In vitro Studies of the Anti-Leukaemic Activity of Bezafibrate & Medroxyprogesterone Acetate against Chronic Lymphocytic Leukaemia

by

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Abstract

Chronic lymphocytic leukaemia (CLL) is the most common leukaemia in the western world, and continues to have very low cure rates. The bulk of the tumour cells are non-cycling peripheral cells that accumulate from a minority of dividing cells within proliferation centres (PCs), located in the lymph nodes (LN) and spleen. The PCs are rich in primed T helper (T_H) cells that express CD40L and secrete IL-4. Like normal B cells, CLL cells interact with T_H via their expression of CD40 and receive signals from IL4. This stimulation provides the signal for B cells to divide, as well as protecting them from apoptotic stimuli. Consequently, many current therapies are unable to sufficiently target the cells in the PC, resulting in therapy resistance, residual disease (RD) and relapse. In addition, most therapies have associated toxicities, restricting their use to younger, fitter patients. Thus, there is an urgent need for new therapies with low toxicity, especially for older patients. New drug discovery is time consuming and expensive. Redeployment of existing drugs is one way to potentially combat this issue. The lipid lowering drug bezafibrate (BEZ) and the sex steroid, medroxyprogesterone acetate (MPA) have been shown to have anti-leukaemic properties in other settings. In this study the in vitro efficacy of BEZ and MPA on resting CLL cells (representing resting cells in the periphery) and when stimulated to proliferate by CD40L (representing cells in the PCs) have been investigated. Both BEZ and MPA exerted pro-apoptotic actions against resting CLL cells and reduced CD40L stimulated proliferation. These actions were increased when both agents were combined and demonstrated
selectivity against CLL cells compared to normal peripheral blood mononuclear
cells. Combined BEZ+MPA, was as effective as the commonly used
chemotherapeutic chlorambucil, with the combination of all 3 agents exerting the
greatest effects. However none of the combinations induced apoptosis of CLL cells
protected by CD40L. Investigations into the possible mechanisms of drug action
revealed that MPA was unlikely to be working either by steroid receptors or by
inhibition of the enzyme AKR1C3 and the mechanism remains unknown. BEZ
alone and in the presence of MPA induced the production of prostaglandin D₂
(PGD₂) and exogenously applied PGD₂ was found to exert apoptosis in a similar
manner to BEZ. Both BEZ and MPA generated reactive oxygen species (ROS) in
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alone. In the absence of CD40L, mitochondrial superoxide (MSO) was also
produced but not in CD40L stimulated cells. This finding suggested that CD40L is
able to prevent or protect against MSO production and, consequently, apoptosis.
Attempts at overcoming this effect revealed that the plant derived compound
lycorine exerted minimal effects alone but, when combined with BEZ+MPA,
reinstated the induction of MSO and recapitulated BEZ+MPA induced apoptosis
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I’m going to try and not make my acknowledgements read too much like a Halle Berryesque Oscars speech but I can’t make any promises......

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I don’t know why people always leave their partner to last it always seemed to me to be a bit mean but now I realise it’s just a case of saving the best till the end. So massive and biggest thanks of all to my husband J for (and this list is not exhaustive) having faith in me, making me be brave, endless encouragement, mopping up the copious tears and always, without fail, being able to make me laugh (even it was sometimes at your own expense). Thankyou.

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54 Lycorine potentiates BEZ+MPA to induce apoptosis in the presence of CD40L in a dose dependent manner

55 Lycorine potentiates BEZ+MPA to produce MSO in the presence of CD40L in a dose dependent manner

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60 If the CD40L is not removed DSN does not sensitise CLL cells to F-ara-A

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<td>MR</td>
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<td>OR</td>
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<td>PC</td>
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<td>TLC</td>
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Abbreviations

TLR       toll like receptor
TNF       tumour necrosis factor
TNFR      tumour necrosis factor receptor
TP53      tumour protein 53
VCAM      vascular cell adhesion molecule
WHO       world health organisation
WT        wild type
ZAP-70    zeta associated protein-70
zVADfmk   benzylloxycabonyl-Val-Ala-Asp-fluoromethylketone
1. General Introduction
1.1. The leukaemias.

The term leukaemia refers to a group of malignancies that involve the progressive clonal proliferation of abnormal leukocytes in the bone marrow (BM), which can then be observed in the peripheral blood and other organs. Leukaemia can be of myeloid or lymphoid origin and either acute or chronic. Therefore, there are broadly 4 types of leukaemia commonly termed: acute myeloid (AML), acute lymphoid (ALL), chronic myeloid (CML) and chronic lymphocytic (CLL). The first three of these types arise from haemopoietic progenitor cells whilst CLL is derived from mature B lymphