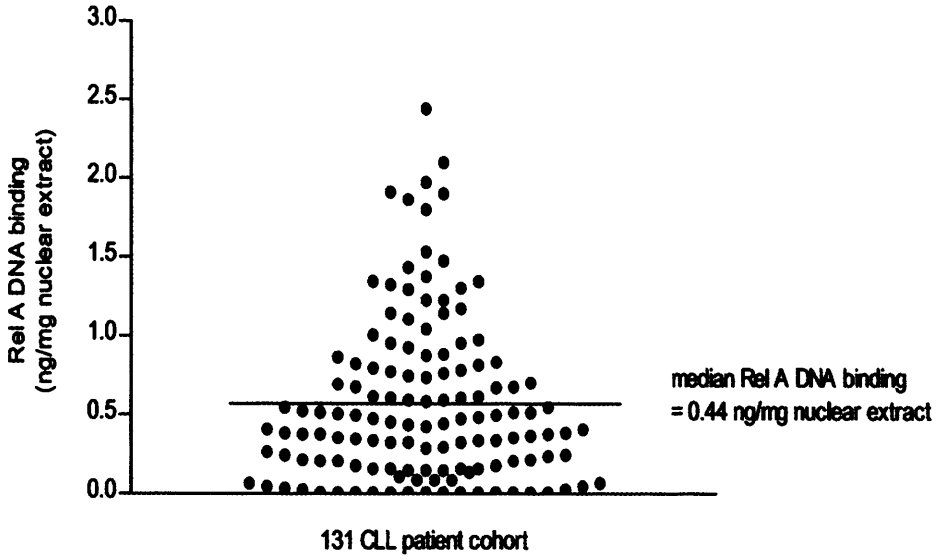

Figure 5.1 Variation in Rel A. Samples from all 131 patients were analysed for Rel A DNA binding using an ELISA-based assay.

5.2.3 Rel A DNA binding and prognostic markers of CLL

The correlation between Rel A and other prognostic markers was analysed in this part of the study. Rel A was markedly elevated in patients with advanced disease ($P<0.0001$, Figure 5.2) and those who required treatment ($P<0.0001$, Figure 5.3) but was not associated with CD38 expression ($P = 0.87$, Figure 5.4), IgV_H mutation status ($P = 0.25$, Figure 5.5) or ZAP-70 expression ($P = 0.55$, Figure 5.6).

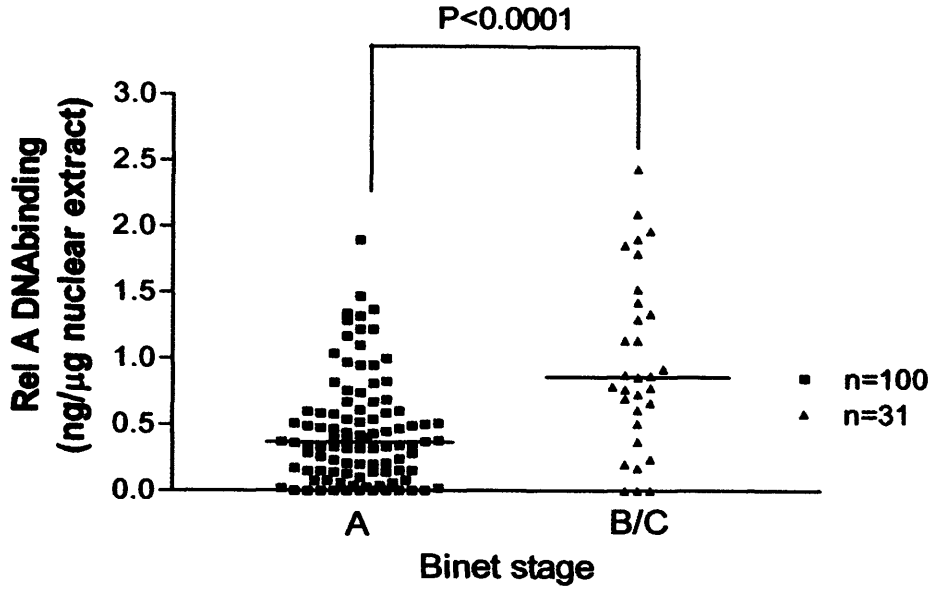

Figure 5.2 Comparison of Rel A DNA binding in Binet subsets. Rel A DNA binding was compared in different subsets of CLL patients categorised according to Binet stage. Elevated Rel A was strongly associated with advanced Binet stage (P<0.0001).

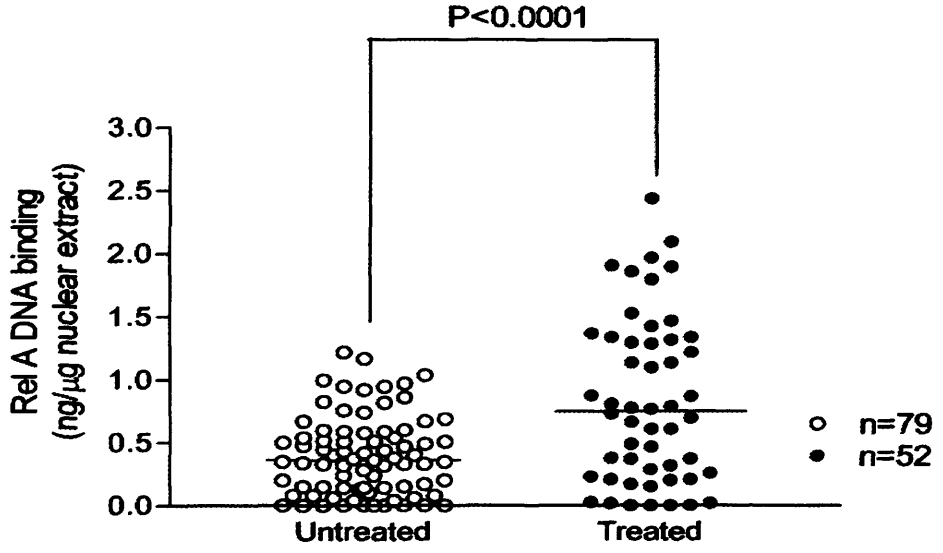

Figure 5.3 Comparison of Rel A DNA binding in treated and untreated groups. Rel A DNA binding was compared in samples derived from untreated patients and those who had previously recieved treatment. Elevated Rel A was strongly associated with the need for prior treatment (P<0.0001). No samples were analysed within 3 months of therapy.

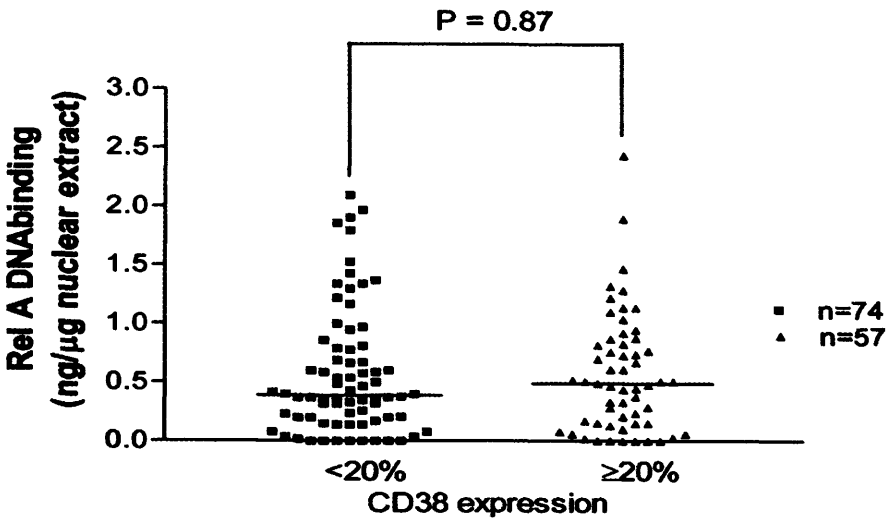

Figure 5.4 Comparision of Rel A DNA binding in CD38 subsets. Rel A DNA binding was compared in different subsets of CLL patients categorised according to CD38 expression. Elevated Rel A was not associated with CD38 expression (P=0.87).

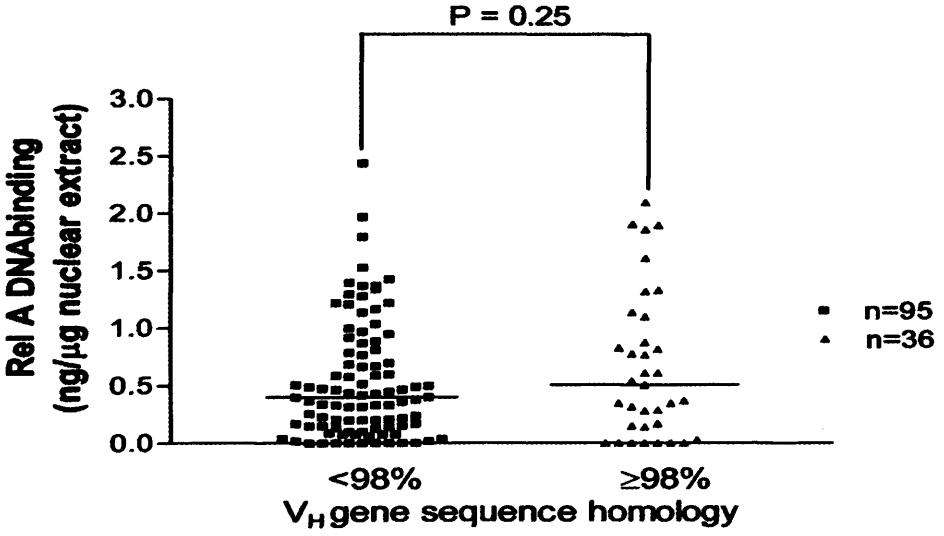

Figure 5.5 Comparison of Rel A DNA binding in IgV_H subsets. Rel A DNA binding was compared in different subsets of CLL patients categorised according to IgV_H mutational status. Elevated Rel A was not associated with the IgV_H status (P=0.25).

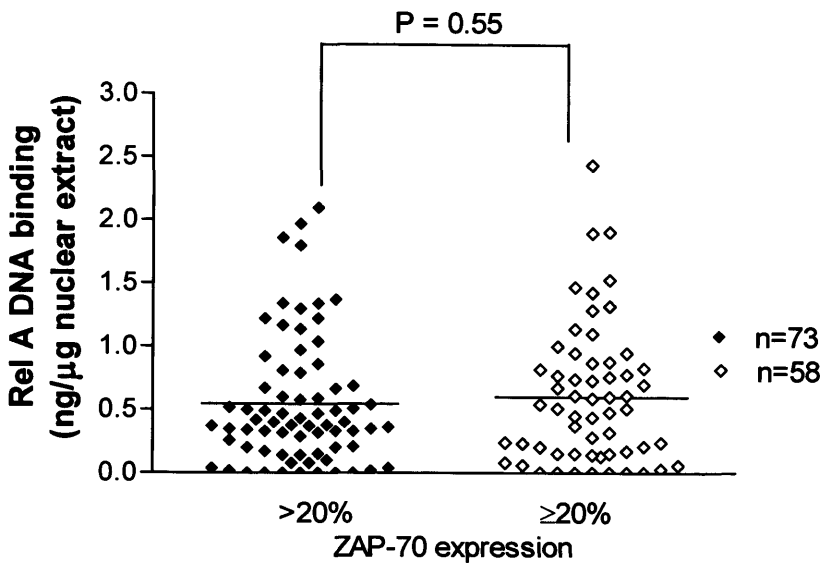

Figure 5.6 Rel A DNA binding in ZAP-70 subgroups Rel A DNA binding was compared in different subsets of CLL patients categorised according to ZAP-70 expression. Elevated Rel A was not associated with ZAP-70 expression (P=0.55).

5.2.4 Association between Rel A DNA binding and time to initial therapy

Time to first treatment is defined as the time from diagnosis to the first treatment intervention (TTFT). It is considered to be an important clinical end-point in assessing disease progression and is less subjective than an assessment of progression-free survival. Patients in this cohort were treated according to accepted guidelines (Cheasonet at al., 1996; Hallek *et al.*, 2008); therefore the need for treatment denotes progressive disease or patients becoming symptomatic due to effects of their leukaemia. Rel A levels were analysed in relation to TTFT in 131 patient samples. 19 (14.5%) had previously received treatment and a further 33 (25.2%) required therapy

during the course of the study. Patients were categorised according to their Rel A DNA binding (above and below median values). The median TTFT in the low Rel A subset was 13.6 years versus 6.8 years in the high Rel A subset (P = 0.01, Figure 5.7). Since Rel A DNA binding was independent of CD38, IgV_H mutation status and ZAP-70, I examined the ability of these established prognostic parameters to define TTFT in our cohort (Figure 5.8, 5.9 and 5.10 respectively). In keeping with previous reports, unmutated IgV_H genes, ≥20% CD38 expression and ≥20% ZAP-70 expression were all associated with significantly shorter TTFT (P = 0.0005, P = 0.04 and P = 0.05 respectively) indicating that this study was carried out on a representative CLL cohort.

In multivariate analysis, the inclusion of Rel A in the model showed independent prognostic significance for TTFT (P = 0.01) and its inclusion diminished the prognostic value of CD38 (P = 0.25), IgV_H gene mutation status (P = 0.05) and ZAP-70 (P = 0.28).

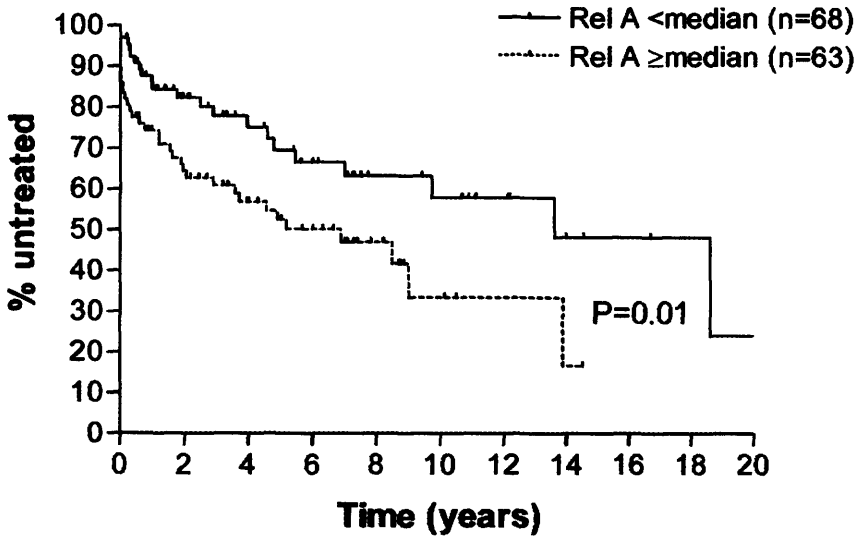

Figure 5.7 Time to first treatment according to Rel A DNA binding Time to first treatment was assessed from the date of diagnosis. Rel A DNA binding above the median value was able to define a population at increased risk of requiring treatment in unit time (P = 0.01).

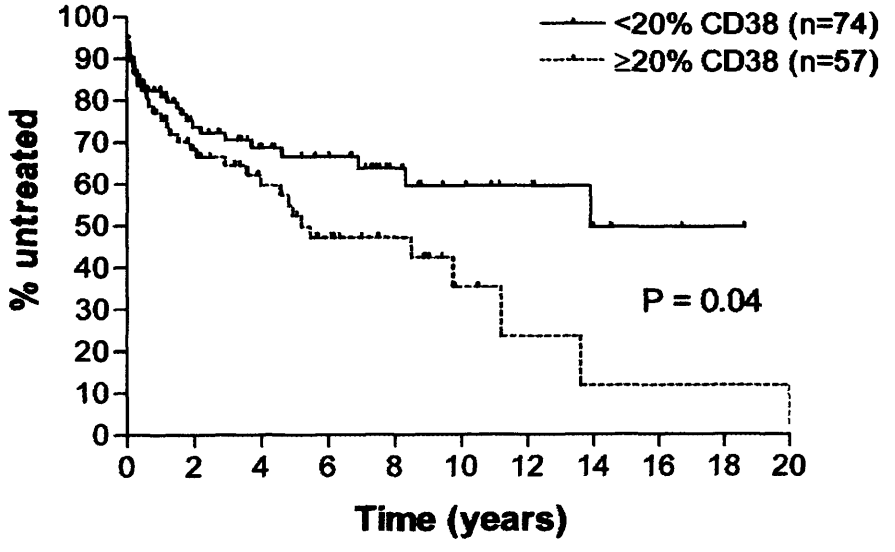

Figure 5.8 Time to first treatment according to CD38 expression Time to first treatment was assessed from the date of diagnosis. CD38 expression was able to define a population at increased risk of requiring treatment in unit time (P = 0.04).

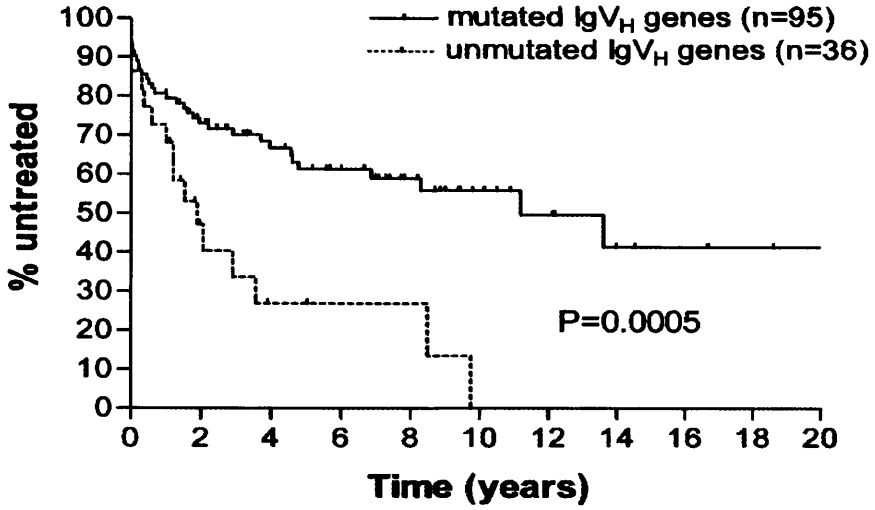

Figure 5.9 Time to first treatment according to IgV$_H$ status Time to first treatment was assessed from the date of diagnosis. IgV$_H$ mutational status was able to define a population at increased risk of requiring treatment in unit time (P = 0.0005).

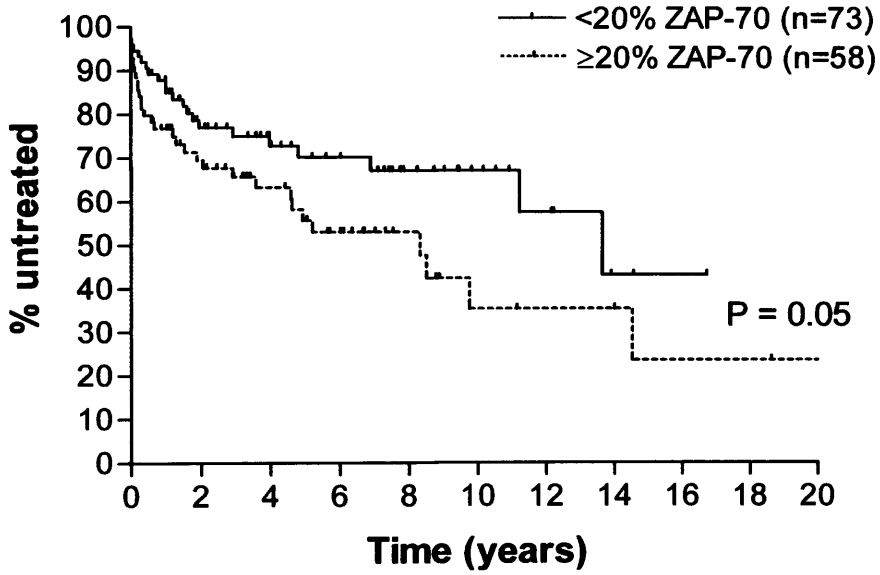

Figure 5.10 Time to first treatment according to ZAP-70 expression Time to first treatment was assessed from the date of diagnosis. ZAP-70 expression was able to define a population at increased risk of requiring treatment in unit time (P = 0.05).

5.2.5 Rel A is a predictor of time to subsequent treatment

Not all of the samples analysed for Rel A were taken at diagnosis. Some patients have had treatment before their inclusion in the study. As a next step of the study prognostic value of Rel A was more accurately assessed by controlling for previous treatment effect by analysing the predictive value for treatment after entering in to the study. The time to subsequent treatment (TTST) was defined as the date of entry into the study to the date requiring treatment. Rel A values were analysed in relation to TTST. Figure 5.11 shows the predictive value of Rel A in this CLL patient cohort. Patients with high Rel A DNA binding showed a median TTST of 22 months. In contrast, the median TTST of the low Rel A subset was not reached as only 5/68 patients with low Rel A DNA binding required treatment during the 24 month follow-up period (P = 0.0001). The ability of IgV_H mutation, CD38 and ZAP-70 to predict TTST during the study period was also evaluated (5.12, 5.13 and 5.14 respectively). Only IgV_H mutation status had prognostic value in this context (P = 0.04); CD38 (P = 0.17) and ZAP-70 (P = 0.13) were not predictive.

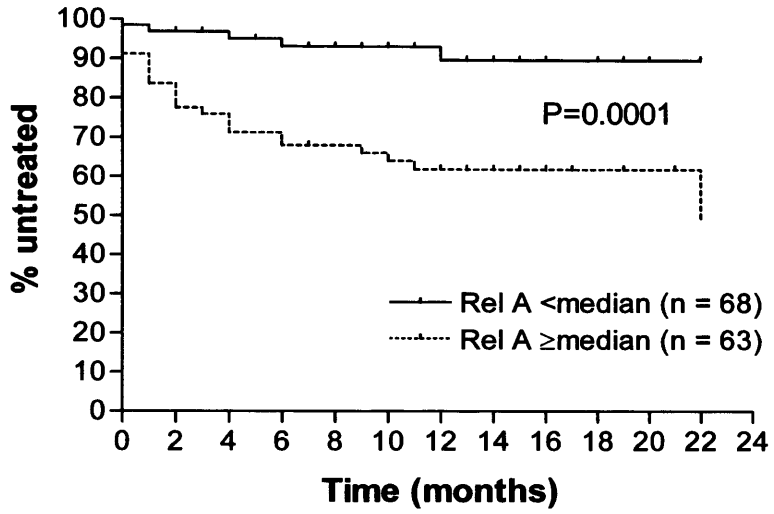

Figure 5.11 Time to subsequent treatment according to Rel A DNA binding Time to subsequent treatment was measured from the date of entry into the study to the treatment intervention. Rel A DNA binding above the median value was able to define a population at greater risk of requiring treatment during the 24 month follow-up period (P = 0.0001).

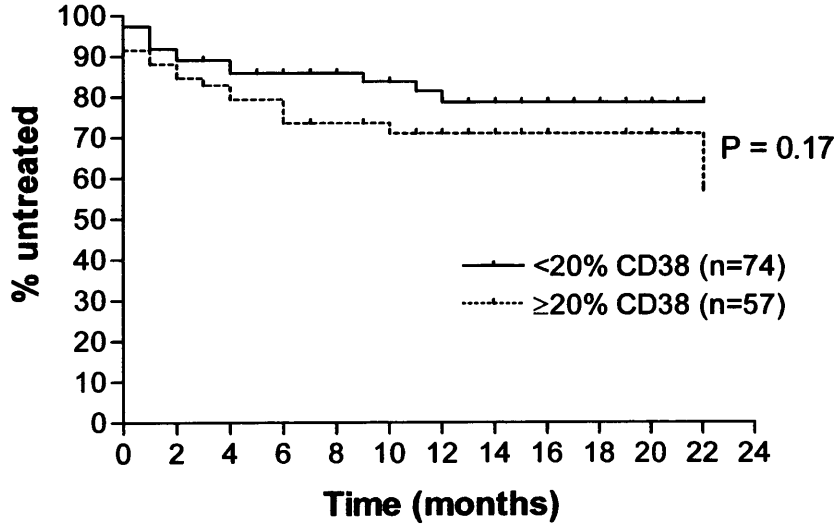

Figure 5.12 Time to subsequent treatment according to CD38 expression Time to subsequent treatment was measured from the date of entry into the study to the treatment intervention. CD38 expression could not differentiate the population needing treatment during the 24 month follow-up period (P = 0.17).

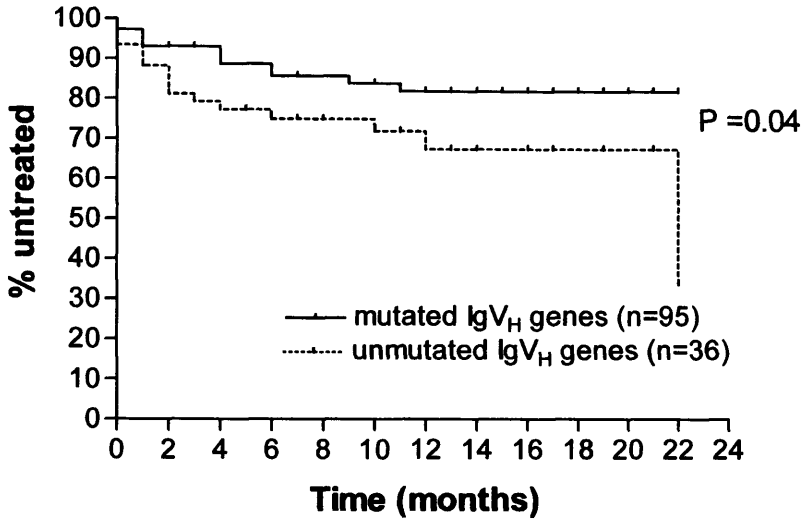

Figure 5.13 Time to subsequent treatment according to IgV$_H$ status. Time to subsequent treatment was measured from the date of entry into the study to the treatment intervention. V$_H$ mutational status could differentiate the population needing treatment during the 24 month follow-up period (P = 0.04).

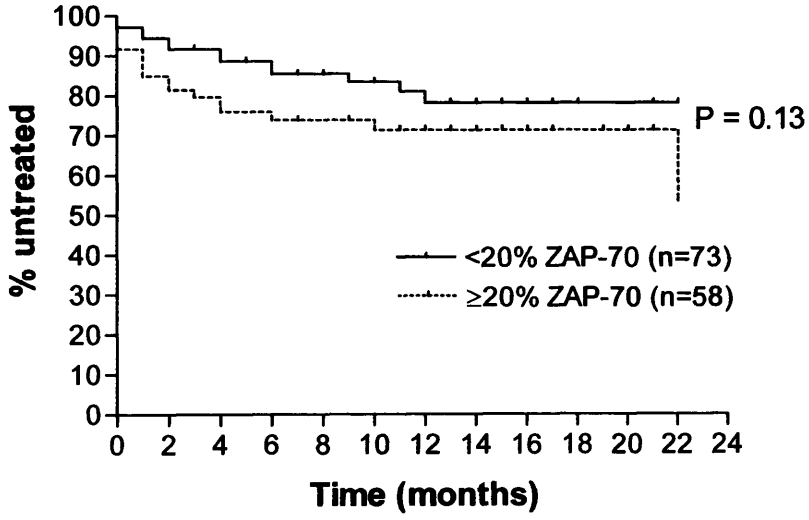

Figure 5.14 Time to subsequent treatment according to ZAP-70 expression. Time to subsequent treatment was measured from the date of entry into the study to the treatment intervention. ZAP-70 expression could not differentiate the population needing treatment during the 24 month follow-up period (P = 0.13).

5.2.6 Rel A is a predictor of overall survival

Overall survival is defined as time to death from date of diagnosis (OS). This is used as a clinical end-point in assessing treatment modalities and prognostic markers. The ability of Rel A to predict patient OS was assessed as the next step of this study. Rel A DNA binding was predictive of survival from date of diagnosis (P = 0.01, Figure 5.15). Similarly, IgV_H mutation status, CD38 and ZAP-70 were all predictive of survival from date of diagnosis (Figure 5.16, 5.17 and 5.18). In multivariate analysis, the inclusion of Rel A in the model showed independent prognostic significance for overall survival (P = 0.04) and its inclusion removed the prognostic value of CD38 (P = 0.38), IgV_H gene mutation status (P = 0.22) and ZAP-70 (P = 0.14).

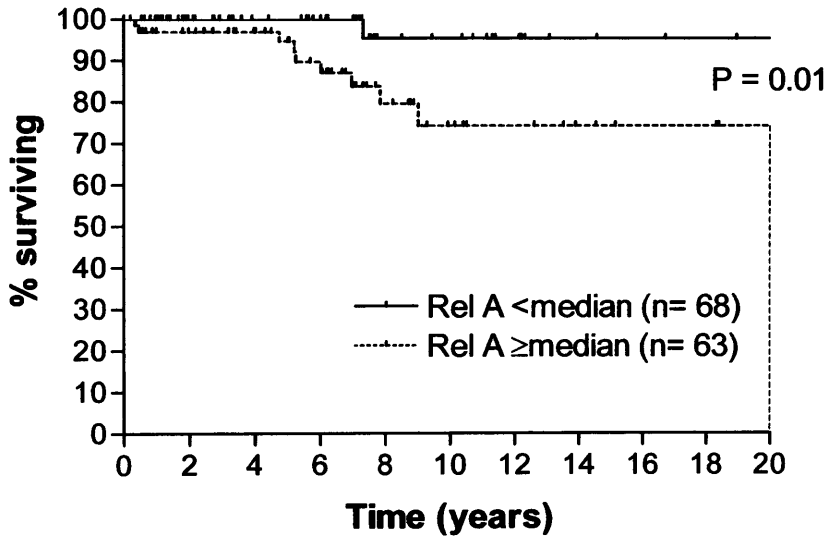

Figure 5.15 Rel A DNA binding and overall survival. Comparison of Kaplan Meier curves for survival demonstrated that Rel A was prognostic for survival from date of diagnosis (P = 0.01).

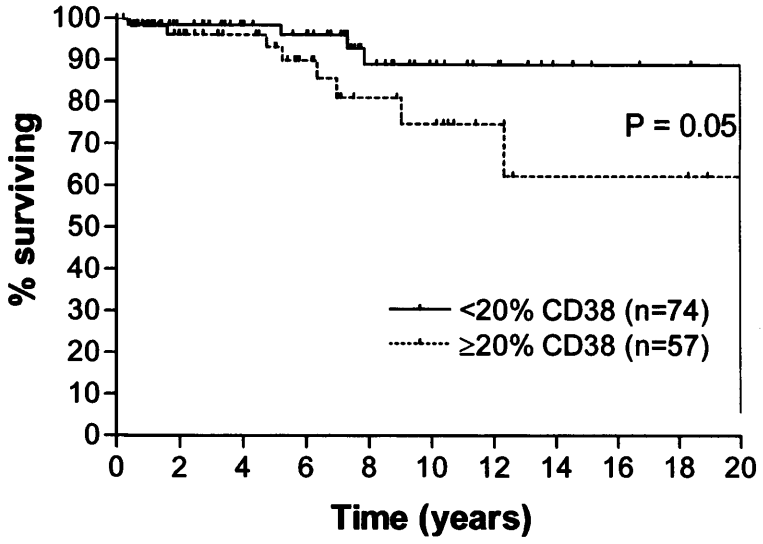

Figure 5.16 CD38 expression and overall survival. Comparison of Kaplan Meier curves for survival demonstrated that CD38 was prognostic for survival from date of diagnosis (P = 0.05).

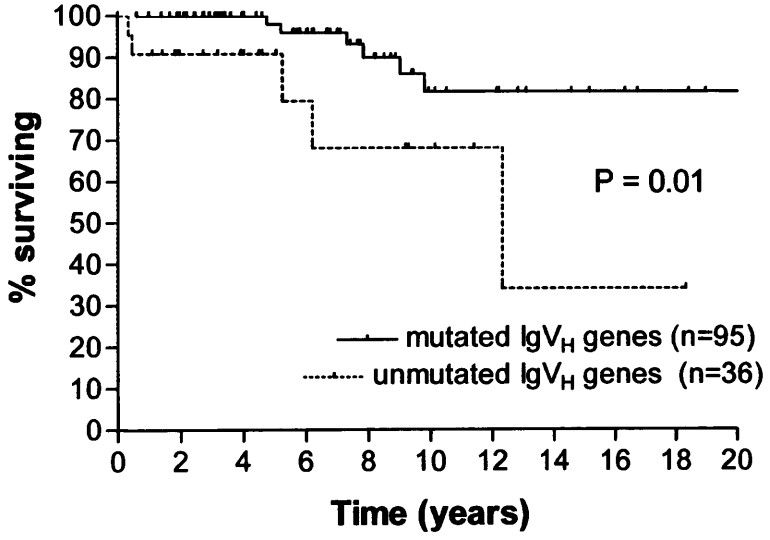

Figure 5.17 IgV$_H$ status and overall survival. Comparison of Kaplan Meier curves for survival demonstrated that IgV$_H$ mutational status was prognostic for survival from date of diagnosis (P=0.01).

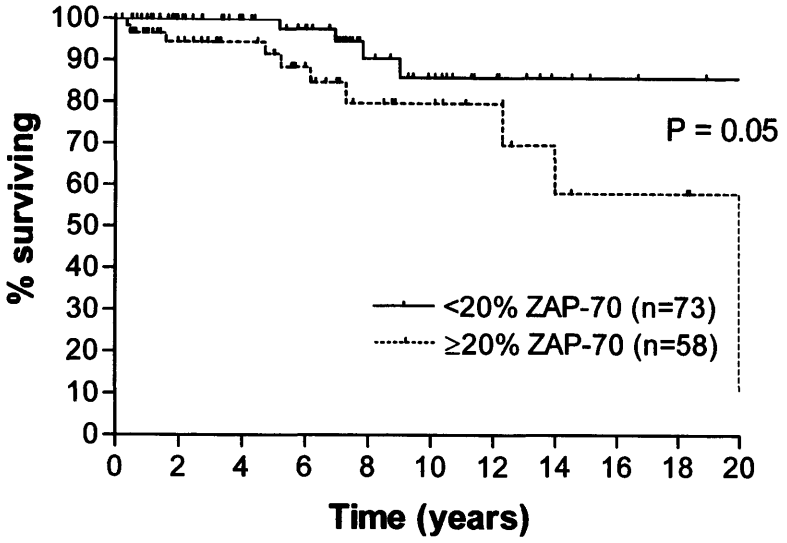

Figure 5.18 ZAP-70 expression and overall survival. Comparison of Kaplan Meier curves for survival demonstrated that ZAP-70 expression was prognostic for survival from date of diagnosis (P = 0.05).

5.2.7 Rel A is a predictor of survival during the period of study

I also assessed the ability of Rel A to predict survival during the study period. The survival during the study was defined as the date of entry into the study to the date of death. Patients with high Rel A DNA binding showed a significantly shorter survival even over the relatively short study period (P= 0.05, Figure 5.19). In contrast CD38 expression (P=0.66), IgV_H mutational status (P=0.71) or ZAP-70 (P=0.39) expression could not predict survival during this short 24 months follow-up.

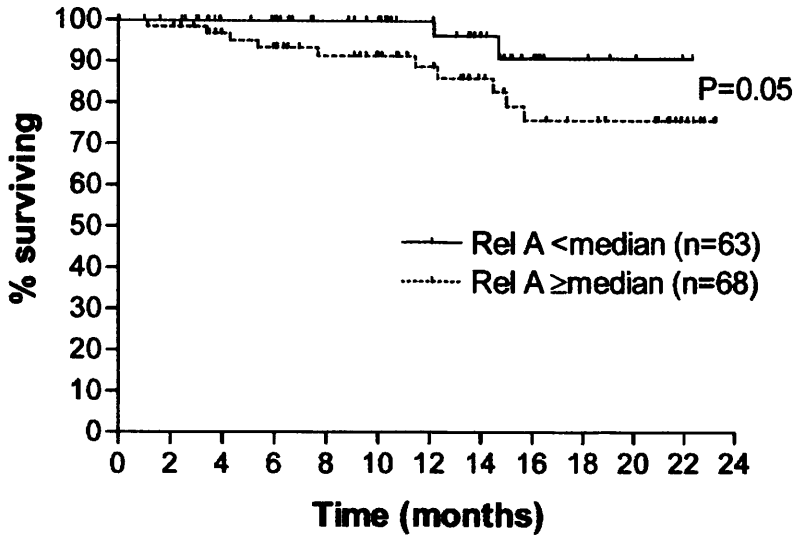

Figure 5.19 Rel A DNA binding and survival from date of entry into the study. Comparison of Kaplan Meier curves for survival demonstrated that Rel A was prognostic for survival from date of entry into the study (P = 0.05).

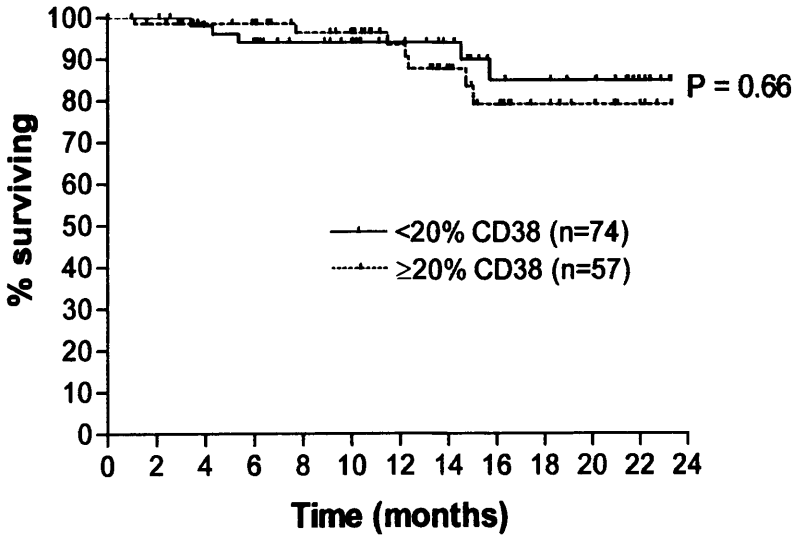

Figure 5.20 CD38 expression and survival from date of entry into the study. Comparison of Kaplan Meier curves for survival demonstrated that CD38 expression was not prognostic for survival from date of entry into the study (P = 0.66).

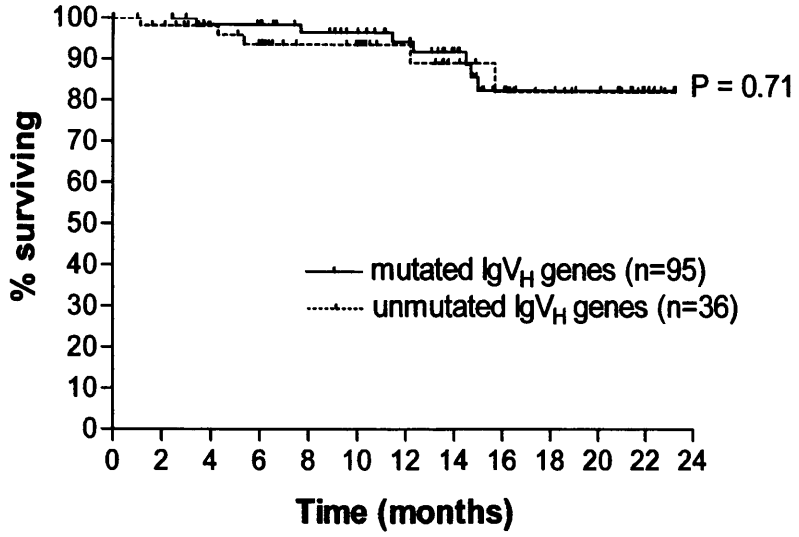

Figure 5.21 IgV$_H$ status and survival from date of entry into the study. Comparison of Kaplan Meier curves for survival demonstrated that IgV$_H$ mutational status was not prognostic for survival from date of entry into the study (P = 0.71).

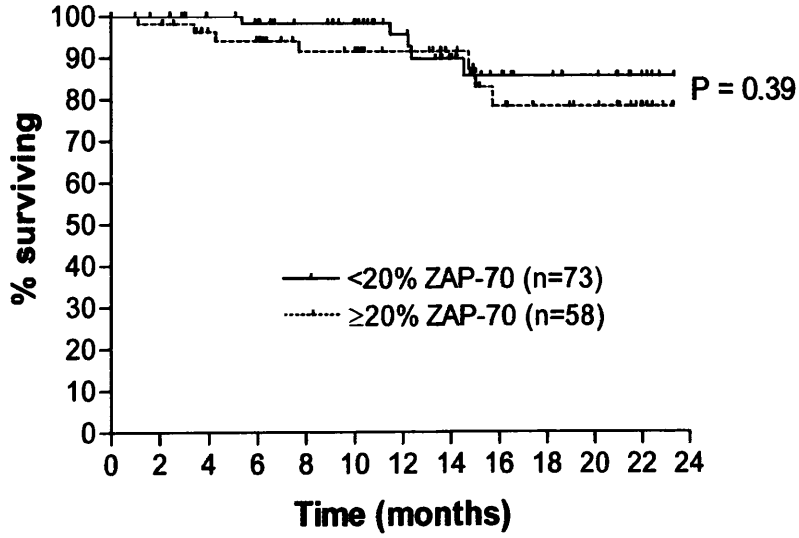

Figure 5.22 ZAP-70 expression and survival from date of entry into the study. Comparison of Kaplan Meier curves for survival demonstrated that ZAP-70 expression was not prognostic for survival from date of entry into the study (P = 0.39).

5.2.8 Rel A is constitutively higher in patients who require treatment

In order for Rel A to be considered a useful marker of TTFT in CLL it seemed important to demonstrate that Rel A was constitutively higher in untreated patients who went on to require therapy. I therefore compared Rel A DNA binding in samples derived from patients who never required therapy (n = 79) with those who went on to receive their first treatment after I had measured their Rel A DNA binding (n = 33). Figure 5.23 shows that patients who require therapy have significantly higher Rel A DNA binding than those patients who do not (P<0.0001).

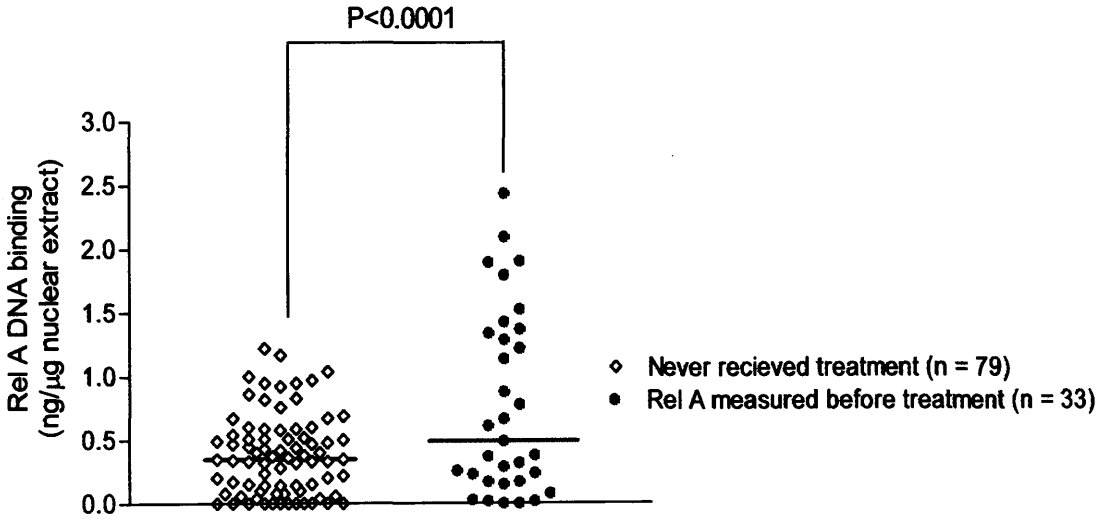

Figure 5.23 Rel A DNA binding in patients needing treatment. Rel A DNA binding was significantly higher in samples derived from previously treated patients (P<0.0001).

5.2.9 Is Rel A modulated by therapy?

Studies in other diseases have shown that Rel A DNA binding can increase in response to chemotherapeutic drugs (Denlinger *et al.*, 2004; Rundall *et al.*, 2004; Artl *et al.*, 2001). I therefore set out to determine if this was also true in CLL. This question was examined in two ways. Firstly, Rel A DNA binding was compared in samples derived from patients who received therapy after I had measured their Rel A with those who received therapy before I measured their Rel A (Figure 5.24). Although there was no statistical difference in Rel A DNA binding between these two groups (P = 0.41) the median Rel A DNA binding was higher in the previously treated subset (median Rel A 0.81ng/μg versus 0.49ng/μg). Secondly, intra-patient Rel A DNA binding was evaluated in a longitudinal manner in patients before and after treatment and in selected patients that did not require therapy. Figure 5.25 shows a clear increase in Rel A DNA binding following treatment (P=0.04). In contrast serial samples from patients not requiring therapy showed no significant change in Rel A DNA binding (P=0.89). None of the samples tested were derived from patients within 3 months of completion of a cycle of therapy in order to avoid the possibility of a transient modulation in Rel A DNA binding induced by chemotherapeutic drugs. The median interval between measurements was similar for the treated and untreated patient groups (15 months vs 13 months respectively).

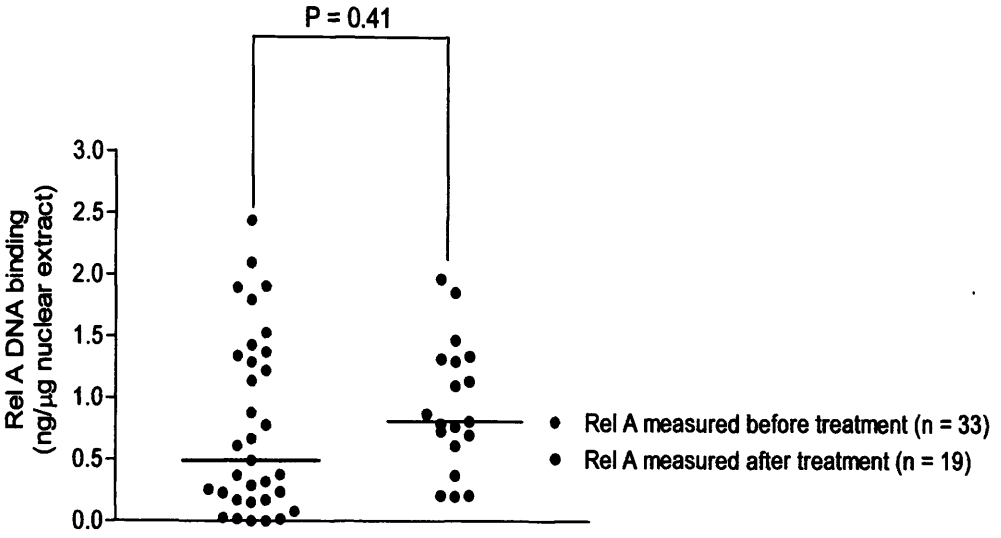

Figure 5.24 Modulation of Rel A DNA binding with treatment. There was no statistical difference in Rel A DNA binding in samples analysed before treatment and those in whom Rel A was measured after treatment (P = 0.41).

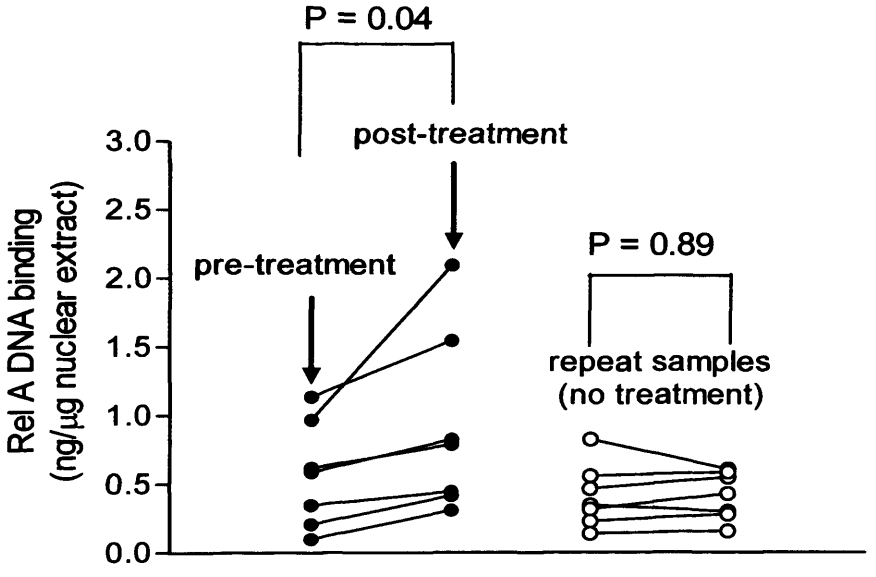

Figure 5.25 Rel A DNA binding in individual samples before and after treatment. Serial intra-patient analysis of Rel A in samples derived from patients who received treatment during the course of the study and those who did not revealed that there was a significant increase in Rel A DNA binding following therapy (P = 0.04). In contrast, samples from patients who did not require treatment remained stable (P = 0.89).

5.3 DISCUSSION

A number of previous studies have shown that NF-κB is constitutively activated in CLL patients (Furman *et al.*, 2000; Cuni *et al.*, 2004). In chapter 3 of this thesis, I demonstrated that NF-κB subunit Rel A DNA binding was highly variable in CLL samples (n = 30) and it is higher compared with normal B-cells. Here I have confirmed the heterogeneity of Rel A DNA binding in 131 unselected CLL patients. High Rel A DNA binding was strongly associated with advanced stage of disease but remarkably, was not associated with IgV_H gene mutation status, CD38 expression or ZAP-70 expression; indicating that Rel A could contribute independent prognostic information in CLL. This is compatible with our initial findings that total NF-κB DNA binding was not associated with IgV_H gene mutation status, CD38 expression or ZAP-70 expression (Hewamana *et al.*, 2008 and Chapter 3).

Furthermore, I went on to assess the ability of Rel A to predict for time to first treatment (TTFT), time to subsequent treatment (TTST) and overall survival in our patient cohort. Elevated Rel A DNA binding was predictive of significantly shorter TTFT (P = 0.01, hazard ratio 1.9). As NF-κB has been implicated in the development of drug resistance in other tumour settings (Artl *et al.*, 2002; Artl *et al.*, 2003), I assessed whether Rel A could predict for the relative effectiveness of therapy in CLL patients. In order to do this TTST was used from the date of entry into the study as a measure of the depth of remission / stable disease. Rel A was more predictive of TTST than any of the other prognostic markers assessed (P = 0.0001, hazard ratio 5.2) indicating that it is an excellent biomarker of response as well as a predictor of needing treatment in the CLL patients studied.

Most strikingly, Rel A was the best predictor of overall survival from date of diagnosis in this study (P = 0.01, hazard ratio 9.1) adding to our hypothesis that Rel A is a key regulator of CLL survival both *in vitro* and *in vivo*. In keeping with this notion, Rel A was the only parameter tested that showed a significant difference in survival over the 24 month follow-up period of this study (P = 0.05, hazard ratio 3.9). Furthermore, the inclusion of Rel A in multi-variate modelling showed the independent importance of Rel A in determining overall survival in our cohort (P = 0.05, hazard ratio 8.0); CD38 expression, IgV_H mutation status and ZAP-70 expression all failed to influence survival in the presence of Rel A.

NF-κB can be activated in response to chemotherapy and can promote drug resistance through the induction of anti-apoptotic proteins like Bcl-2, survivin and c-FLIP as well as the activation of the AKT survival pathway (Ozes *et al.*, 1999; Granziero *et al.*, 2001; Aron *et al.*, 2003; Schimmer *et al.*, 2003a; 2003b). In this cohort, Rel A DNA binding was significantly higher in samples derived from previously treated patients (P<0.0001) and clearly increased in serial samples tested pre- and post-therapy. However, constitutive Rel A was significantly higher in patients who went on to require treatment when compared to those who did not. Furthermore, high Rel A was also associated with a shorter TTST in this cohort. Taken together, this data indicates that Rel A is constitutively elevated in patients with more aggressive disease but is also induced by conventional chemotherapy which in turn appears to contribute to the depth of response to subsequent treatment cycles.

One of the biggest drawbacks of this study was of the variability of treatment modalities used and the inability of assessing the response to treatment. Therefore it is important to prospectively assessing Rel A in a randomised clinical trial in order to address this issue in the future.

In conclusion, Rel A is a superior prognostic marker for survival in CLL and is the only prognostic marker currently available that appears to have the capacity to predict the duration of response to therapy (Grever *et al.*, 2007). In addition, this finding strongly supports the search for more accessible surrogate markers of NF-κB activation in this disease. Vascular endothelial growth factor (VEGF) and interleukin 6 (IL-6) are probable candidates for this purposes. The fact that Rel A DNA binding is independent of IgV_H mutation status, CD38 expression and ZAP-70 expression offers the possibility that the prognostic information derived from some or all of these factors could be combined to provide even more accurate prediction of clinical outcome for CLL patients in the future.

6.0 CONCLUSIONS AND FINAL DISCUSSION

6.1 SUMMARY OF FINDINGS

1. Total NF-κB activity, measured by EMSA, is heterogeneous in CLL patient samples.
2. Total NF-κB activity does not correlate with known prognostic markers like IgV_H mutational status, ZAP-70 expression and CD38 expression.
3. Rel A, p50 and c Rel are the main NF-κB subunits in CLL.
4. Rel A ELISA is a reliable method for assessment of NF-κB activity with good inter-assay variability.
5. NF-κB subunit Rel A activity measured by ELISA correlates with total NF-κB measured by EMSA.
6. NF-κB subunit Rel A activity measured by ELISA correlates with *in vitro* CLL cell survival, tumour bulk and disease progression and provides prognostic information that is independent of IgV_H mutational status, ZAP-70 expression and CD38 expression.
7. LC-1 is a potent NF-κB inhibitor and effective in all prognostic subsets of CLL samples.
8. LC-1 activates intrinsic and extrinsic apoptotic pathways.
9. LC-1 induced cell killing is preceded by time and dose- dependent reduction of Rel A and down regulation of NF-κB dependent anti-apoptotic genes.

10. LC-1 has the ability to overcome pro-survival signalling by CD40 ligation and IL-4.
11. LC-1 synergises with fludarabine and synergy is maintained in poor prognostic subsets of CLL. This combination allows fludarabine to be used at lower concentrations than clinically used today.
12. Rel A is a promising new clinical prognostic factor with ability to predict TTFT, TTST and OS.

6.2 FINAL DISCUSSION

In this study, I identified and validated NF-κB signalling as a key pathway in CLL and set out to conduct a detailed analysis of NF-κB activity, its biological relevance and its clinical implications. In addition, I also investigated the potential for using NF-κB inhibitors in the treatment of CLL.

One of the key achievements was to move beyond the simple concept that NF-κB is constitutively elevated in CLL. This study showed considerable variation in total NF-κB and the specific subunits of NF-κB, using more than one technique. We associated this variability with the biology of CLL cells. According to these results, only 20% of the variability of spontaneous apoptosis can be explained by Rel A DNA binding. It would be interesting to know what is controlling the rest of *in vitro* cell survival in these leukaemia cells. As NF-κB is a transcription factor, it may have a broader role in the CLL cells by altering the expression of genes under its control. Therefore, a logical next step would be to assess the differential expression of genes that are transcriptionally regulated by NF-κB.

Data from our research group clearly demonstrates that there is an association between the change in NF-κB DNA binding post stimulation with anti-IgM and *in vitro* cell survival. In this context, some cells showed increased and others showed decreased total NF-κB DNA binding. This suggests a functional importance of change of (inducible) NF-κB DNA binding in CLL cells and is supported by other published work. According to Mockridge *et al* (2007) cases responding to sIgM ligation usually expressed higher levels of CD38 and ZAP-70 and had unmutated IgV_H genes. This data implies downstream pathways are intact in CLL and it is interesting to investigate this further and identify the clinical differences between responders and non-responders. Again this would be an interesting part of a next step in our research.

Although it has long been known that NF-κB can be constitutively activated in CLL, the mechanism(s) involved in this are poorly understood. Signalling via BAFF and APRIL is thought to play a major role in NF-κB activation and survival of CLL (Nishio *et al.*, 2005; Tomoyuki E, 2006; Shinners *et al.*, 2007; Endo *et al.*, 2007). In addition, certain micro-environmental factors are thought to modulate NF-κB (Burger *et al.*, 2000; Tsukada *et al.*, 2002). In this study, I showed that NF-κB is not uniformly elevated in CLL but is very heterogeneous across the patient population studied. The question is what causes the differential signalling? Is it due to variability in micro-environmental factors or due to the inherent signalling capacity of CLL cells? In addition, there is some evidence to suggest that genetic mutations of the NF-κB signalling pathway change the expression of downstream molecules in haematological malignancies (Keats *et al.*, 2007). A reasonable next step in this line of

research would be to study the impact of micro-environmental factors on the regulation of NF-κB expression in CLL patients.

I have shown that Rel A DNA binding has the ability to predict a number of relevant clinical end-points in CLL including TTFT, TTST and OS. Furthermore, in agreement with the published data in other tumour settings (Nikanishi *et al.*, 2005), NF-κB increased post treatment in some of the patients studied. Also Rel A DNA binding was shown to influence the depth of remission as evidenced by shorter TTST in patients with high Rel A DNA binding. This finding has wider clinical implications in relation to the role that Rel A plays in drug resistance in CLL. However, the next challenge will be to test these findings in prospective clinical trials. One of the key factors in this regard will be the standardisation of the Rel A assessment process. We assessed Rel A in 131 patients with excellent inter-assay variability. However, there are a number of significant problems associated with this assay. Firstly, there may be batch to batch differences in antibody affinity (both primary and secondary). Secondly, there may also be variation in the concentration / strength of recombinant Rel A used to quantify Rel A DNA binding. In addition, the quality and absolute amount of nuclear extract can significantly vary between patients and so characterisation of these parameters is also essential. We managed to overcome a number of these issues by performing Rel A DNA binding using the same batch of ELISA kit and recombinant proteins. However if Rel A is to be used as a prognostic parameter in the clinical setting more stringent quality control measures are required. This could be done by developing locally or internationally accepted median levels and controls for antigen affinity and recombinant protein concentrations.

NF-κB is often differentially expressed in cancer cells and this can enhance the expression of MDR-1, whose product, P-glycoprotein, a plasma membrane transporter, mediates chemoresistance by inducing the efflux of chemotherapeutic molecules (Bentires-Ali *et al.*, 2003). In addition, p53 stabilization is decreased upon NF-κB activation (Tergaonka *et al.*, 2002). Taken together this makes NF-κB a promising drug target in cancer (Braun *et al.*, 2006). There is a wide array of small molecules targeting NF-κB under development. Some examples are sulphasalazine analogues, BAY-117082, Deguelin, Curcumin, DHMEQ and AT514 (Habens *et al.*, 2005; Escobar-Diaz *et al.*, 2005; Everett *et al.*, 2006; Horie *et al.*, 2006; Pickering *et al.*, 2007; Greeraerts *et al.*, 2007). In this study I demonstrated that the novel parthenolide analogue LC-1 is capable of induction of CLL cell killing irrespective of prognostic markers or *in vitro* resistance to conventional chemotherapeutics.

This highlights the potential for using drugs like LC-1 as a salvage treatment particularly in poor prognostic subsets of CLL. Unusually, LC-1 induced cell killing by activating both the intrinsic and extrinsic apoptotic pathways. In contrast, the purine nucleotide analogue fludarabine has been shown to induce apoptosis through the intrinsic apoptosis pathway only (Klopfer *et al.*, 2004). This observation led to my hypothesis that LC-1 and fludarabine may synergise in apoptosis induction. This was tested in 26 CLL patient samples and strong synergy was observed between these two agents. In addition, the synergy was undiminished in poor prognostic subsets of CLL, further supporting the use of LC-1, and NF-κB inhibitors in general in this subset of CLL patients.

The Bcl-2 family and IAP family proteins are implicated in apoptosis regulation and drug resistance in CLL and at least some of these proteins are regulated by NF-κB. In this study I examined the gene expression using RT-PCR of a limited number of proteins from these families (Bcl-2, BIRC5 and C-FLIP) and showed that LC-1 induced a significant reduction in gene transcription. Also this supports the results seen in apoptosis induction by LC-1 via both the extrinsic (CFLAR being an inhibitor of the extrinsic apoptotic pathway) and the intrinsic (BCL-2 being inhibitor of intrinsic apoptotic pathway) pathways.

In conclusion, the results derived from this study implicate a role for NF-κB in disease progression and resistance to treatment in CLL. Furthermore, NF-κB was shown to be a valid target for the treatment of this incurable disease and the pre-clinical agent LC-1 showed promising *in vitro* synergy with conventional chemotheraputic agents. Further work is clearly now warranted to validate these findings and to more fully understand how NF-κB is regulated in CLL cells.

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The NF-κB subunit Rel A is associated with in vitro survival and clinical disease progression in chronic lymphocytic leukemia and represents a promising therapeutic target

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In this study, we characterized nuclear factor κB (NF-κB) subunit DNA binding in chronic lymphocytic leukemia (CLL) samples and demonstrated heterogeneity in basal and inducible NF-κB. However, all cases showed higher basal NF-κB than normal B cells. Subunit analysis revealed DNA binding of p50, Rel A, and c-Rel in primary CLL cells, and Rel A DNA binding was associated with in vitro survival ($P = .01$) with high white cell count ($P = .01$) and shorter lymphocyte doubling time ($P = .01$). NF-κB induction after in vitro stimulation with anti-IgM was associated with increased in vitro survival ($P < .001$) and expression of the signaling molecule ZAP-70 ($P = .003$). Prompted by these data, we evaluated the novel parthenolide analog, LC-1, in 54 CLL patient samples. LC-1 induced apoptosis in all the samples tested with a mean LD_{50} of 2.8 μM after 24 hours; normal B and T cells were significantly more resistant to its apoptotic effects ($P < .001$). Apoptosis was preceded by a marked loss of NF-κB DNA binding and sensitivity to LC-1 correlated with basal Rel A DNA binding ($P = .03$, $r^2 = 0.15$). Furthermore, Rel A DNA binding was inversely correlated with sensitivity to fludarabine ($P = .001$, $r^2 = 0.3$), implicating Rel A in fludarabine resistance. Taken together, these data indicate that Rel A represents an excellent therapeutic target for this incurable disease. (Blood. 2008;111: 4681-4689)

Introduction

B-cell chronic lymphocytic leukemia (CLL) is a malignancy characterized by the accumulation of CD5, CD19, and CD23 positive lymphocytes. Diagnosis is aided by the CLL immunophenotyping score which includes assessment of CD5 and CD23, FMC7, CD79b, and surface IgM.[1] Although CLL is the commonest leukemia in the Western world, it manifests a very heterogeneous clinical course, with some patients having normal age-adjusted survival, whereas the median survival for those patients with advanced stage disease is only 3 years.[2] The factors that contribute to the pathogenesis and progression of this disease are poorly understood, but decreased susceptibility to apoptosis[3] and dysregulated proliferation have been implicated.[4] Clinical studies have shown that high ZAP-70 expression, high CD38 expression, unmutated V_H genes, and cytogenetic abnormalities (especially deletions of 11q and 17p) are all associated with a poor prognosis.[5-9]

Nuclear factor κB (NF-κB) is a collective name for a group of inducible homodimeric and heterodimeric transcription factors made up of members of the Rel family of DNA-binding proteins. In humans, this family is composed of c-Rel, Rel B, p50, p52, and Rel A (p65) which, when bound in the cytoplasm to inhibitor of NF-κB (IκB) proteins, are inactive.[10,11] Various factors, including ligation of CD40 or the B-cell receptor (BCR), result in proteosomal degradation of IκB releasing NF-κB, which then translocates to the nucleus.[10,11] Once in the nucleus, NF-κB can enhance survival by inducing apoptosis inhibitory proteins, including inhibitor of apoptosis proteins (IAPs), Fas-associated death domain (FADD)-like interleukin (IL)-1β–converting enzyme (FLICE), and FADD–like IL-1β–converting enzyme-inhibitory protein (FLIP).[12-14] CLL cells have been reported to exhibit high constitutive NF-κB activation compared with normal B lymphocytes.[15-17] Although the exact factors responsible for the constitutive expression of NF-κB are not fully resolved, many factors, including Akt activation, BCR signaling, CD40 ligation, IL-4, and B-cell activating factor (BAFF), have been shown to increase NF-κB activity and enhance CLL cell survival, with members of the Bcl-2 family being principal transcriptional targets.[18-22] Several recent studies have demonstrated the proof of concept of the effectiveness of targeting NF-κB in hematologic malignancies, including CLL[23,24] and acute myeloid leukemia.[25,26]

In this study, we first set out to determine the range of constitutive DNA binding of NF-κB within our patient cohort and to characterize the specific subunits of NF-κB in these primary CLL cells. We then went on to investigate the ability of freshly isolated CLL cells to induce NF-κB expression in response to BCR

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stimulation with anti-IgM and correlated our findings with proximal and distal signaling events, as defined by tyrosine phosphorylation, and changes in in vitro apoptosis. Basal and induced NF-κB was subsequently correlated with other known clinical prognostic markers. Finally, we investigated the effects of a novel NF-κB inhibitor LC-1 in primary CLL cells to identify potential biologic response markers to be used in an imminent clinical trial.

Methods

This study was approved by the South East Wales local research ethics committee (LREC #02/4806).

Patients

Peripheral blood samples from CLL patients were obtained with their written informed consent in accordance with the Declaration of Helsinki. CLL was defined by clinical criteria as well as cellular morphology and the coexpression of CD19 and CD5 in lymphocytes simultaneously displaying restriction of light-chain rearrangement. Comprehensive clinical information, including treatment histories, was available for all patients, and none of the previously treated patients had received chemotherapy within 3 months before sample collection. Staging was based on the Binet classification system.[27] The novel agent under investigation in these studies, LC-1, is a dimethylamino analog of parthenolide generated from the reaction of parthenolide with dimethylamine and was formulated as a water-soluble fumarate salt (by S.N. and P.A.C.). Details of the synthesis and structural identity of LC-1 are currently being prepared for publication. LC-1 was recently shown to selectively eradicate acute myelogenous leukemia stem cells in vitro via the induction of stress responses and the inhibition of NF-κB.[25]

ZAP-70 and CD38 expression

Cytoplasmic ZAP-70 expression was determined by using a modification of a previously published flow cytometry method.[28] Cells were fixed and permeabilized using the Fix and Perm kit (Caltag, Botolph Claydon, United Kingdom) according to the manufacturer's instructions. Permeabilized cells were labeled with the following antibodies: ZAP-70-Alexa fluor 488 (Caltag), CD3-phycoerythrin (PE), CD56-PE (BD Biosciences, Cowley, United Kingdom), and CD19-Allophycocyanin (APC) (Caltag). After appropriate lymphocyte gating, cytoplasmic ZAP-70 expression was determined in $CD19^+$ CLL cells by gating on the $CD3^+$ cells. Patients were considered to be ZAP-70 positive when more than or equal to 20% of the $CD19^+$ CLL cells had equal or greater expression of ZAP-70 than the gated $CD3^+$ cells. Cell surface expression of CD38 was examined by flow cytometry using a standard 3-color flow cytometry approach using CD5-fluorescein isothiocyanate (FITC; Dako UK, Ely, United Kingdom), CD38-PE (Caltag), and CD19-APC (Caltag). The cutoff point for CD38 positivity in CLL cells was more than or equal to 30%.

Intracellular expression of tyrosine phosphorylated proteins

CLL lymphocytes were analyzed by 3-color immunofluorescent staining using CD5 and CD19 surface antigenic markers in conjunction with a FITC-labeled phosphorylated-tyrosine antibody. The cells were analyzed on a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA) and at least 10 000 events were acquired from each sample. The mean fluorescent intensity for phosphorylated tyrosine was calculated using WinMDI software (J. Trotter, Scripps Research Institute, La Jolla, CA).

V_H gene mutation analysis

V_H gene mutational status was determined for all patients using the method described previously.[29] The resulting polymerase chain reaction products were sequenced and were considered unmutated if they showed 98% or greater homology with the closest germ line sequence.

Cell culture

Peripheral blood lymphocytes from 54 CLL patients and 10 age-matched normal control patients were isolated from freshly collected blood samples by density centrifugation. B cells were subsequently positively isolated using $CD19^+$ Dynabeads (Invitrogen, Paisley, United Kingdom), and the beads were then detached using DETACHaBEAD CD19 reagent (Invitrogen). The purity of the $CD5^+/CD19^+$ CLL cells was more than 96% and normal B cells ($CD19^+$) more than 90% as assessed by flow cytometry. $CD19^+$ lymphocytes were then cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (Invitrogen), 100 U/mL penicillin, and 100 μg/mL streptomycin in the presence of parthenolide or LC-1 (10^{-7} to 10^{-5} M) for up to 48 hours. Parallel experiments using fludarabine (10^{-7} to 10^{-5} M) were also performed to assess the comparative intrasample and intersample in vitro cytotoxicity. Control cultures were also carried out to which no drug was added to normal and leukemic lymphocytes. Cells were subsequently harvested by centrifugation and were analyzed by flow cytometry. Experiments were performed in either duplicate or triplicate.

Measurement of apoptosis by annexin V/propidium iodide dual labeling

Apoptosis was assessed by dual-color immunofluorescent flow cytometry as described previously. Briefly, 10^6 cells were washed in ice-cold phosphate buffered saline and were incubated for 15 minutes in the dark in 200 μL of binding buffer contain 4 μL annexin V-FITC and 10 μg/mL propidium iodide (PI; Caltag). Cells were analyzed on a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA).

Caspase-3 activation assay

CLL cells were incubated at 37°C in a humidified 5% carbon dioxide atmosphere in the presence of LC-1 (10^{-7} to 10^{-5} M) for 24 hours. Cells were then harvested by centrifugation and labeled with CD19-APC antibody. Subsequently, the cells were incubated for 1 hour at 37°C in the presence of the PhiPhiLux G_1D_2 substrate (Calbiochem, Nottingham, United Kingdom). The substrate contains 2 fluorophores separated by a quenching linker sequence that is cleaved by active caspase-3. Once cleaved, the resulting products fluoresce green and can be quantified using flow cytometry.

NF-κB detection by electrophoretic mobility shift assay

Nuclear extracts were generated from CLL cells (> 90% $CD5^+/CD19^+$ lymphocytes as assessed by flow cytometry) using a modification of a previously described protocol.[30] The protein from nuclear extracts was quantified by the Bradford method. Equivalent quantities of nuclear protein (1-4 μg) were incubated with ^{32}P radiolabeled probes, generated by T4 polynucleotide kinase labeling. The probes used corresponded to a consensus NF-κB binding site (Promega, Southampton, United Kingdom). The binding reaction was performed in binding buffer (4% glycerol, 1 mM ethylenediaminetetraacetic acid, 10 mM Tris, pH 7.5, 100 mM NaCl, 0.1 mg/mL nuclease-free bovine serum albumin, 50 mM dithiothreitol) with the addition of 2 ng of poly dIdC (GE Healthcare, Little Chalfont, United Kingdom) for 30 minutes at room temperature before separation of protein DNA complexes using a 4% native acrylamide gel. NF-κB DNA binding was quantified by scanning densitometry. NF-κB inhibition studies were also carried out in CLL cells exposed to LC-1 for 2, 4, 8, 16, and 24 hours.

Rel A NF-κB DNA binding detection by enzyme-linked immunosorbent assay

Nuclear extracts from CLL patients and B and T lymphocytes derived from age-matched controls were assayed for Rel A activity with a TransAM NF-κB transcripton factor assay kit according to the manufacturer's instructions (Active Motif, Carlsbad, CA). The principle behind the assay involves the capture of NF-κB by an oligonucleotide containing an NF-κB consensus site, which is immobilized in each well of a 96-well plate. The Rel A subunit was subsequently detected using a

Rel A-specific antibody. The normal lymphocytes were cell sorted (purity > 95% for each lymphocyte population), and these samples were compared with the CLL samples. The optical density reading at 450 nm (OD_{450}) was read on a microtiter plate reader (Bio-Rad, Hemel Hempstead, United Kingdom). OD_{450} values were converted into nanograms per microgram of nuclear extract for each sample tested from a standard curve constructed using known quantities of recombinant Rel A NF-κB.

Statistical analysis

All of the datasets derived in this study were tested for normality using the Kolmogorov-Smirnov test. Where the data were deemed to be Gaussian or approximately Gaussian, the datasets were compared using parametric assays (equal variance or paired Student *t* test); in all other cases, a Mann-Whitney test was used. Correlation coefficients were calculated from least squares linear regression plots, and LD_{50} values were calculated from line of best-fit analysis of the sigmoidal dose response curves. All statistical analyses were performed using Graphpad Prism 3.0 software (GraphPad Software, San Diego, CA).

Results

NF-κB is heterogeneous in CLL patient samples

CLL cells have been shown to exhibit high levels of nuclear NF-κB compared with nonmalignant B cells.[16,31] However, a detailed analysis of the variation in levels of NF-κB across multiple patients or a dissection of the constituent subunits in primary CLL cells has not been previously described. We investigated NF-κB DNA binding in 30 unselected CLL patients using electrophoretic mobility shift assay (EMSA). All of the isolated mononuclear samples tested had more than 90% $CD5^+/CD19^+$ lymphocytes as assessed by flow cytometry. We chose 2 cell lines as reference for the assay: BL41, a Burkitt lymphoma line with relatively low NF-κB activity, and IARC-171, an Epstein-Barr virus (EBV) immortalized line derived from the same patient. EBV activates NF-κB during B-cell immortalization, so IARC-171 cells represent a positive control for a B-cell high in NF-κB.

Figure 1A shows a representative sample of nuclear extracts derived from 12 patients demonstrating the differential expression of NF-κB DNA binding. DNA binding varied from almost undetectable (patients b, d, g, and i) to levels comparable to or higher than those found in the EBV-immortalized cells (patients c, h, and k) with other samples showing intermediate NF-κB DNA binding. The specificity of the NF-κB binding was confirmed by an EMSA cold competitor assay (Figure 1B). A nonradioactively labeled NF-κB DNA oligonucleotide eliminated binding, whereas an oligonucleotide for another transcription factor, AP1, left DNA binding unaffected.

Specific NF-κB subunits have different abilities to activate gene expression. For this reason, the next step of our study was a qualitative analysis of individual NF-κB subunits in leukemic CLL B cells. For this we performed super-shift EMSA using antibodies against specific NF-κB proteins (Rel A, p50, p52, c-Rel, and Rel-B) with nuclear extracts prepared from CLL cells (Figure 1C). A rabbit serum control was used in each case. Rel A, p50, and c-Rel subunits were detected in all the samples tested, as evidenced by shifts in their respective bands following antibody binding, but with marked variation in the amounts of each in the different patient samples.

NF-κB is a key link between BCR signals and cell apoptosis

We next investigated the ability of CLL cells to induce NF-κB as a downstream consequence of BCR ligation. Proximal signaling

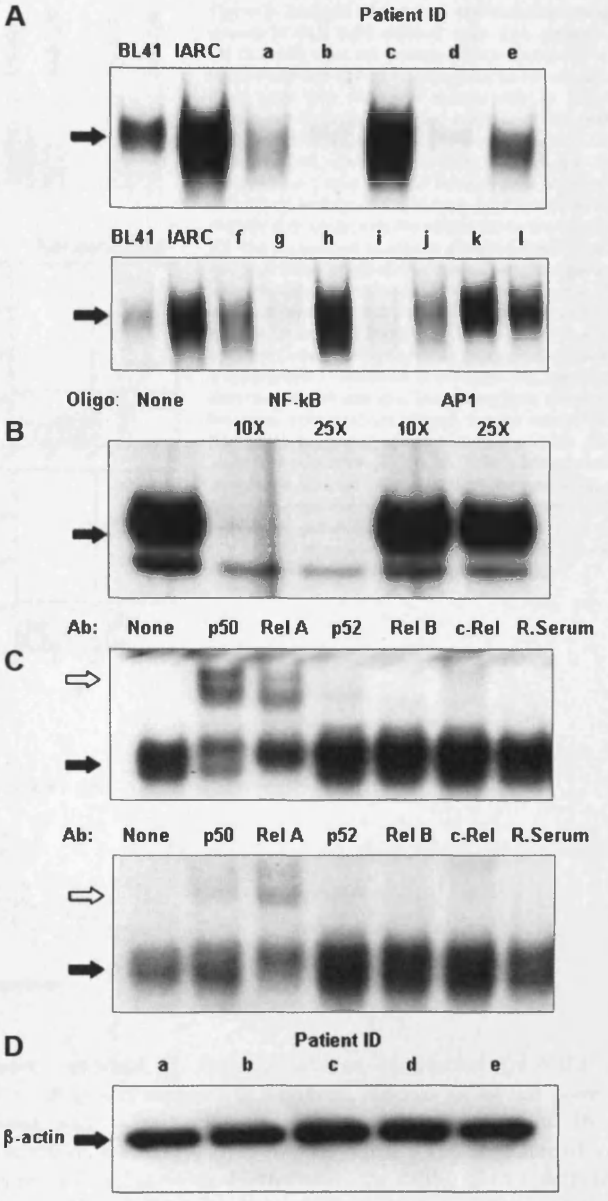

Figure 1. CLL patients show heterogeneity of NF-κB DNA binding. (A) NF-κB DNA binding activity in cell nuclear extracts was measured using electrophoretic mobility shift assays. An oligonucleotide corresponding to the consensus sequence to NF-κB was radiolabeled and incubated with 2 μg nuclear extract. The DNA-polynucleotide complex was restored by electrophoresis in a 4% native polyacrylamide gel in 0.5× Tris-borate/EDTA buffer. The gels were dried, and protein binding was visualized by autoradiography. To demonstrate specificity, a cold competitor assay was performed on 2 μg nuclear extracts of CLL patient k (B). Cold, nonradiolabeled NF-κB and a nonspecific oligonucleotide, AP1, was added at 10× and 25× the concentration of radiolabeled NF-κB and incubated for 30 minutes, before radiolabeled NF-κB incubation. (C) For qualitative analysis of NF-κB subunits, super-shift analysis was performed on NF-κB using p50, Rel A, p52, Rel B, and c-Rel antibodies and normal rabbit sera; 2 μg nuclear extracts from CLL nuclear extracts were used for these experiments. The different lanes marked, none, p50, Rel A, p52, Rel B, c-Rel, and Rab ser (pre-immune rabbit sera) represent incubation with different antibodies. They were then incubated with a radiolabeled oligonucleotide corresponding to the consensus sequence of NF-κB for 30 minutes. Ab indicates the different antibodies used; None, no antibody was incubated. White arrows indicate the antibody-protein-DNA complexes; black arrows, the protein-DNA complexes. Free DNA has been omitted. (D) Nuclear extracts from sample a through sample e were Western blotted for β-actin to assess the relative integrity of the nuclear protein extracts between samples and to confirm equal loading of nuclear proteins in the experiments.

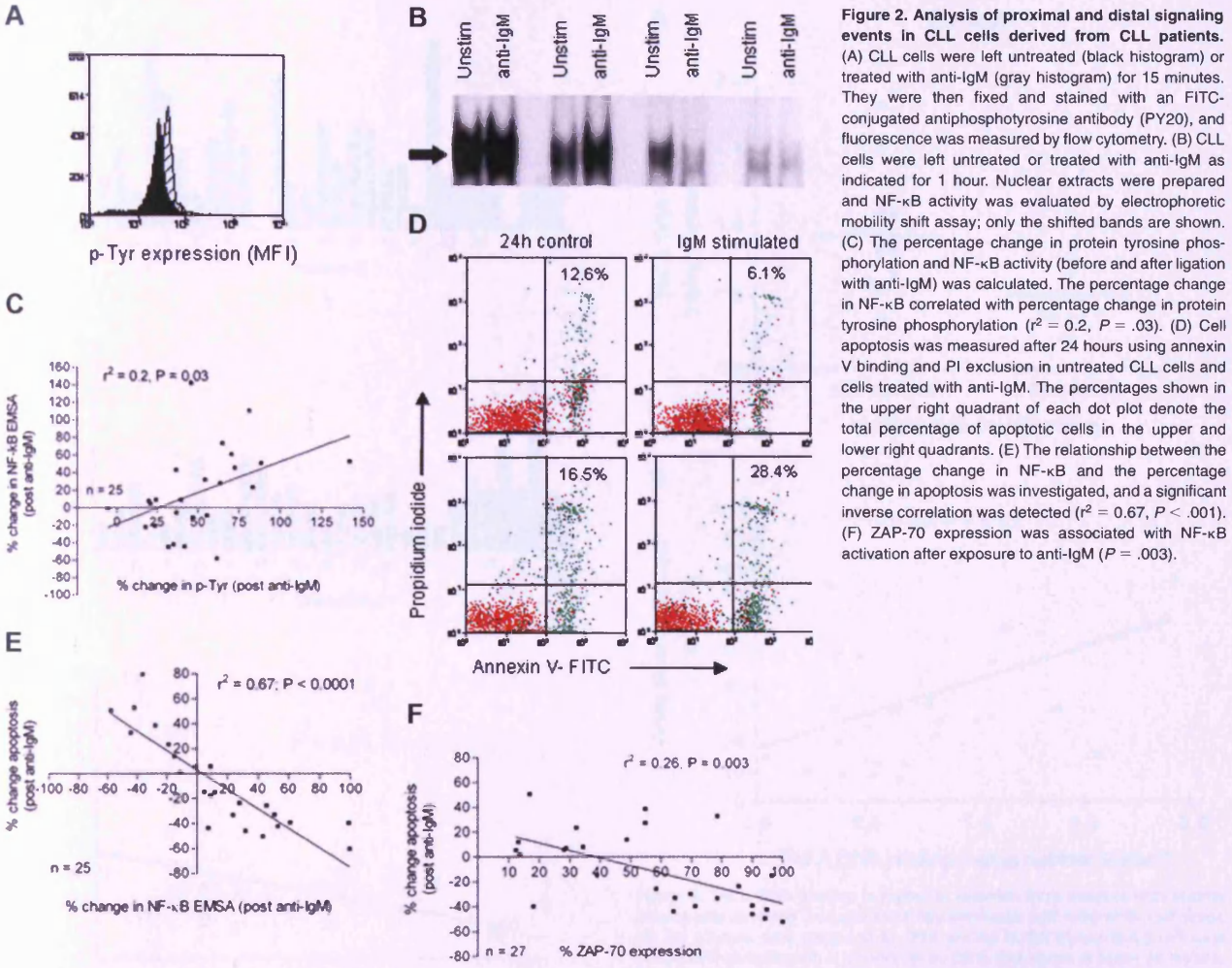

Figure 2. Analysis of proximal and distal signaling events in CLL cells derived from CLL patients. (A) CLL cells were left untreated (black histogram) or treated with anti-IgM (gray histogram) for 15 minutes. They were then fixed and stained with an FITC-conjugated antiphosphotyrosine antibody (PY20), and fluorescence was measured by flow cytometry. (B) CLL cells were left untreated or treated with anti-IgM as indicated for 1 hour. Nuclear extracts were prepared and NF-κB activity was evaluated by electrophoretic mobility shift assay; only the shifted bands are shown. (C) The percentage change in protein tyrosine phosphorylation and NF-κB activity (before and after ligation with anti-IgM) was calculated. The percentage change in NF-κB correlated with percentage change in protein tyrosine phosphorylation ($r^2 = 0.2$, $P = .03$). (D) Cell apoptosis was measured after 24 hours using annexin V binding and PI exclusion in untreated CLL cells and cells treated with anti-IgM. The percentages shown in the upper right quadrant of each dot plot denote the total percentage of apoptotic cells in the upper and lower right quadrants. (E) The relationship between the percentage change in NF-κB and the percentage change in apoptosis was investigated, and a significant inverse correlation was detected ($r^2 = 0.67$, $P < .001$). (F) ZAP-70 expression was associated with NF-κB activation after exposure to anti-IgM ($P = .003$).

was assessed by quantifying protein tyrosine phosphorylation before and after cross-linking of the BCR. Subsequently, NF-κB induction and cellular apoptosis were measured in paired CLL samples. Freshly isolated CLL cells from 24 patients were stimulated with anti-IgM for 15 minutes, and protein tyrosine phosphorylation was analyzed by flow cytometry. Figure 2A shows a typical example of an overlaid histogram plot of tyrosine phosphorylation in CLL cells derived from an individual patient before and after stimulation with anti-IgM. Using the same experimental paradigm, NF-κB was analyzed by electrophoretic mobility shift in samples with and without BCR stimulation. Figure 2B shows representative data from 4 different patient samples. To analyze the effects of BCR stimulation on NF-κB nuclear expression and because of the inherent variation from experiment to experiment when using EMSA, we internally controlled the experiments by expressing the data as percentage change in NF-κB after BCR stimulation (stimulated − control/control × 100). We identified a correlation ($r^2 = 0.2$, $P < .05$) between the percentage change in protein tyrosine phosphorylation and the percentage change in NF-κB (Figure 2C). We observed that anti-IgM treatment of CLL cells caused an increase in apoptosis in some samples and a decrease in apoptosis in others (Figure 2D). Importantly, this was reflected by a change in NF-κB; and when data from 24 patients were analyzed, a striking inverse correlation ($r^2 = 0.67$, $P < .001$) was detected in which an increase in NF-κB correlated with a reduction in cellular apoptosis (Figure 2E). Therefore, the data suggest that NF-κB is a key regulator of in vitro CLL cell survival. Furthermore, the ability of CLL cells to induce NF-κB DNA binding was closely associated with the expression of ZAP-70 (Figure 2F). These data support a previous report finding that forced overexpression of ZAP-70 in CLL cells enhanced BCR signaling.[32]

The Rel A NF-κB subunit DNA binding shows marked variability, which correlates with CLL cell survival

The variation in basal NF-κB prompted us to quantify the Rel A component of the complex. This component was of particular interest because it contains a transactivation domain absent in the p50 component of NF-κB.[33] Using a DNA binding ELISA-based method, we analyzed Rel A in nuclear extracts derived from freshly isolated CLL cells. We generated a standard curve (data not shown) using recombinant Rel A, which showed excellent linearity ($r^2 = 0.98$), and this allowed us to express the values for Rel A as nanograms per microgram of nuclear extract. Data from 30 patients are shown in Figure 3A, which shows the variability in Rel A from undetectable levels up to 1.909 ng/μg of nuclear extract. Samples derived from normal B and

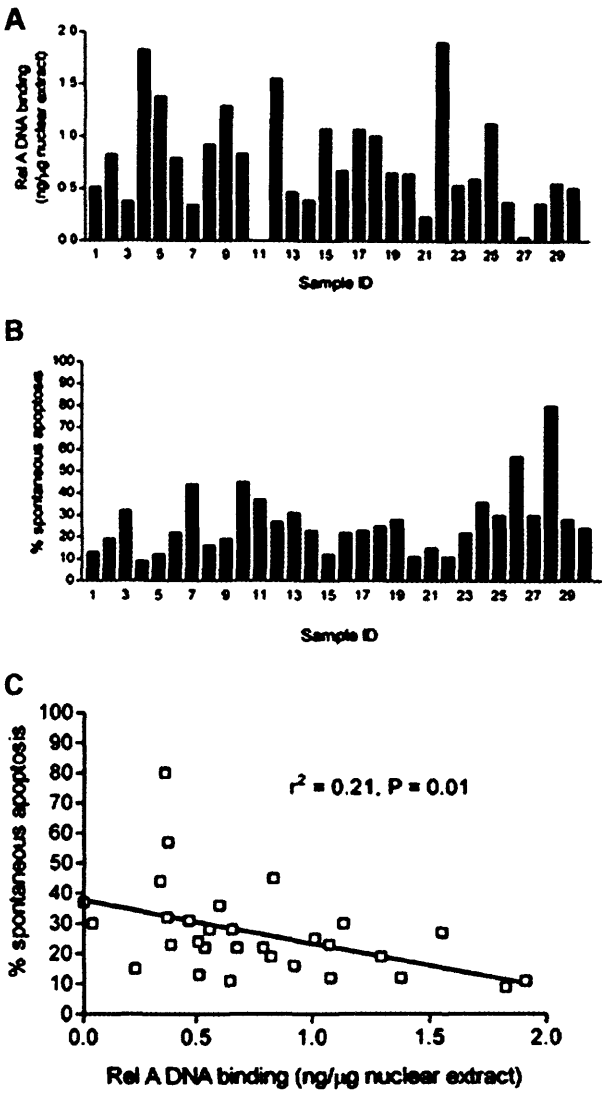

Figure 3. Quantitative analysis of rel A DNA binding in CLL B cells and its relationship to apoptosis. (A) Subunit-specific Rel A ELISA (Active Motif) assays were performed using 2 µg of nuclear extracts derived from 30 freshly isolated ex vivo CLL samples. Absorbance was measured at 450 nm. Results were compared with a standard curve generated using recombinant protein that allowed the calculation of the amount of p65 present in CLL cells expressed relative to total nuclear protein from CLL patient samples. (B) Parallel experiments were performed in which aliquots of CLL B cells derived from the same 30 CLL patients were set up in in vitro culture for 48 hours, and spontaneous apoptosis was then measured using annexin V and PI staining. FACS plots were analyzed using Summit 4.3 software (Dako). Percentage apoptosis was defined as the total annexin V^+ cells (PI^+ and PI^-) after 48 hours in culture. (C) The level of apoptosis showed a significant negative correlation with nuclear Rel A DNA binding activity in the nucleus of freshly isolated CLL cells.

T lymphocytes had levels of Rel A below the threshold of detection (data not shown). In parallel experiments, samples from each of these 30 patients were also analyzed for in vitro apoptosis after 48 hours in cell culture using annexin V and PI labeling (Figure 3B); again, the samples showed a wide variation ranging from 9% to 80%. We then compared the ex vivo Rel A DNA binding activity of each sample with their propensity to undergo in vitro apoptosis after 48 hours in culture. Figure 3C shows that there was a significant inverse correlation between CLL cell in vitro apoptosis and Rel A DNA binding activity ($r^2 = 0.21$, $P = .01$).

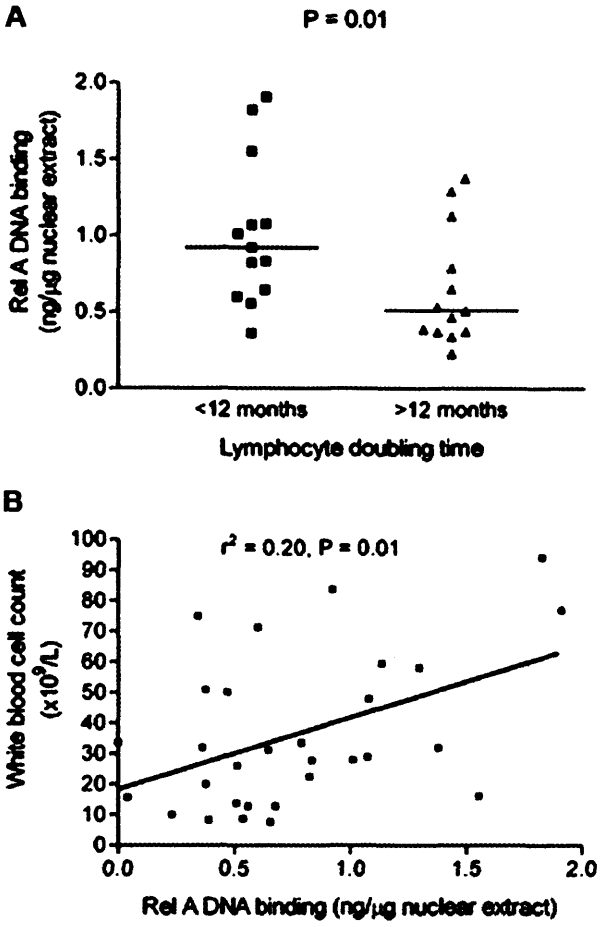

Figure 4. Rel A DNA binding is higher in samples from patients with shorter lymphocyte doubling time and positively correlates with total white cell count. (A) Rel A levels were measured by DNA binding ELISA (Active Motif) and were compared in patients with a lymphocyte doubling time above or below 12 months. Patients with lymphocyte doubling time of less than 12 months show a significantly higher level of Rel A ($P < .01$). (B) There was a significant positive correlation between Rel A DNA binding activity in cells from patients and their total white cell count ($r^2 = 0.2$, $P = .01$).

Basal Rel A DNA binding is higher in patients with shorter lymphocyte doubling times and positively correlates with total white cell count

Having implicated NF-κB subunit Rel A as important for in vitro CLL cell survival, we next investigated Rel A in relation to clinical markers of tumor burden and disease activity in CLL. Clinical laboratory parameters, such as lymphocyte count or total white cell count, bone marrow infiltration pattern, and lymphocyte doubling time (LDT), reflect the tumor burden and/or disease activity in CLL.[34] Rel A DNA binding was measured using subunit-specific ELISA in 30 freshly isolated patient samples and results were compared with LDT, calculated from patient records, and total white cell count on the date of sample collection. Rel A was significantly higher in patients with an LDT less than 12 months ($P < .01$; Figure 4A). There was also a significant correlation ($r^2 = 0.2$, $P = .01$) between Rel A DNA binding and total white cell count (Figure 4B). Taken together, these data suggest that Rel A contributes to tumor burden and disease activity in CLL. It is of interest that basal Rel A expression did not correlate with V_H gene mutation status, CD38 expression, or ZAP-70 expression in this cohort.

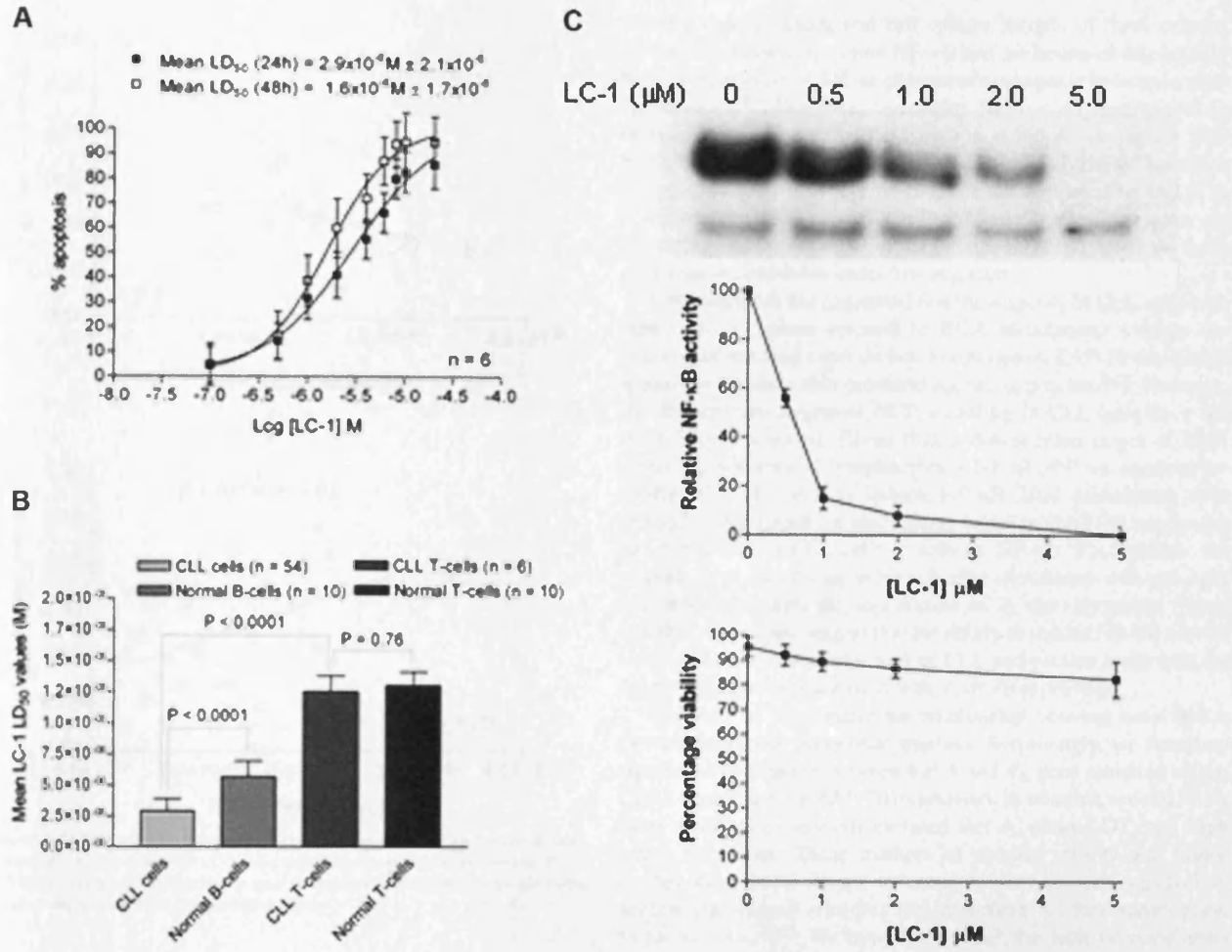

Figure 5. LC-1 and parthenolide induce time- and concentration-dependent apoptosis in primary CLL cells and follow the reduction of NF-κB. (A) $CD19^+$ CLL cells were treated with LC-1 (0-10 μM) for 24 and 48 hours and were then labeled with annexin V and PI. The percentage of apoptotic cells was calculated as the sum of the upper and lower right quadrants (ie, annexin V^+/PI^+ and annexin V^+/PI^-). A concentration-dependent increase in annexin V^+ cells was observed. (B) Normal $CD19^+$ B lymphocytes derived from nonleukemic, age-matched, donors were more than 2-fold less sensitive to the apoptotic effects of LC-1 than $CD19^+$ CLL cells ($P < .001$). (C) CLL cells were treated with LC-1 (0-5 μM) for 4 hours before harvesting and nuclear protein extraction. EMSA analysis (top panel) and quantification by densitometry (middle panel) revealed a dose-dependent decrease in NF-κB activity in the absence of detectable apoptosis (bottom panel) measured by annexin V and PI.

LC-1 inhibits NF-κB and induces apoptosis of CLL cells

We next investigated the in vitro effects of a putative NF-κB inhibitor, LC-1, in 54 freshly isolated CLL patient samples. LC-1 is a derivative of the sesquiterpene lactone parthenolide, which has been shown to selectively kill CLL cells in vitro[23] and targets the transcription factor NF-κB. This has been shown to be mediated through the inhibition of IκB kinase in HeLa cells,[35] but this has not been definitively shown in primary CLL cells. Parthenolide has poor solubility and unfavorable pharmacokinetics; in contrast, LC-1 has superior pharmacokinetic properties and more than 1000-fold greater solubility than the parent compound and therefore represents an excellent candidate drug. As part of our evaluation of the molecule, we wanted to establish whether Rel A DNA binding could predict for in vitro sensitivity to LC-1 and fludarabine.

CLL cells were incubated with a range of concentrations of LC-1 or parthenolide (0.1-10 μM) for up to 48 hours, and apoptosis was quantified using annexin V and PI labeling. Both LC-1 (Figure 5A) and parthenolide (data not shown) induced apoptosis was dose- and time-dependent. The 24-hour mean LD_{50} value (± SD) for LC-1 in the 54 CLL patient samples tested was 2.8 μM (± 1.0 μM). LC-1-induced apoptosis was associated with a concomitant increase in the activation of caspase-3 (not shown). LC-1 and parthenolide showed a time- and concentration-dependent increase in apoptosis with equimolar concentrations of LC-1 significantly more cytotoxic than parthenolide at both 24 hours ($P = .02$) and 48 hours ($P = .03$). Importantly, the mean LC-1 LD_{50} value was significantly lower for CLL cells than that derived from normal B and T cells and the T-cell derived from CLL patients (Figure 5B). This suggests a specificity of this inhibitor for leukemic cells. Figure 5C demonstrates that LC-1 inhibits NF-κB DNA binding in a dose-dependent manner. The inhibition is detectable at 4 hours and precedes any loss of cell viability; hence, the effect is not merely a consequence of cell death induction.

Correlation between Rel A DNA binding and in vitro drug sensitivity

We next examined the relationship between Rel A DNA binding and sensitivity to LC-1 and the purine nucleoside analog, fludarabine. Basal nuclear Rel A DNA binding was inversely correlated with LC-1 LD_{50} values (Figure 6A), suggesting that CLL cells that relatively overexpress Rel A are more dependent on NF-κB signaling to

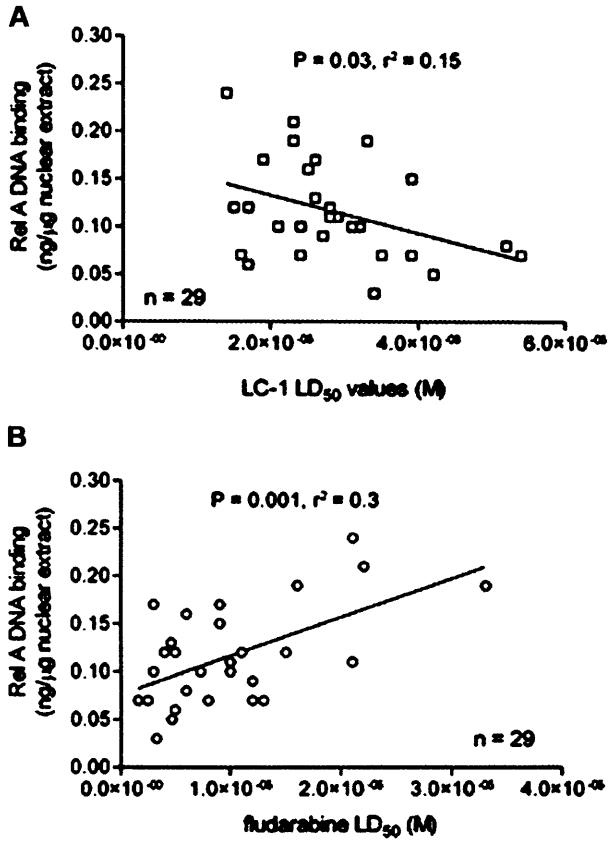

Figure 6. Rel A DNA binding correlates with sensitivity to LC-1 and resistance to fludarabine. (A) Sensitivity to LC-1 was inversely correlated with nuclear Rel A NF-κB DNA binding. (B) In contrast, in vitro resistance to fludarabine was positively correlated with nuclear Rel A NF-κB DNA binding.

maintain in vitro cell viability. In contrast, fludarabine LD_{50} values positively correlated with nuclear Rel A expression (Figure 6B), suggesting that drug resistance to fludarabine may be mediated, at least in part, through the transcriptional effects of Rel A.

Discussion

One of the key achievements of this study was to move beyond the relatively simplistic concept that NF-κB is elevated in CLL patient samples toward a more detailed qualitative and quantitative study of this important group of transcription factors. We have shown that CLL cells typically express p50, Rel A, and c-Rel but in various amounts. In agreement with previous reports,[15,16] even the CLL samples that had low NF-κB expression had higher levels than primary B cells in which no NF-κB could be detected by EMSA or Rel A-specific ELISA. NF-κB has been implicated in tumorigenesis and survival of a growing list of leukemias and lymphomas, including Hodgkin lymphoma, diffuse large B-cell lymphoma, multiple myeloma, acute lymphoblastic leukemia, and chronic myeloid leukemia.[10] However, no previous study has quantified NF-κB subunits in primary patient samples. Furthermore, this is the first study to relate this quantification to in vitro cell survival, tumor burden, and disease activity (LDT), thus demonstrating that an individual component of the NF-κB complex can contribute to the regulation of human disease.

This study builds directly on others that have shown NF-κB provides prosurvival signals to several different cell types. Both primary cancer tissues and cell culture models of these cancers exhibit constitutive activated NF-κB and inhibition of this activity by overexpression of IκB or pharmacologic agents induces in vitro apoptosis.[24,36,37] Our data, correlating Rel A with spontaneous in vitro apoptosis, show that the variation in Rel A can explain 21% ($r^2 = 0.21$) of the variation in apoptosis of CLL B cells. This raises 2 important questions: (1) what genes are regulated by Rel A to promote increased CLL cell survival? and (2) what molecules are regulating the other 79% of CLL cell survival? Both of these questions are currently under investigation.

Previous work has suggested that the majority of CLL cells with unmutated V_H genes respond to BCR stimulation, whereas the majority of mutated cases do not. Furthermore, ZAP-70 expression appears to enhance this proximal signaling process.[32,38] However, the downstream targets of BCR signaling in CLL cells have not been fully elucidated. Given that a downstream target of BCR signaling in normal B lymphocytes is NF-κB,[20,39] we assessed the ability of CLL cells to induce NF-κB after stimulation with anti-IgM. We found an association between ZAP-70 expression and the ability of CLL cells to activate NF-κB. Furthermore, the magnitude of the change in NF-κB after stimulation with anti-IgM was associated with the suppression of in vitro apoptosis. Taken together, these data suggest that the ability to induce NF-κB may be a critical factor in the pathology of CLL and present a rationale for the poor prognosis associated with ZAP-70 expression.

We went on to evaluate the relationship between basal Rel A DNA binding and prognostic markers. Surprisingly, we found no significant correlation between Rel A and V_H gene mutation status, CD38 expression, or ZAP-70 expression. In contrast, we did find a clear association between elevated Rel A, short LDT, and high white cell count. These markers of disease activity and tumor burden have been shown to correlate with overall survival in several studies and reinforce the importance of this transcription factor in CLL.[40-42] We hypothesize that the lack of correlation between basal Rel A DNA binding and V_H gene status, CD38, and ZAP-70 is because these factors contribute to the ability of CLL cells to induce NF-κB after stimulation rather than constitutive expression. In support of this, the data presented in this manuscript clearly show an association between ZAP-70 expression and the ability of CLL cells to activate NF-κB after stimulation with anti-IgM. Given the association between basal Rel A and high white cell count and LDT and the striking association between ZAP-70 and the ability to induce NF-κB, it seems that both basal and inducible NF-κB is important in the pathology of CLL.

Finally, this report represents the first preclinical evaluation of LC-1, a novel dimethylamino analog of parthenolide, in primary human CLL cells. LC-1 was more than 2-fold more potent in vitro than molar equivalents of parthenolide and has greater solubility and superior pharmacokinetics than the parent compound. Interestingly, LC-1 uniformly inhibited nuclear NF-κB activity in all the CLL samples tested, but strikingly cells derived from patients with the highest nuclear NF-κB activity were most sensitive to LC-1–induced apoptosis, suggesting some aspect of preferential NF-κB "oncogene addiction." Given the heterogeneity of NF-κB in the CLL patient cohort and our observation that Rel A is elevated in patients with a shorter lymphocyte doubling time, this study indicates that targeting NF-κB could be most valuable for patients with a poor prognosis. In this context, it is noteworthy that several other NF-κB inhibitors have also shown promise in preclinical evaluation in primary CLL cells.[23,24,43-46]

The development of drug resistance is a major clinical problem in CLL.[47-51] Therefore, it is important to note that sensitivity to LC-1 was

undiminished in patients who had received prior chemotherapy. Furthermore, 7 patients included in this study had marked clinical resistance to fludarabine but retained in vitro sensitivity to LC-1. In addition, a patient with a known p53 deletion showed similar sensitivity to LC-1 but, in contrast, showed marked in vitro resistance to fludarabine. At present, only 3 agents, alemtuzumab, flavopiridol, and corticosteroids, appear to retain clinical activity in patients with p53 deletions/mutations so new effective agents are urgently required for the treatment of these clinically problematic subsets.[52-60] LC-1, with its unique cytotoxic profile, could be considered for clinical trials for the treatment of poor prognosis CLL patients, including those with clinical resistance to fludarabine.

In conclusion, the data presented highlight the importance of both constitutive and inducible NF-κB in CLL. In particular, it indicates that the specific NF-κB subunit, Rel A, is associated with survival and possibly proliferation of CLL cells. The regulation of this transcription factor is complex and requires further elucidation to understand the critical signaling events that control NF-κB activation in vivo. However, our data suggest that Rel A may contribute to fludarabine resistance; therefore, molecules designed to inhibit NF-κB, and Rel A in particular, may be clinically useful both as single agents and in combination with chemotherapeutic drugs.

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Authorship

Contribution: S.H. performed research, analyzed data, and wrote the paper; S.A. performed research; T.T.L. performed research and analyzed data; M.C., C.J., and M.L.G. performed research; C.T.J. contributed vital new reagents and revised the manuscript; S.N., P.A.C., and A.K.B. contributed vital reagents; G.P. contributed vital new reagents and revised the manuscript; C.F. contributed vital new reagents, analyzed data, and revised the manuscript; C.R. contributed vital new reagents and revised the manuscript; P.B. and C.P. designed research, performed research, analyzed data, and wrote the paper.

Conflict-of-interest disclosure: C.T.J., S.N., and P.A.C. hold stock in Leuchemix, Inc. The other authors declare no competing financial interests.

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The Novel Nuclear Factor-κB Inhibitor LC-1 Is Equipotent in Poor Prognostic Subsets of Chronic Lymphocytic Leukemia and Shows Strong Synergy with Fludarabine

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Abstract **Purpose:** We have recently shown that the novel nuclear factor-κB (NF-κB) inhibitor LC-1 is effective in primary chronic lymphocytic leukemia (CLL) cells. Here we elucidated the mechanism of action of LC-1, evaluated its relative cytotoxicity in prognostic subsets, and investigated its potential synergistic interaction with fludarabine.

Experimental Design: Ninety-six fully characterized CLL cases were assessed for *in vitro* sensitivity to LC-1 and fludarabine. In selected cases, caspase activation, inhibition of Rel A DNA binding, and the transcription of *CFLAR, BIRC5*, and *BCL2* were measured before and after exposure to LC-1. In addition, the efficacy of LC-1 was assessed in the presence of the survival factors CD154 and interleukin-4, and the potential synergistic interaction between LC-1 and fludarabine was evaluated.

Results: Cell death was associated with caspase-3 activation mediated via activation of both caspase-8 and caspase-9. Apoptosis was preceded by a reduction of nuclear Rel A DNA binding and inhibition of *CFLAR, BIRC5*, and *BCL2* transcription. Importantly, LC-1 overcame the cytoprotective effects by interleukin-4 and CD40 ligand and was equipotent in CLL cells derived from good and bad prognostic subsets. LC-1 exhibited strong synergy with fludarabine, and the combination produced a highly significant mean dose reduction index for fludarabine of >1,000.

Conclusions: In view of imminent first-in-man study of LC-1 in Cardiff, these data show an important mechanistic rationale for the use of LC-1 in this disease. Furthermore, it validates the concept of targeting nuclear factor-κB in CLL and identifies the therapeutic potential of LC-1 in combination with fludarabine even in patients with fludarabine resistance.

B-cell chronic lymphocytic leukemia (CLL) is the most common form of leukemia in adults in the western world and is characterized by the accumulation of CD5-, CD19-, and CD23-positive lymphocytes in the peripheral blood, lymph nodes, bone marrow, liver, and spleen. Factors contributing to the pathogenesis and progression of this disease are poorly understood, but decreased susceptibility to apoptosis (1) and altered control of proliferation have been implicated (2). CLL manifests a heterogeneous clinical course, with some patients having normal age-adjusted survival whereas the median survival for those patients with advanced-stage disease is only 3 years (3). Clinical treatment of CLL is limited by the development of progressive drug resistance and the non-selectivity of most drugs toward malignant cells. Chemotherapeutic drugs, such as fludarabine, chlorambucil, prednisolone, and certain monoclonal antibodies are able to induce CLL cell apoptosis *in vivo*, but complete remissions are rare and all patients eventually relapse (4). Therefore, new treatment options based on the biological characteristics of CLL cells are required to improve the prognosis of this incurable leukemia.

The nuclear factor-κB (NF-κB) cell signaling pathway is an attractive therapeutic target as NF-κB proteins have been implicated in the regulation of genes controlling apoptosis, proliferation, angiogenesis, and metastasis in various cancers (5–8). Consequently, numerous small molecular inhibitors of NF-κB have undergone preclinical investigation (9–12) but none has yet reached clinical trials. In the context of CLL, a number of studies have suggested that CLL cells exhibit constitutive NF-κB activation (13–15). More recently, however, we showed that there is considerable variation in the degree of activation of NF-κB in CLL patient samples (16). We and others have shown that parthenolide and its analogue LC-1 are able to induce apoptosis *in vitro* and this seemed to be mediated via the inhibition of NF-κB (16, 17). In view of an imminent phase 1 clinical trial of LC-1 in patients with CLL and other

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Translational Relevance

Our study contains important new evidence on the mechanism of action of the nuclear factor (NF)-κB inhibitor LC-1 and represents the most thorough investigation to date of the biological consequences of NF-κB inhibition in chronic lymphocytic leukemia. Our findings clearly show that LC-1 inhibits Rel A DNA binding and consequently down-regulates the transcription of NF-κB target genes. These events precede the induction of apoptosis. Importantly, LC-1 is able to overcome the prosurvival effects of CD40 ligand and interleukin-4; both thought to be important in maintaining chronic lymphocytic leukemia cells *in vivo*. In addition, we showed that LC-1 is equipotent in good and poor prognostic subsets and showed profound synergy with fludarabine, suggesting that this therapeutic strategy may have clinical application particularly in drug resistant/poor prognostic disease. Given that LC-1 will undergo first-in-man studies in Cardiff later this year, our data are both timely and relevant.

hematologic malignancies, we set out to fully elucidate the mechanism(s) of action of LC-1, to assess its relative efficacy in poor prognostic subsets, and to examine the potential for synergy with fludarabine in CLL patient samples. We also assessed the ability of LC-1 to overcome survival signals thought to be critical for *in vivo* CLL cell survival.

Patients, Materials, and Methods

Peripheral blood samples from 96 patients with CLL were obtained with the patients' written informed consent (LREC 02/4806). CLL was defined by clinical criteria as well as cellular morphology and the coexpression of CD19 and CD5 in lymphocytes simultaneously displaying restriction of light-chain rearrangement. Clinical information including treatment histories was available for all patients, and none of the previously treated patients had received chemotherapy within 3 mo before sample collection. The novel compound LC-1 is a dimethylamino derivative of the naturally occurring small molecule parthenolide. LC-1 (also known as DMAPT) was prepared from the reaction of parthenolide with dimethylamine, and the resulting dimethylamino analogue was then converted to its water-soluble fumarate salt. The irreversible inhibitor of IκB kinase (IκK), BAY 11-7082, was purchased from Calbiochem. The Rel A inhibitory peptide set (Ser276) was purchased from Imgenex.

Isolation of mononuclear cells. Human peripheral blood was layered onto Histopaque 1077 (Sigma-Aldrich) and centrifuged at 300 × *g* for 30 min. After centrifugation, the opaque interface containing mononuclear cells was aspirated and transferred into a clean sterile centrifuge tube. The mononuclear cells were then washed with PBS (pH 7.2; Sigma-Aldrich) at 300 × *g* for 5 min. After washing, the supernatant was aspirated and discarded. The pellet containing mononuclear cells was then washed with 0.87 mol/L of ammonium chloride (Sigma-Aldrich) solution to lyse contaminating erythrocytes at 300 × *g* for 5 min. After centrifugation, the supernatant was aspirated and discarded, and the pellet was again washed with PBS (pH 7.2; Sigma-Aldrich) at 300 × *g* for 5 min and repeated. After final wash, the mononuclear cells were resuspended in PBS (pH 7.2; Sigma-Aldrich) and counted using a Vi-cell XR cell counter (Beckman Coulter).

In vitro *cytotoxicity assays.* Peripheral blood lymphocytes from 96 CLL patients were isolated from freshly collected blood samples by density centrifugation. Mononuclear cells from CLL patients were cultured in RPMI 1640 supplemented with 10% heat-inactivated FCS (Invitrogen), 100 units/mL penicillin, and 100 μg/mL streptomycin in the presence of LC-1 (0.5-8 μmol/L) and fludarabine (0.25-16 μmol/L). Control cultures were also carried out to which no drug was added. Cells were subsequently harvested by centrifugation and were analyzed by flow cytometry for apoptosis or used for protein extraction. Similar experiments were also done using BAY 11-7082 and the Rel A inhibitory peptide.

Measurement of apoptosis by Annexin V/propidium iodide dual labeling. Apoptosis was assessed by dual-color immunofluorescent flow cytometry as described previously (18). Briefly, 1×10^6 cells were washed in ice-cold PBS and were incubated for 15 min in the dark in 196 μL of binding buffer and 4 μL of Annexin V-FITC. Propidium iodide (10 μg/mL; Invitrogen) was added and cells were immediately analyzed on a FACS Calibur flow cytometer (Becton Dickinson).

In vitro *synergy assessment.* Peripheral blood lymphocytes from 24 CLL patients were isolated from freshly collected blood samples by density centrifugation. After the optimal molar ratio for the combination of LC-1 and fludarabine (2:1) was experimentally determined, CLL lymphocytes were cultured in the presence of LC-1 (0.5-8 μmol/L) and/or fludarabine (0.25-4 μmol/L) for up to 48 h. Control cultures were also carried out in which no drug was added. Apoptosis was assessed by Annexin V/propidium iodide dual labeling.

Caspase-3 activation assay. CLL cells were incubated at 37°C in a humidified 5% carbon dioxide atmosphere in the presence of LC-1 (0.5-8 μmol/L) for up to 48 h. Cells were then harvested by centrifugation and labeled with CD19-APC antibody. Subsequently the cells were incubated for 1 h at 37°C in the presence of the PhiPhiLux G_1D_2 substrate (Calbiochem). The substrate contains two fluorophores separated by a quenching linker sequence that is cleaved by active caspase-3. Once cleaved, the resulting products fluoresce green and can be quantified using flow cytometry. Also cells were further analyzed using caspase inhibition with caspase-8 inhibitor (ZIETD.fmk; 50 μmol/L), caspase-9 inhibitor (ZLEHD.fmk; 50 μmol/L), or pan-caspase inhibitor (ZVAD.fmk; 50 μmol/L).

Nuclear and cytosolic protein extraction. Nuclear extracts were prepared using a method previously described (19). Cells were first washed in 1 mL of hypotonic buffer [10 mmol/L HEPES (pH 7.9), 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl] and centrifuged at 10,000 × *g* for 1 min. Cells were then lysed in 100 μL hypotonic buffer supplemented with 1 mmol/L phenylmethosulfonylfluoride, 1:100 dilutions of phosphatase inhibitor cocktails I and II (Sigma P-2850 and P-5726), and 0.1% (v/v) NP40 detergent and placed on ice for 5 min. Following centrifugation (10,000 × *g*, 5 min, 4°C), the supernatant was retrieved and this represented a cytosolic extract. Before extraction, the nuclear extract was washed by the addition of 1 mL of hypotonic buffer followed by centrifugation. Nuclear extracts were prepared by incubating the remaining pellet for 15 min in 50 μL high salt buffer [20 mmol/L HEPES (pH 7.9), 420 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 25% (v/v) glycerol] supplemented with 1 mmol/L phenylmethosulfonylfluoride and 1:100 dilutions of phosphatase inhibitor cocktails I and II immediately before use. Following centrifugation (10,000 × *g*, 5 min, 4°C), the supernatant was collected representing the nuclear extract. Quantification of protein was by Bradford-based assay (Bio-Rad).

ZAP-70 and CD38 expression. The V_H gene mutational status, and ZAP-70 and CD38 expression were assessed as previously described (16). V_H sequences with a germline homology of ≥98% were regarded as unmutated. The cutoff point for ZAP-70 and CD38 positivity was ≥20% expression.

Rel A detection by ELISA. Nuclear proteins were assayed for Rel A DNA binding with a TransAM NF-κB kit according to the manufacturer's instructions (Active Motif). NF-κB binding assays in microwells are reported to be at least 10 times more sensitive than electrophoretic mobility shift assay (20). The consensus oligonucleotide used for NF-κB binding was 5′-GGGACTTTCC-3′. A wild-type and mutated consensus oligonucleotides were used to monitor the

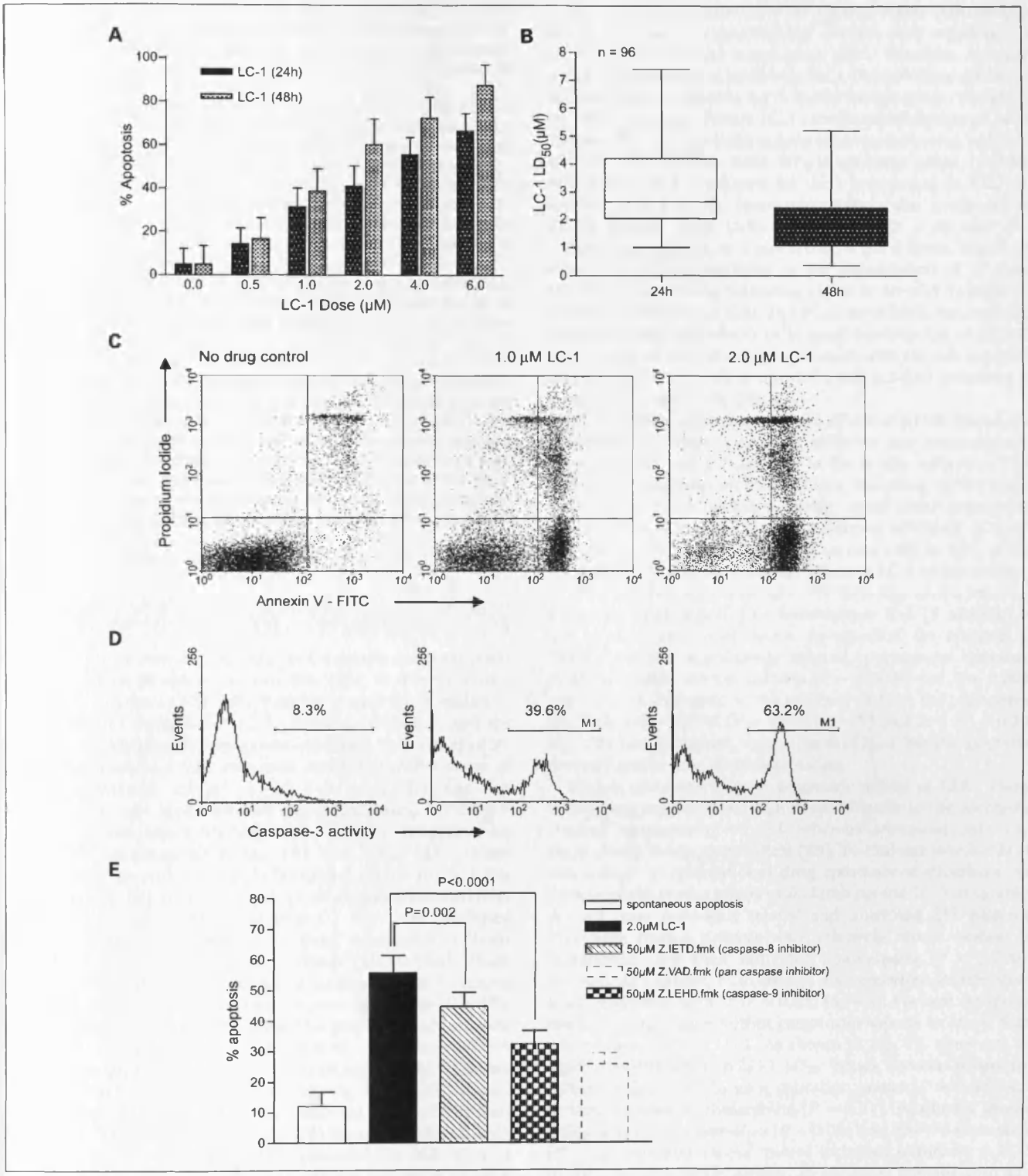

Fig. 1. LC-1 induces both the intrinsic and extrinsic apoptotic pathways. *A,* LC-1 induced a time- and dose-dependent increase in apoptosis in CLL cells. Here a composite graph of six representative patients is shown. *B,* the cytotoxic effects of LC-1 were consistent across the whole cohort (n = 96). *C,* CLL cells were treated with LC-1 (0-8 μmol/L) for 24 h and 48 h and were then labeled with Annexin V and propidium iodide. A concentration-dependent increase in Annexin V – positive cells was observed. The percentage of apoptotic cells was calculated as the sum of the upper and lower right quadrants, i.e., Annexin V – positive/propidium iodide – positive and Annexin V – positive/propidium iodide – negative. *D,* under the same conditions, caspase-3 activity was determined by using a fluorogenic substrate molecule that once cleaved by active caspase-3, fluoresced green and was detected by flow cytometry. The increase in caspase-3 activation was concentration-dependent and was correlated with an increase in apoptotic cell death. *E,* cells were cultured with caspase-8 inhibitor (50 μmol/L), caspase-9 inhibitor (50 μmol/L), or pan caspase inhibitor (50 μmol/L) with or without LC-1. Caspase-3 activation was measured as in *B*.

specificity of the assay. The absorbance reading at 450 nm (A_{450}) was read on a microtiter plate reader (Bio-Rad). A_{450} values were converted into ng Rel A NF-κB per microgram of nuclear protein for each sample tested from a standard curve constructed using known quantities of recombinant Rel A.

Real-time reverse transcription-PCR. The amount of *CFLAR*, *BIRC5*, and *BCL2* mRNA was assessed using real-time reverse transcription-PCR (RT-PCR) using the Light Cycler System (Roche Diagnostics). Total RNA was extracted, reverse-transcribed with random hexamers, and amplified using *CFLAR*-specific primers 5′-AGAGTGAGGCGATTTGACCTG-3′ (forward) and 5′-AAGGTG-AGGGTTCCTGAGCA-3′ (reverse); *BIRC5*-specific primers 5′-TGTTGGGAATCTGGAGATGA-3′ (forward) and 5′-CGGATGAACTCCTGTCCTTT-3′ (reverse); and *BCL2*-specific primers 5′-GGTCATGTGTGTGGAGAGCG-3′ (forward) and 5′-GGTGCCGGTTCAGGTACTCA-3′ (reverse) purchased from Eurogentec S.A. In addition, the amount of *RPS14* mRNA was quantified in all samples as an internal housekeeping control using 5′-GGCAGACCGA-GATGAACTCT-3′ (forward) and 5′-CCAGGTCCAGGGGTCTTGGT-3′ (reverse) and SYBRGreen as a detection method. The results of the real-time RT-PCR were expressed as normalized target gene values, e.g., the ratio between *CFLAR* and *RPS14* transcripts calculated from the crossing points of each gene. All experiments were done in duplicate.

Statistical analysis. All statistical analyses were done using Graphpad Prism 4.0 software (Graphpad Software, Inc.). Drug sensitivity for each individual drug and drug combination was evaluated using nonlinear regression and line of best fit dose-response curves. Curves were then analyzed using the median effect method to determine the degree of synergy (21). Combination indices and dose reduction indices were assessed using Calcusyn software (Biosoft).

Results

LC-1 induces both the intrinsic and extrinsic apoptotic pathways. We have previously shown that LC-1 is able to induce apoptosis in primary CLL cells. Here we extend those studies to investigate the dynamics of LC-1–induced cell killing and the specific mechanism(s) of apoptosis induction in a cohort of 96 CLL patient samples. CLL cells were incubated with a range of concentrations of LC-1 (0.5-8 μmol/L) for up to 48 hours and apoptosis was quantified using Annexin V and propidium iodide labeling. LC-1–induced apoptosis was dose- and time-dependent (Fig. 1A) with mean LD_{50} values (±SD) of 2.9 μmol/L ± 1.4 at 24 hours and 1.8 μmol/L ± 1.0 at 48 hours (Fig. 1B). LC-1–induced apoptosis was associated with caspase-3 activation (Fig. 1C and D) that was inhibited by the pan-caspase inhibitor (zVAD.fmk), a caspase-9 inhibitor (zLEDH.fmk), and a caspase-8 inhibitor (zIETD.fmk). These data indicate that LC-1 induces apoptosis through the induction of both the intrinsic and extrinsic apoptotic pathways (Fig. 1E).

LC-1 inhibits Rel A DNA binding. Our previous study showed that LC-1 inhibited NF-κB as evidenced by decreased DNA binding in electrophoretic mobility shift assays (16). Here we extend these observations by examining its effects on the specific NF-κB subunit Rel A. LC-1 induced a time-dependent (Fig. 2A) and dose-dependent (Fig. 2B) decrease in Rel A DNA binding. Importantly, these effects preceded the induction of apoptosis (Fig. 2C and D). To show that the inhibition of Rel A was important for the induction of CLL cell apoptosis, we used a previously described Rel A inhibitory peptide (22) to evaluate the consequences of specific Rel A inhibition. The Rel A inhibitory peptide induced significantly more cell death in CLL cells than both the no-peptide controls and the control peptide–treated cells (Fig. 2E).

LC-1–induced suppression of NF-κB target gene transcription. Rel A contains a transactivating domain that regulates the transcription of NF-κB target genes (22). Therefore, a downstream consequence of inhibiting Rel A DNA binding should be the inhibition of specific Rel A transactivated genes. Therefore, we next examined whether LC-1–mediated inhibition of Rel A had an effect on downstream gene transcription using real-time RT-PCR. We studied three NF-κB-regulated genes (*CFLAR*, *BIRC5*, and *BCL2*) selected for their importance in CLL cell survival (15, 23, 24). Gene transcription was compared in paired samples from individual patients (n = 6) with and without the addition of 2 μmol/L LC-1 for 4 hours. Figure 2F shows significant reductions in the transcription of all three NF-κB-regulated genes following exposure to LC-1 (*CFLAR* by 2.7-fold, *BIRC5* by 5.3-fold, and *BCL2* by 3-fold). Furthermore, we showed similar reductions in gene transcription of *CFLAR*, *BIRC5*, and *BCL2* following incubation with the IκK inhibitor BAY 11-7082 (25) with a 1.3-, 2.5-, and 2.5-fold reduction in expression, respectively (Fig. 2G).

LC-1 overcomes the cytoprotective effects of CD40 ligand and interleukin-4. There is growing evidence that microenvironmental factors play a major role in the *in vivo* survival of CLL cells (26). A number of these factors, including CD40 ligand (CD154) and interleukin-4 (IL-4), cause their prosurvival effects, at least in part, via the induction of NF-κB (14, 27, 28). As these signals seem crucial to CLL cells *in vivo*, it was important to assess the cytotoxic effects of LC-1 in the presence of these survival signals *in vitro*. We therefore added recombinant CD154 (1 μmol/L) or recombinant IL-4 (5 nmol/L) to our *in vitro* cytotoxicity assays. As expected, the addition of CD154 and IL-4 significantly reduced spontaneous apoptosis in the untreated control cultures (P = 0.0006 and P = 0.004, respectively). However, in the presence of LC-1 the prosurvival effect of both CD154 (P = 0.49; Fig. 3B) and IL-4 (P = 0.33; Fig. 3D) was abrogated, suggesting that LC-1 has the potential to overcome *in vivo* survival signaling. F3

LC-1 is equipotent in poor prognostic subsets of CLL. Pleiotropic drug resistance is a significant obstacle to the successful clinical management of CLL whereby treatment with one agent causes resistance to others (29). To evaluate whether LC-1 was subject to conventional drug resistance mechanisms, we compared the *in vitro* effects of fludarabine and LC-1 in samples derived from previously treated and untreated CLL patients. Previously treated patients were relatively more resistant to fludarabine than their untreated counterparts (P < 0.0001; Fig. 4A). In contrast, both treated and untreated groups show equal sensitivity to LC-1 (P = 0.53; Fig. 4A). We next compared the LC-1 LD_{50} values within prognostic subsets to assess their relative sensitivity to LC-1. As shown in Fig. 4B, there was no significant difference in LC-1 LD_{50} values between prognostic subsets defined by V_H gene mutation status (P = 0.21) and *in vitro* response to fludarabine (P = 0.21). Prognostic groups defined by CD38 expression (P = 0.06) and ZAP-70 expression (P = 0.05) showed a trend toward increased sensitivity to LC-1 in the poor prognostic subsets. In addition, patients with 17p and 11q abnormalities also showed similar sensitivity to the apoptotic induction by LC-1. Taken together, these data show the potential value of inhibiting NF-κB as a therapeutic strategy in CLL, particularly in resistant and relapsed disease. F4

LC-1 shows strong synergy with fludarabine in primary CLL cells. Having shown the cytotoxic profile of LC-1 in poor

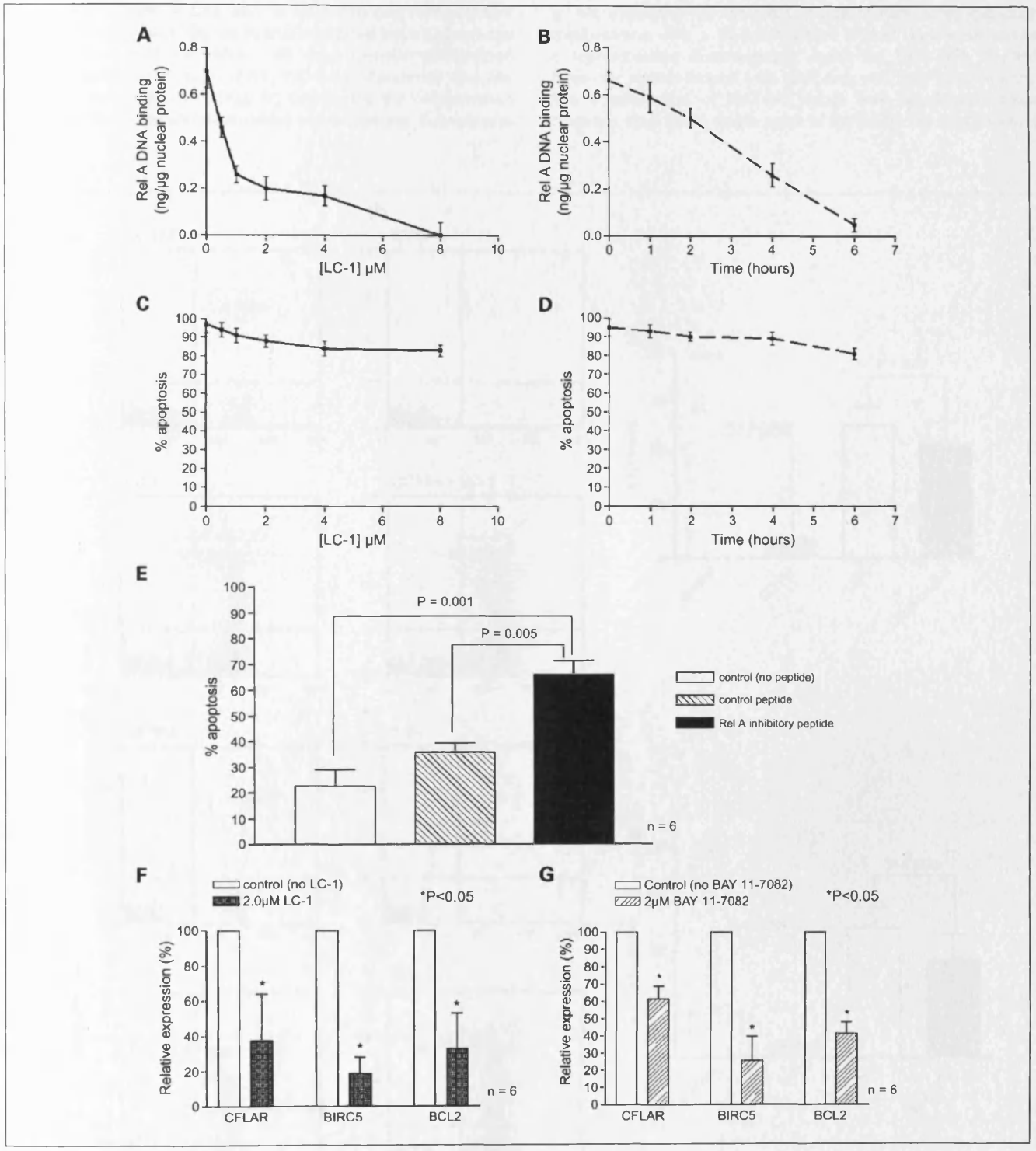

Fig. 2. Effects of LC-1 on constitutive Rel A activity and antiapoptotic genes in primary CLL cells. *A,* CLL cells were treated with 2 μmol/L of LC-1 for 1, 2, 4, and 6 h before apoptosis assay and nuclear protein extraction. ELISA analysis revealed a time-dependent decrease in Rel A DNA binding. *B,* CLL cells were treated with LC-1 (0-8 μmol/L) for 4 h before apoptosis assay and nuclear protein extraction. ELISA analysis revealed a dose-dependent decrease in Rel A DNA binding. *C* and *D,* up to 4 h time point there was little evidence of apoptosis induction as measured by flow cytometry using the Annexin V/propidium iodide assay. Therefore, the loss of NF-κB activity preceded the induction of apoptosis and was not merely a consequence of the commitment to cell death. CLL cells showed a time- and dose-dependent reduction in Rel A DNA binding following exposure to LC-1. *E,* we confirmed the importance of Rel A for the maintenance of CLL cell survival by specifically inhibiting this transcription factor with a Rel A inhibitory peptide. The peptide (50 μmol/L) induced apoptosis in all of the samples tested ($n = 6$) following exposure for 24 h. *F,* we also examined the transcription of downstream NF-κB target genes by real-time RT-PCR. CLL cells were treated with or without 2 μmol/L of LC-1 for 4 h and (*G*) with or without 2 μmol/L of BAY 11-7082 for 4 h. The expression of *CFLAR, BIRC5*, and *BCL2* were quantified using real-time RT-PCR and were normalized to the housekeeping gene *RPS14*. Both LC-1 and BAY 11-7082 induced similar reductions in these genes over the same time period, suggesting a common mechanism of action. All experiments were carried out in duplicate.

prognostic subsets of CLL and its ability to overcome *in vitro* resistance to fludarabine, we next investigated potential synergy between LC-1 and fludarabine. We experimentally determined the optimal molar ratio (2:1, LC-1: fludarabine) for the combination of the two drugs by comparing the combination index derived from different molar combinations. Subsequently, we measured the cytotoxic effects of each drug and their combination over a 24-hour culture period. Figure 5A shows a representative dose-response curve for CLL cells derived from one patient treated with each drug and their combination. The combination of the two drugs was significantly more cytotoxic than either single agent as shown by the median effect

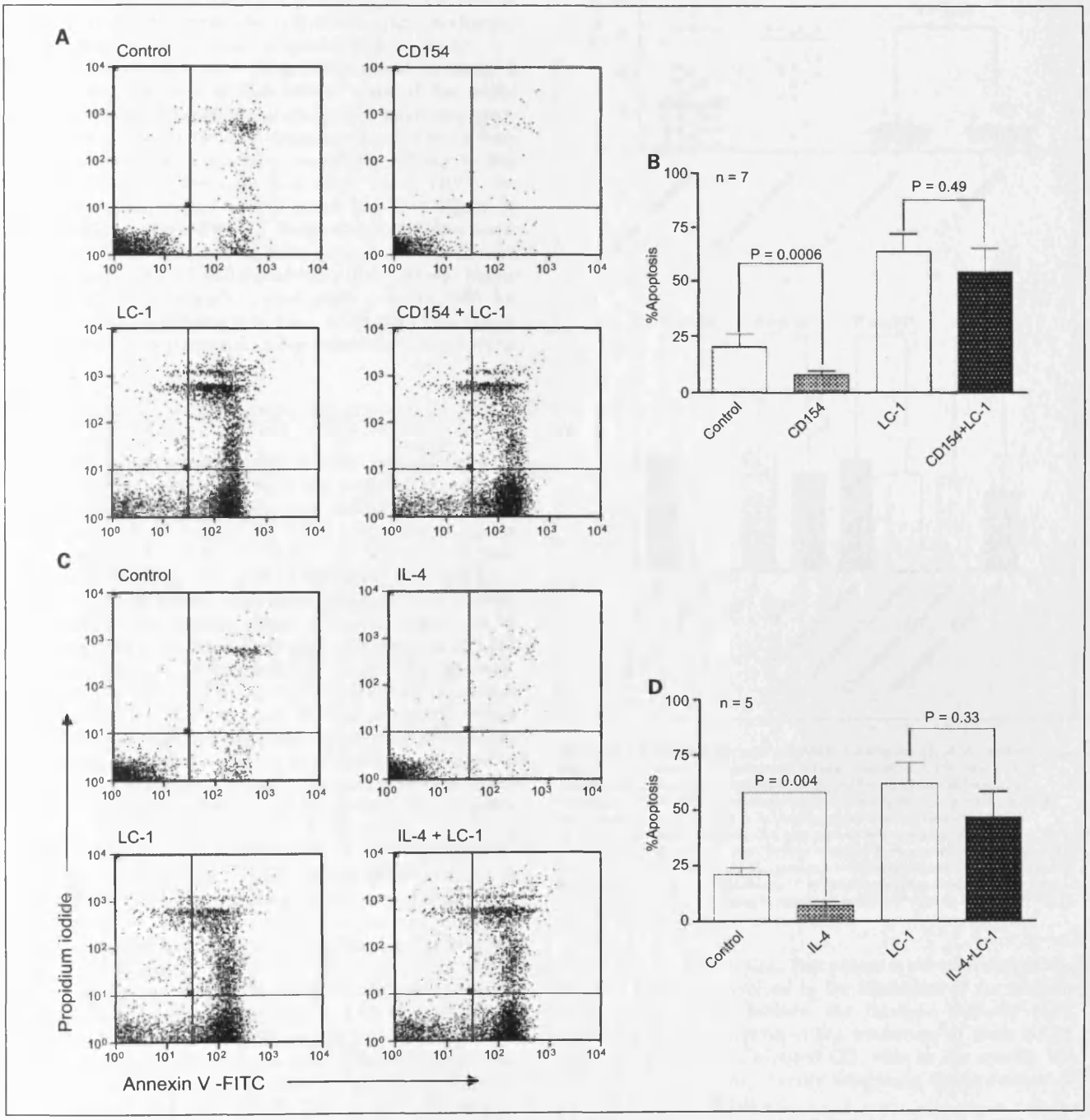

Fig. 3. LC-1 overcomes the cytoprotective effects of CD40 ligand and IL-4. *A,* mononuclear cells from seven patient samples were cultured with or without CD154 (1 μmol/L) for up to 48 h. There was a significant cytoprotective effect by CD154 when compared with control cultures ($P = 0.0006$). *B,* addition of LC-1 (2 μmol/L) overcomes the prosurvival effect of CD154 ($P = 0.49$). *C,* mononuclear cells from five patient samples were cultured with or without IL-4 (5 nmol/L) for 24 h. There was a significant cytoprotective effect by IL-4 when compared with control cultures ($P = 0.004$). *D,* addition of LC-1 (2 μmol/L) overcomes the prosurvival effect of IL-4 ($P = 0.33$).

plots (Fig. 5B). Furthermore, the combination index plot (Fig. 5C) showed synergism across a wide range of cell killing (fractional effect). The combination index values revealed strong synergistic effects in the LC-1/fludarabine combination with a mean combination index of 0.26 (Fig. 5D). In addition, we showed undiminished synergy in samples from poor prognosis subsets (Fig. 5E). Our finding that the LC-1/fludarabine combination exerts synergistic cytotoxicity in fludarabine-resistant cells raises the possibility that the combination of NF-κB inhibitors and conventional chemotherapy may be clinically useful in poor prognosis groups of CLL.

Combination of LC-1 with fludarabine would facilitate a significant dose reduction of fludarabine. One of the major benefits of using combinations of drugs that have synergistic effects is that doses of individual drugs can be reduced to limit dose-dependent side effects, while maintaining efficacy. In this study we calculated the dose reduction index (DRI) for fludarabine in each patient sample tested (n = 24). Figure 5F shows a representative DRI for fludarabine (DRI/fractional effect curve) in CLL cells derived from one patient treated with the combination of LC-1 and fludarabine. The DRI was highly favorable in all the samples tested with a mean DRI for fludarabine when combined with LC-1 of >1,000. This means that there could be a theoretical 3 log reduction in the dose of fludarabine.

Discussion

Molecular targeted therapies can alter the natural course of malignant disease because they have the potential to preferentially target the cancer cells (30). Our previous studies have shown that the NF-κB subunit Rel A is a biomarker of disease progression in CLL (16). Furthermore, we showed that inhibition of NF-κB by the novel parthenolide analogue LC-1 resulted in CLL cell-killing. Here we extend these observations by characterizing the cytotoxic effects of LC-1 in a cohort of 96 CLL patient samples. All of the samples were sensitive to LC-1 with a mean LD_{50} at 24 hours of 2.9 μmol/L. Furthermore, there was no significant difference in sensitivity to LC-1 within prognostic subsets. Indeed, LC-1 showed a trend toward increased efficacy in poor prognostic groups even in cells that showed marked *in vitro* resistance to fludarabine. Unusually, LC-1–mediated apoptosis resulted in the activation of both the intrinsic and extrinsic pathways, and this provides one possible explanation for the efficacy of this agent in samples with drug resistance to conventional therapy.

We went on to show that LC-1 specifically inhibited the DNA binding of Rel A in a dose- and time-dependent manner. This inhibition preceded the induction of apoptosis, indicating that it may be a critical regulator of LC-1–mediated cell-killing. We confirmed this by using a specific Rel A inhibitory peptide; the peptide induced significant cell death in primary CLL cells, showing that Rel A plays a key role in CLL survival and is therefore an important drug target in this condition. We next explored the downstream consequences of Rel A inhibition by quantifying the transcription of three NF-κB-regulated genes, *CFLAR*, *BCL2*, and *BIRC5*, which have been implicated in CLL cell survival and resistance to chemotherapy (15, 23, 24, 31–34). Transcription of all three genes was significantly suppressed by LC-1, suggesting that NF-κB plays a role in preventing the activation of both the intrinsic and extrinsic

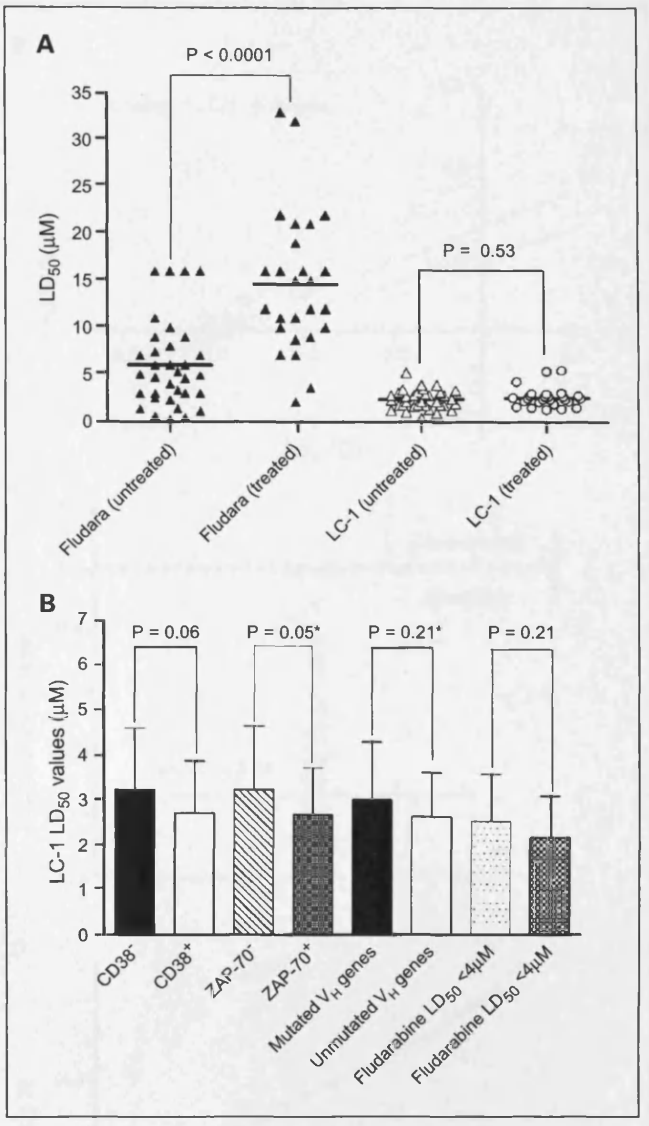

Fig. 4. LC-1 is equipotent in poor prognostic subsets of CLL. *A*, fludarabine LD_{50} and LC-1 LD_{50} values were compared between treated and untreated patient groups. Previously treated patients showed significant *ex vivo* resistance to fludarabine (P < 0.0001), but treated and untreated patient groups were equally sensitive to LC-1 (P = 0.53). *B*, LC-1 LD_{50} values were compared between prognostic subsets of patients. CLL cells derived from patients with CD38 expression ≥20% (n = 40) and <20% (n = 50), ZAP-70 expression ≥20% (n = 37) and <20% (n = 53), unmutated V_H genes (n = 23) and mutated V_H genes (n = 50), and *ex vivo* fludarabine resistance (n = 16) and fludarabine sensitivity (n = 44) showed no significant difference in sensitivity to LC-1 (P = 0.06, P = 0.05, P = 0.21, and P = 0.21, respectively).

apoptotic pathways in CLL. This notion is substantiated by the fact that *CFLAR* is involved in the inhibition of the extrinsic pathway and *BCL2* inhibits the intrinsic pathway (35). Importantly, we recapitulated the inhibition of these NF-κB target genes when we exposed CLL cells to the specific IκK inhibitor BAY 11-7082, thereby reinforcing the credentials of LC-1 as a NF-κB inhibitor.

Despite their prolonged survival *in vivo*, most CLL cells undergo spontaneous apoptosis when cultured *in vitro* (36), suggesting that humoral factors and cellular interactions provide critical survival signals *in vivo*. There is growing

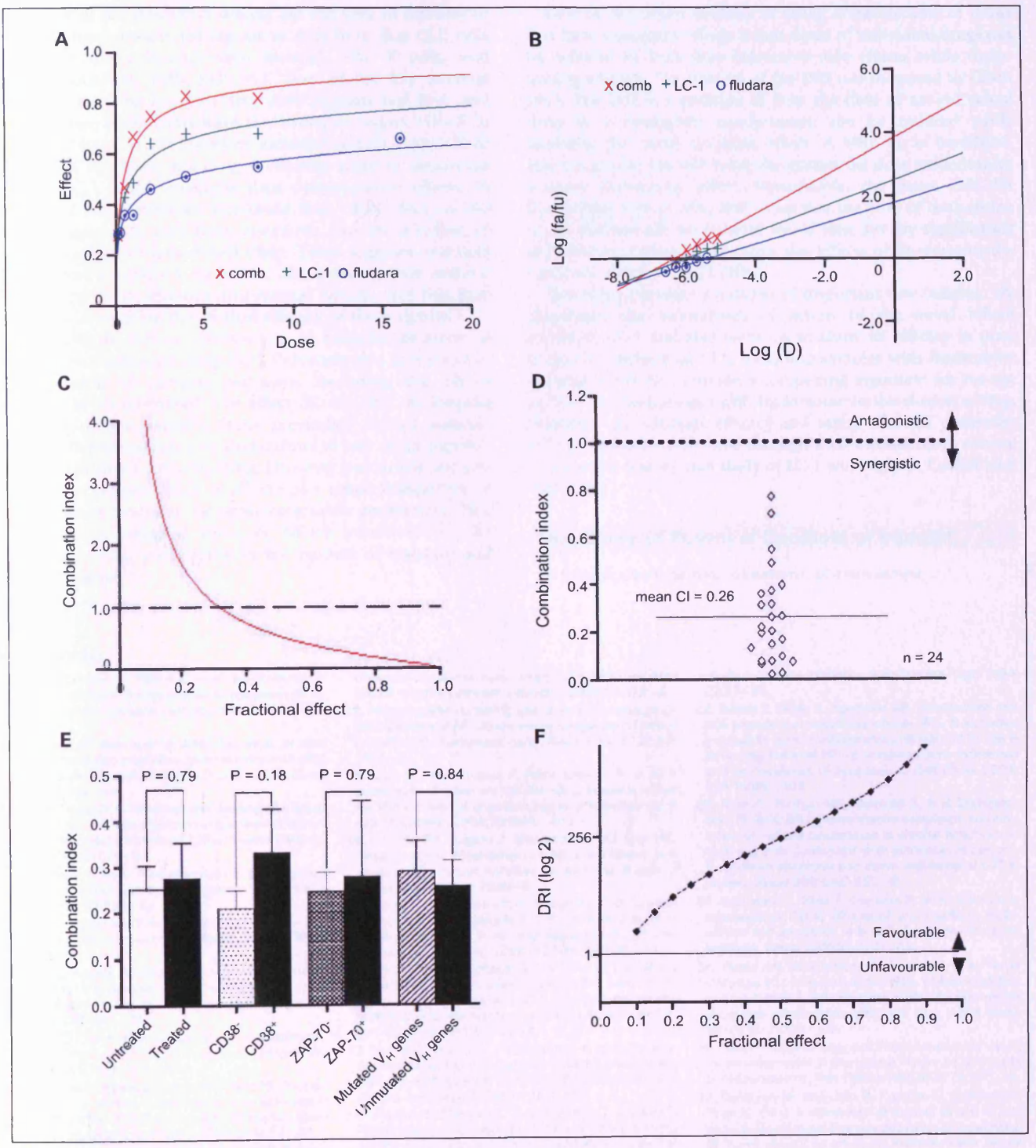

Fig. 5. Synergistic effect of LC-1 with fludarabine in primary CLL cells. CLL cells were treated for up to 48 h with LC-1 (0.5-8 μmol/L) and/or fludarabine (0.25-4 μmol/L) at a fixed molar ratio of 2:1. Cytotoxicity was quantified using an Annexin V/propidium iodide assay. The median-effect plot was constructed using Calcusyn software, where Fa = fraction affected and Fu = fraction unaffected. *A*, dose-response curve for CLL cells treated with LC-1 and/or with fludarabine. *B*, median-effect plot for CLL cells treated with LC-1 and/or with fludarabine. *C*, the combination index (CI) plot was constructed by computer analysis of the data in (*B*) using the conservative isobologram. CI values of <1 occurred at a wide range of drug concentrations. *D*, distribution of CI among 24 patients tested with mean value of 0.26 ± 0.20. *E*, analysis of samples from prognostic subsets revealed that there was no significant difference in synergy in samples derived from patients who had previously been treated, those who show *ex vivo* resistance to fludarabine, those with >20% CD38 expression, those with >20% ZAP-70 expression, or those with unmutated V_H genes ($P = 0.79$, $P = 0.49$, $P = 0.18$, $P = 0.79$, and $P = 0.84$, respectively). *F*, CLL cells were treated for 48 h with LC-1 (0.5-8 μmol/L) and/or fludarabine (0.25-4 μmol/L) at a fixed molar ratio of 2:1.The cytotoxicity was quantified using an Annexin V/propidium iodide assay. Dose reduction index (DR) – Fraction affected plots were calculated using CalcuSyn software. DRI values of >1 for fludarabine were observed over a wide range of inhibition levels when combined with LC-1.

evidence that the lymphoid organs are the sites of delivery of many of these prosurvival signals as it is here that CLL cells come into direct contact with stromal cells, T cells, and follicular dendritic cells (37–39). Two of the key survival stimuli seem to be derived from CD40 ligation and IL-4, and both of these interactions have the ability to induce NF-κB in CLL cells (40, 41). We therefore included recombinant CD40 ligand and IL-4 into our drug sensitivity assay to determine whether LC-1 could overcome their cytoprotective effects. In accordance with previously published data, CD40 ligation and IL-4 prevented CLL cells from apoptosis, but the addition of LC-1 abrogated this prosurvival effect. Taken together, our data clearly show that NF-κB inhibition can overcome the survival signals induced by microenvironmental factors, and this may be crucial in ensuring the clinical efficacy of these agents.

The ability to induce apoptosis is an essential property of most chemotherapeutic drugs (42). Perversely this may result in the activation of survival pathways, including the NF-κB pathway, as an unwanted side effect (5, 43–45). In keeping with this notion, samples from previously treated patients showed relative resistance to fludarabine in our study together with elevated Rel A DNA binding. However, we clearly showed the equal potency of LC-1 in all samples tested irrespective of their previous treatment or other prognostic parameters. This highlights the potential value of NF-κB inhibitors for the treatment of CLL, particularly in the context of resistant and relapsed disease.

One of the major benefits of using combinations of drugs that have synergistic effects is that doses of individual drugs can be reduced to limit dose-dependent side effects, while maintaining efficacy. The concept of the DRI was proposed by Chou (46). The DRI is a measure of how the dose of an individual drug in a synergistic combination can be reduced while retaining the same cytotoxic effect. A DRI >1 is beneficial, and the greater the DRI value the greater the dose reduction for a given therapeutic effect. Remarkably, the mean DRI for fludarabine was >1,000, indicating that the dose of fludarabine could theoretically be reduced by >3 logs thereby significantly reducing its immunosuppressive side effects while retaining the cytotoxic effects on CLL cells.

This study provides a number of important new insights. We elucidated the mechanism of action of the novel NF-κB inhibitor LC-1 and also went on to show its efficacy in poor prognostic subsets of CLL, including samples with fludarabine resistance. We also provide a compelling argument for the use of LC-1 in combination with fludarabine in the clinical setting. However, the ultimate efficacy and safety of small molecular NF-κB inhibitors will come through their evaluation in clinical trials, and a first-in-man study of LC-1 will begin in Cardiff later this year.

Disclosure of Potential Conflicts of Interest

C.T. Jordan, patent and shares in Leuchemix, LC-1 manufacturer. Q4

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Rel A is an independent biomarker of clinical outcome in chronic lymphocytic leukemia

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ABSTRACT

PURPOSE

We recently demonstrated the biological importance of the NF-kB subunit Rel A in chronic lymphocytic leukaemia (CLL) and hypothesized that Rel A DNA binding would have prognostic significance in this disease.

PATIENTS AND METHODS

Rel A DNA binding was quantified in nuclear extracts derived from 131 unselected CLL patient samples using a quantitative DNA binding ELISA-based method. We then investigated the ability of Rel A to predict for the requirement for treatment and survival and compared our findings with other established prognostic markers.

RESULTS

Rel A DNA binding was strongly associated with advanced Binet stage ($P<0.0001$) but did not correlate with IgV_H mutation status ($P = 0.25$), CD38 expression ($P = 0.87$) or ZAP-70 expression ($P = 0.55$). It was predictive of time to first treatment ($P = 0.02$) and time to subsequent treatment ($P = 0.0001$). In addition, Rel A was the most predictive marker of survival both from date of diagnosis (hazard ratio 9.1, $P = 0.01$) and date of entry into the study (hazard ratio 3.9, $P = 0.05$) and retained prognostic significance in multi-variate analysis for both time to first treatment and overall survival in the presence of Binet stage, IgV_H mutation status, CD38 and ZAP-70.

CONCLUSIONS

Rel A is an independent prognostic marker of survival in CLL and appears to have the unique capacity to predict the duration of response to therapy. Prospective assessment of Rel A as a marker of clinical outcome and as a therapeutic target are now warranted.

INTRODUCTION

Chronic lymphocytic leukaemia (CLL) is the commonest leukaemia in the Western world but it manifests a very heterogeneous clinical course.[1;2] Some patients show rapid disease progression with a mean survival of less than 36 months, whereas others exhibit a more indolent disease profile with a significantly better prognosis.[3] The biological factors that contribute to the progression of this disease are poorly understood but decreased susceptibility to apoptosis[4-6] and dysregulated proliferation undoubtedly play important roles.[7-9] This marked variability in clinical course has stimulated the search for prognostic markers that can predict patient outcome. The need for these biomarkers is particularly important now because of the introduction of more effective therapies that might be optimally employed in the early phase of the disease before the problems associated with high tumor burden and drug resistance are encountered. Clinical studies have consistently shown that unmutated IgV_H genes, high ZAP-70 expression, high CD38 expression and cytogenetic abnormalities (especially deletions of 11q and 17p) are all associated with a poor prognosis.[10-14] However, none of these markers are able to accurately predict the clinical course of individual patients or their likely response to therapy. This limits their use in clinical management particularly in deciding if and when to treat. Therefore, the search for better markers is both timely and relevant.

NF-kB proteins have long been known to be key regulators of cell survival, cell growth, immune response and inflammation and have been shown to be elevated in CLL cells.[15-17] This family of transcription factors transactivate more than 200 genes[18] including a panel of anti-apoptotic gene products e.g. Survivin, Bcl-2 and FLICE inhibitory protein (FLIP) all of which have been implicated in the inhibition of cell death in CLL.[19-21] We recently showed that the NF-kB subunit Rel A is associated with *in vitro* survival and clinical disease progression in CLL.[15] These findings led us to hypothesize that Rel A might be a prognostic marker in this disease. We therefore analysed Rel A DNA binding in 131 patients and correlated the results with the established markers of prognosis and clinical outcome.

MATERIALS AND METHODS

PATIENTS

Peripheral blood samples from 131 CLL patients were obtained following written informed consent (LREC # 02/4806). 32/131 (24.4%) were diagnostic samples and 112/131 (85.5%) were untreated at the start of the study. CLL was defined by clinical criteria as well as cellular morphology and the co-expression of CD19 and CD5 in lymphocytes simultaneously displaying restriction of light-chain rearrangement. Comprehensive clinical information including treatment histories was available for all patients either through their case notes or direct interviews with patients. None of the previously treated patients had received chemotherapy within 3 months prior to sample collection. Staging was based on the Binet classification system[22] and the clinical characteristics of the CLL patient cohort are summarized in Table 1.

ZAP-70 AND CD38 EXPRESSION

Cytoplasmic ZAP-70 expression was determined by using a modification of a previously published flow cytometry method.[15;23] After appropriate lymphocyte gating, cytoplasmic ZAP-70 expression was determined in $CD19^+$ CLL cells by gating on the $CD3^+$ cells. Patients were considered to be ZAP-70 positive when ≥20% of the $CD19^+$ CLL cells had equal or greater expression of ZAP-70 than the gated $CD3^+$ cells. Cell surface expression of CD38 was examined by flow cytometry using a standard three-color flow cytometry approach utilizing CD5-fluorescein isothiocyanate (Dako), CD38-PE (Caltag) and CD19-APC (Caltag). The cut-off point for CD38 positivity in CLL cells was ≥20%.

IgV_H MUTATION STATUS

IgV_H gene mutational status was determined for all patients using the method described previously.[24] The resulting PCR products were sequenced and were considered unmutated if they showed 98% or greater homology with the closest germ line sequence.

SUB-CELLULAR FRACTIONATION

All of the nuclear extracts were prepared in an identical fashion from freshly isolated CLL samples and the extraction method used showed remarkable inter-patient consistency in terms of the amount of nuclear and cytosolic proteins generated from a fixed number of CLL lymphocytes.[25] Details of the methods employed have been described previously.[15] Protein concentrations for each nuclear extract were determined by Nanodrop absorbance (Nanodrop Technologies).

REL A DNA BINDING DETECTION BY ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

Nuclear extracts from CLL patients were assayed for Rel A activity with a TransAM NF-kB transcription factor assay kit according to the manufacturer's instructions (Active Motif). The principle behind the assay involves the capture of NF-kB by an oligonucleotide containing an NF-kB consensus site which is immobilized in each well of a 96 well plate. The Rel A subunit was subsequently detected using a Rel A-specific antibody. The optical density reading at 450nm (OD_{450}) was read on a microtiter plate reader (Bio-Rad). OD_{450} values were converted into ng/µg of nuclear extract for each sample tested from a standard curve constructed using known quantities of recombinant Rel A NF-kB. In the range of Rel A proteins we were measuring there was a linear relationship between Rel A protein amount and OD_{450} (r^2=0.99). All the Rel A measurements were done using the same batch of ELISA kits and the same batch of recombinant Rel A protein was used for the purpose of generating standard curves. Intra- and inter-assay variability was assessed using standardised nuclear extracts derived from a Jurkat cell line. In addition, samples from the same patients were tested on different plates on different days. In every case, there was less than 10% variability between results suggesting that the method is reliable and reproducible.

STATISTICAL ANALYSIS

To assess the relationship between Rel A and known prognostic factors (CD38, ZAP-70, IgV_H mutation status, treatment status and Binet stage) Rel A was examined as a continuous

variable within each categorical group. To examine the relationship between Rel A and clinical outcome, the cohort was divided according to the median expression of Rel A. Overall survival and time to first treatment times were calculated from date of diagnosis. Time to subsequent treatment and survival over the course of the study were calculated from the date that Rel A was measured. Survival curves were constructed using the method of Kaplan and Meier and the Log-Rank test was used to assess any differences between patient and tumor characteristics. Cox regression analysis determined important independent prognostic factors for time to first treatment and overall survival. Statistical analysis was carried out using Prism 3.0 (Graphpad) and SAS statistical software (SAS Institute).

RESULTS

REL A DNA BINDING IS INDEPENDENT OF IgV_H MUTATION STATUS, CD38 EXPRESSION AND ZAP-70 EXPRESSION

We measured Rel A in nuclear extracts derived from 131 serially collected CLL patient samples using a quantitative DNA binding ELISA-based method. Data from all 131 patients is shown in Figure 1A; Rel A DNA binding ranged from undetectable to 2.44 ng/μg of nuclear extract with a median value of 0.44ng/μg (95% CI 0.48-0.66). Rel A was markedly elevated in patients with advanced disease ($P<0.0001$, Figure 1B) and those who had previously received treatment ($P<0.0001$, Figure 1C) but was not associated with CD38 expression ($P = 0.87$, Figure 1D), IgV_H mutation status ($P = 0.25$, Figure 1E) or ZAP-70 expression ($P = 0.55$, Figure 1F).

ASSOCIATION BETWEEN REL A DNA BINDING AND TIME TO INITIAL THERAPY

All patients who required therapy were treated in accordance with the published guidelines.[26] Of the 131 patients analysed, 19 (14.5%) had previously received treatment and a further 33 (25.2%) required therapy during the course of the study. We first examined the relationship between Rel A DNA binding and the time from diagnosis to first treatment (TTFT) using subsets defined by the median Rel A value. The median TTFT in the low Rel A subset was 13.6 years versus 6.8 years in the high Rel A subset ($P = 0.01$, Figure 2A). Since Rel A DNA binding was independent of CD38, IgV_H mutation status and ZAP-70 we examined the ability of these established prognostic parameters to define TTFT in our cohort (Table 2). In keeping with previous reports, unmutated IgV_H genes, ≥20% CD38 expression and ≥20% ZAP-70 expression were all associated with significantly shorter TTFT ($P = 0.0005$, $P = 0.04$ and $P = 0.05$ respectively) indicating that our study was carried out on a representative CLL cohort. In multi-variate analysis, the inclusion of Rel A in the model showed independent prognostic significance for TTFT ($P = 0.01$) and its inclusion diminished the prognostic value of CD38 ($P = 0.25$), IgV_H gene mutation status ($P = 0.05$) and ZAP-70 ($P = 0.28$).

REL A IS A PREDICTOR OF TIME TO SUBSEQUENT TREATMENT

We wanted to more accurately assess the prognostic value of measuring Rel A in our cohort by controlling for previous treatment effects and to acknowledge that not all of the samples analysed for Rel A were taken at diagnosis. Therefore we assessed the time to subsequent treatment (TTST) using the date of entry into the study as a sensor date for each patient sample. Figure 2B shows the predictive value of Rel A in our CLL patient cohort. Patients with high Rel A DNA binding showed a median TTST of 22 months. In contrast, the median TTST of the low Rel A subset was not reached as only 5/68 patients with low Rel A DNA binding required treatment during the 24 month follow-up period (P = 0.0001). We also evaluated the ability of IgV_H mutation, CD38 and ZAP-70 to predict TTST during the study period (Table 2). Only IgV_H mutation status had prognostic value in this context (P = 0.04); CD38 (P = 0.17) and ZAP-70 (P = 0.13) were not predictive.

REL A IS A PREDICTOR OF OVERALL SURVIVAL

We assessed the capacity of Rel A to predict patient survival from date of diagnosis. In addition, we assessed survival over the course of the 24 month study period using date of entry into the study as the sensor point. Rel A DNA binding was predictive of survival from date of diagnosis (P = 0.015, Figure 2C). Similarly, IgV_H mutation status, CD38 and ZAP-70 were all predictive of survival from date of diagnosis (Table 2). In multi-variate analysis, the inclusion of Rel A in the model showed independent prognostic significance for overall survival (P = 0.04) and its inclusion removed the prognostic value of CD38 (P = 0.38), IgV_H gene mutation status (P = 0.22) and ZAP-70 (P = 0.14). Further, only Rel A (P = 0.01, Figure 2D) was predictive of survival during the course of the 24 month follow-up period of this study.

REL A IS CONSTITUTIVELY HIGHER IN PATIENTS WHO REQUIRE TREATMENT

In order for Rel A to be considered a useful marker of TTFT in CLL it seemed important to demonstrate that Rel A was constitutively higher in untreated patients who went on to require therapy. We therefore compared Rel A DNA binding in samples derived from patients who never required therapy (n = 79) with those who went on to receive their first treatment after we had measured their Rel A DNA binding (n = 33). Figure 3A shows that patients who

require therapy have significantly higher Rel A DNA binding than those patients who do not ($P<0.0001$).

IS REL A MODULATED BY THERAPY?

Studies of other diseases have shown that Rel A DNA binding can increase in response to chemotherapeutic drugs.[27-29] We examined this question in two ways. Firstly, we compared Rel A DNA binding in samples derived from patients who received therapy after we had measured their Rel A with those who received therapy before we measured their Rel A (Figure 3B). Although there was no statistical difference in Rel A DNA binding between these two groups ($P = 0.41$) the median Rel A DNA binding was higher in the previously treated subset (median Rel A 0.81ng/μg versus 0.49ng/μg). Secondly, we evaluated intra-patient Rel A DNA binding in a longitudinal manner in patients before and after treatment and in selected patients that did not require therapy. Figure 3C shows a clear increase in Rel A DNA binding following treatment ($P=0.04$). In contrast serial samples from patients not requiring therapy showed no significant change in Rel A DNA binding ($P=0.89$). None of the samples tested were derived from patients within 3 months of completion of a cycle of therapy in order to avoid the possibility of a transient modulation in Rel A DNA binding induced by chemotherapeutic drugs. The median interval between measurements was similar for the treated and untreated patient groups (15 months vs 13 months respectively).

DISCUSSION

A number of previous studies have shown that NF-kB is constitutively activated in CLL patients.[15-17] Here we used an ELISA-based assay to demonstrate marked variation in Rel A DNA binding within a cohort of 131 unselected CLL patients. The Rel A DNA binding assay that we employed was both reliable and reproducible with less than 10% variation in biological replicates performed on different plates on different days. High Rel A DNA binding was strongly associated with advanced stage of disease but remarkably, was not associated with IgV_H gene mutation status, CD38 expression or ZAP-70 expression; indicating that Rel A could contribute independent prognostic information in CLL. We therefore assessed the ability of Rel A to predict for time to first treatment (TTFT), time to subsequent treatment (TTST) and overall survival in our patient cohort.

Elevated Rel A DNA binding was predictive of significantly shorter TTFT (P = 0.01, hazard ratio 1.9). As NF-kB has been implicated in the development of drug resistance in other tumor settings[30;31] we assessed whether Rel A could predict for the relative effectiveness of therapy in CLL patients. In order to do this we used TTST from the date of entry into the study as a measure of the depth of remission. Rel A was more predictive of TTST than any of the other prognostic markers assessed (P = 0.0001, hazard ratio 5.2) indicating that it is excellent biomarker of response in CLL patients. Although this result is encouraging, it will need to be tested more thoroughly in a prospective clinical trial in which all of the patients receive uniform therapy. Most strikingly, Rel A was the best predictor of overall survival from date of diagnosis in our study (P = 0.01, hazard ratio 9.1) adding to our hypothesis that Rel A is a key regulator of CLL survival both in vitro and in vivo.[15] In keeping with this notion, Rel A was the only parameter tested that showed a significant difference in survival over the 24 month follow-up period of this study (P = 0.05, hazard ratio 3.9). Furthermore, the inclusion of Rel A in multi-variate modelling showed the independent importance of Rel A in determining overall survival in our cohort (P = 0.05, hazard ratio 8.0); CD38 expression, IgV_H mutation status and ZAP-70 expression all failed to influence survival in the presence of Rel A.

NF-kB can be activated in response to chemotherapy and can promote drug resistance through the induction of anti-apoptotic proteins like Bcl-2, surviving and FLIP as well as the activation of the AKT survival pathway.[19-21;32] We found that Rel A DNA binding was significantly higher in samples derived from previously treated patients ($P<0.0001$) and clearly increased in serial samples taken tested pre- and post-therapy. However, constitutive Rel A was significantly higher in patients who went on to require treatment when compared to those who did not. Furthermore, high Rel A was also associated with a shorter TTST in our cohort. Taken together, this data indicates that Rel A is constitutively elevated in patients with more aggressive disease but is also induced by conventional chemotherapy which in turn appears to contribute to the depth of response to subsequent treatment cycles.

In conclusion, this study identifies Rel A as a superior prognostic marker for survival in CLL and crucially demonstrates that Rel A has the potential to predict the duration of response to therapy.[33] Although there are undoubtedly challenges involved in the routine measurement of Rel A DNA binding in clinical practice, our work shows that it is possible to generate robust and reliable results that have clinical importance using a widely available measurement platform. In addition, our findings strongly support the search for more accessible surrogate markers of NF-kB activation in this disease. The fact that Rel A DNA binding is independent of IgV_H mutation status, CD38 expression and ZAP-70 expression offers the possibility that the prognostic information derived from some or all of these factors could be combined to provide even more accurate prediction of clinical outcome for CLL patients in the future.

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Table 1. Clinical characteristics of the 131 CLL patient cohort.

Factor	Subset	Number	%
Median Age		67 years	
	Range	36 – 85 years	
Stage at diagnosis	A	100	77
	B	16	12
	C	15	11
Previous treatment	Treated	52	40
	Untreated	79	60
CD38	<20%	74	56
	≥20%	57	44
Genetics	$11q^-$ / $17p^-$	9	7
	N / O	87	66
	Not Determined	35	27
IgV_H gene status	<98%	95	73
	≥98%	36	27
ZAP-70	<20%	73	56
	≥20%	58	44

$11q^-$ and $17p^-$ = any FISH or karyotypic abnormality of 11q or 17p
N = Normal (i.e. no abnormality detected by FISH)
O = Other cytogenetic abnormality (excluding $11q^-$ or $17p^-$)

Table 2. Comparison of prognostic factors in terms of time to treatment and overall survival

	Time to treatment						Survival					
	Time to first treatment			Time to subsequent treatment			From date of diagnosis			From date of entry into study		
	P-value	HR	95% CI	P-value	HR	95% CI	P-value	HR	95% CI	P-value	HR	95% CI
Rel A (<median/≥median)	0.01	1.9	1.15-3.41	0.0001	5.2	2.00-8.61	0.01	9.1	1.45-15.51	0.05	3.9	0.97-9.59
IgV_H gene mutation (<98%/≥98%)	0.0005	2.7	1.84-9.01	0.04	2.1	1.04-4.73	0.01	3.9	1.66-40.29	0.71	1.2	0.38-4.05
CD38 expression (<20%/≥20%)	0.05	1.6	1.00-2.80	0.17	1.6	0.79-3.46	0.05	2.8	0.96-9.82	0.66	1.3	0.25-2.42
ZAP-70 expression (<20%/≥20%)	0.04	1.7	1.00-2.98	0.13	1.7	0.84-3.66	0.05	3.1	1.01-9.17	0.39	1.6	0.53-5.13

HR = Hazard ratio
CI = confidence interval

FIGURE LEGENDS

Figure 1. Correlation of Rel A and other prognostic factors.

(A) Samples from all 131 patients were analysed for Rel A DNA binding using an ELISA-based assay. Rel A was then compared in categorical subsets of the CLL cohort based on (B) Binet stage (C) the requirement for treatment, (D) CD38 expression, (E) IgV_H gene mutation status and (F) ZAP-70 expression. Elevated Rel A was strongly associated with advanced Binet stage and the need for treatment but did not correlate with CD38 expression, IgV_H gene mutation status or ZAP-70 expression.

Figure 2. Rel A as a prognostic marker in our CLL cohort.

Time to first treatment was assessed from the date of diagnosis (A) Rel A DNA binding above the median value was able to define a population at increased risk of requiring treatment in unit time. Time to subsequent treatment was measured from the date of entry into the study to the time of next treatment intervention. (B) Rel A DNA binding above the median value was able to define a population at greater risk of requiring another cycle of treatment. Comparison of Kaplan Meier curves for survival demonstrated that (C) Rel A was prognostic for survival from date of diagnosis and (D) was also predictive of survival from date of entry into the study.

Figure 3. The effect of treatment on Rel A DNA binding.

(A) Rel A DNA binding was significantly higher in samples derived from previously treated patients ($P<0.0001$). (B) However, there was no statistical difference in Rel A DNA binding in samples analysed before treatment and those in whom Rel A was measured after treatment ($P = 0.41$). (C) Serial intra-patient analysis of Rel A in samples derived from patients who received treatment during the course of the study and those who did not revealed that there was a significant increase in Rel A DNA binding following therapy ($P = 0.04$). In contrast, samples from patients who did not require treatment remained stable ($P = 0.89$).

Figure 1

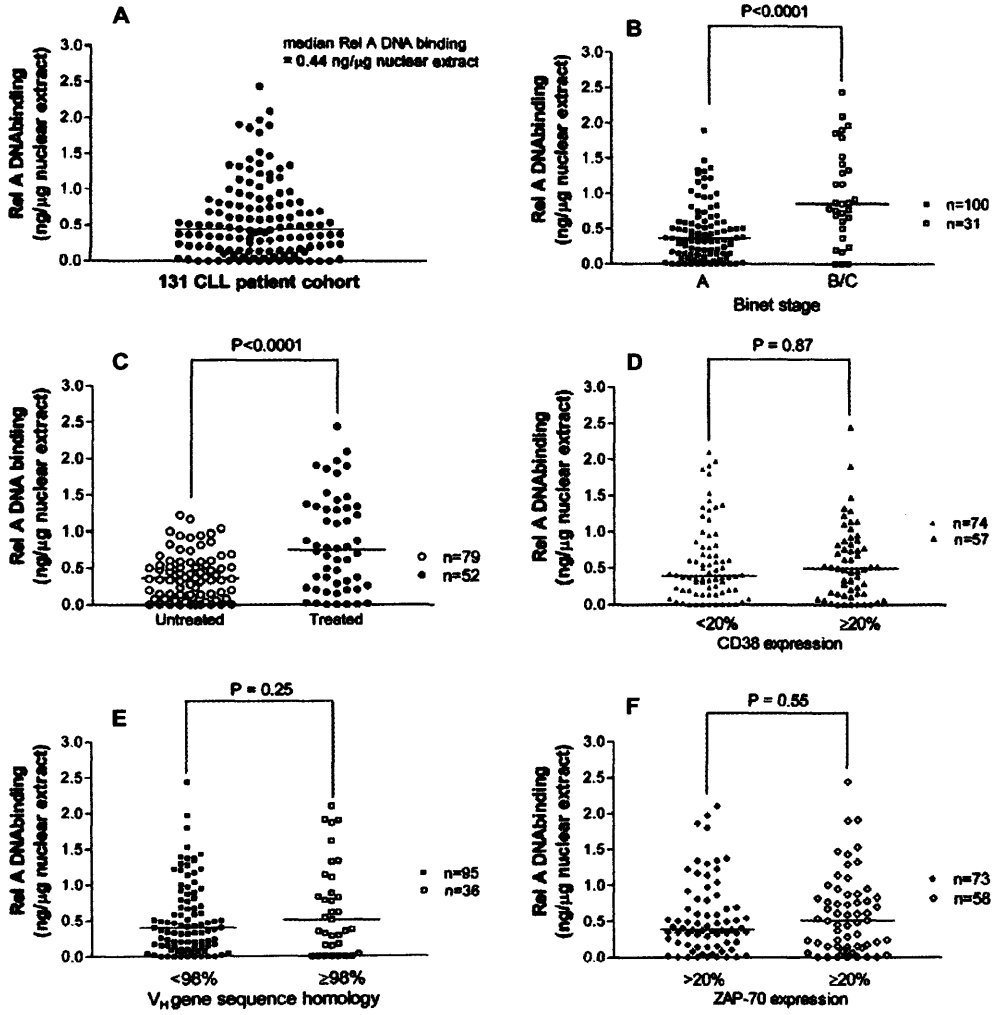

A
median Rel A DNA binding
= 0.44 ng/µg nuclear extract
Rel A DNAbinding (ng/µg nuclear extract)
131 CLL patient cohort
B
P<0.0001
n=100
n=31
A
B/C
Binet stage
C
P<0.0001
Rel A DNA binding (ng/µg nuclear extract)
n=79
n=52
Untreated
Treated
D
P = 0.87
n=74
n=57
<20%
≥20%
CD38 expression
E
P = 0.25
n=95
n=36
<98%
≥98%
V_H gene sequence homology
F
P = 0.55
n=73
n=58
>20%
≥20%
ZAP-70 expression

Figure 2

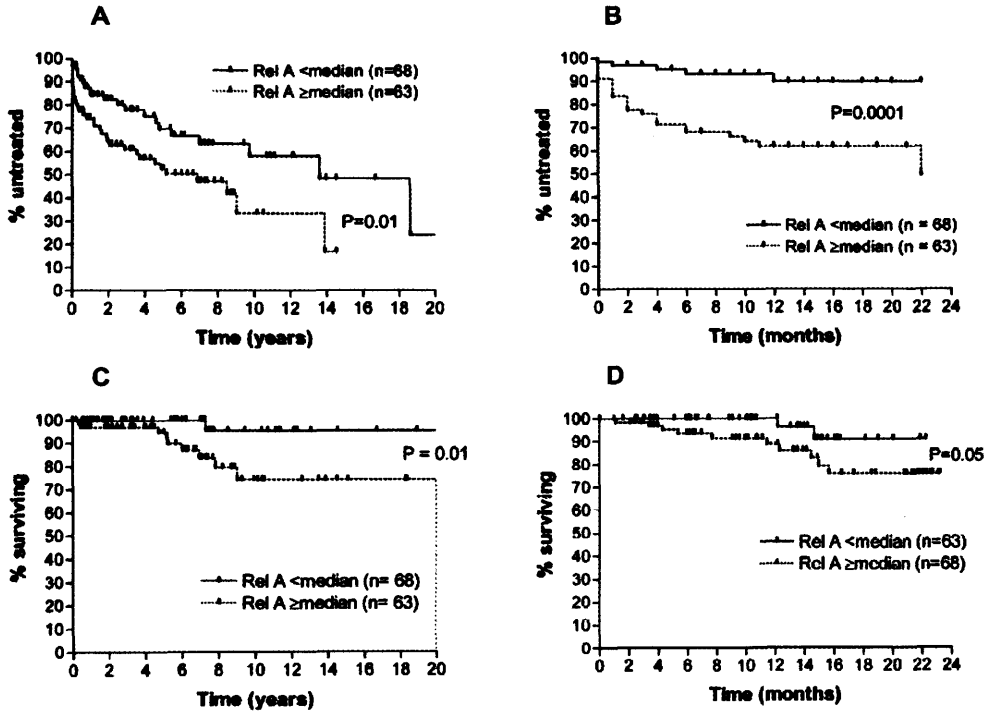
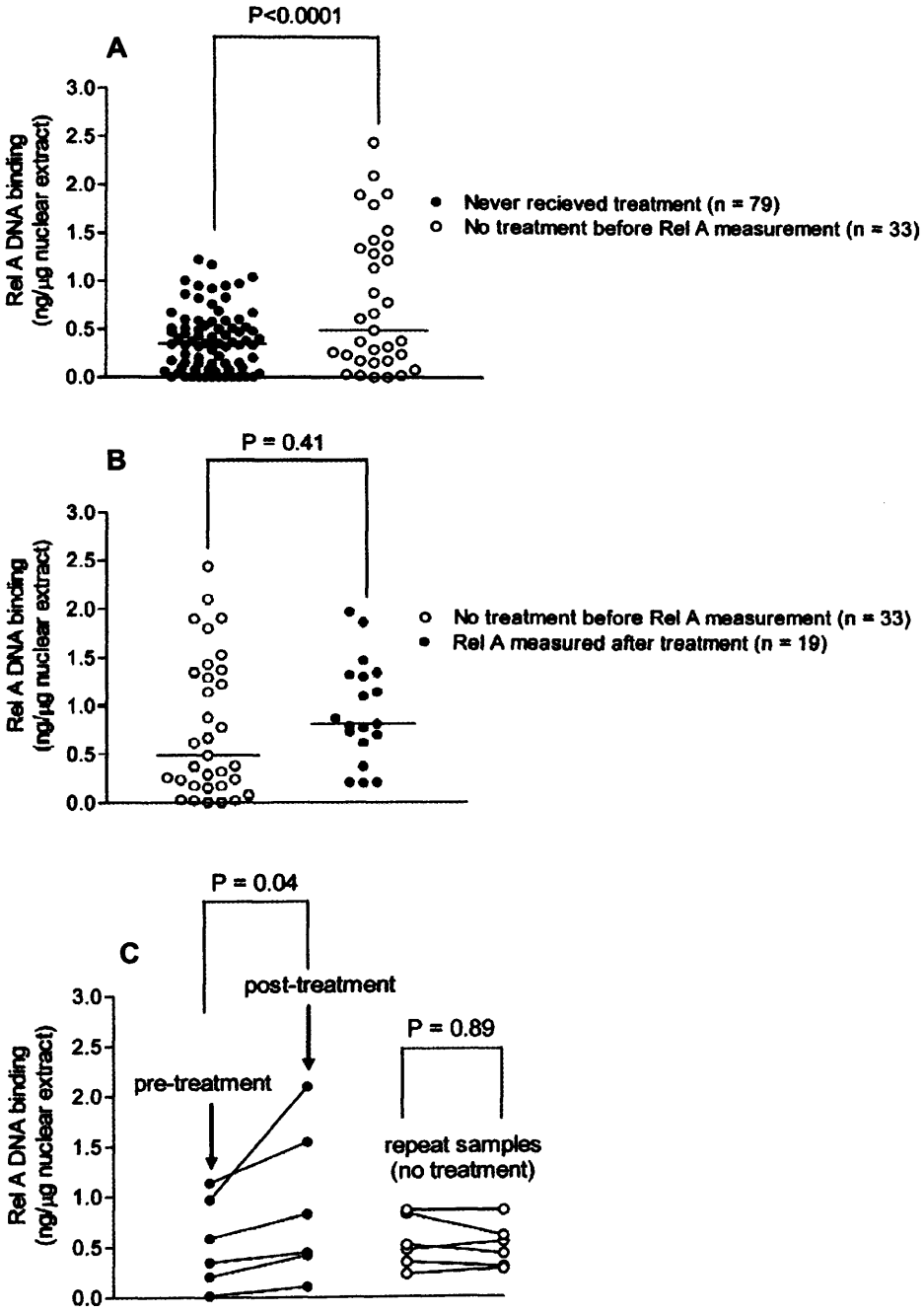

A
Rel A <median (n=68)
Rel A ≥median (n=63)
% untreated
P=0.01
Time (years)
B
P=0.0001
Rel A <median (n = 68)
Rel A ≥median (n = 63)
% untreated
Time (months)
C
P = 0.01
Rel A <median (n= 68)
Rel A ≥median (n= 63)
% surviving
Time (years)
D
P=0.05
Rel A <median (n=63)
Rel A ≥median (n=68)
% surviving
Time (months)

Figure 3


P<0.0001
A
Rel A DNA binding (ng/µg nuclear extract)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Never recieved treatment (n = 79)
No treatment before Rel A measurement (n = 33)
P = 0.41
B
Rel A DNA binding (ng/µg nuclear extract)
No treatment before Rel A measurement (n = 33)
Rel A measured after treatment (n = 19)
P = 0.04
C
post-treatment
P = 0.89
pre-treatment
repeat samples (no treatment)
Rel A DNA binding (ng/µg nuclear extract)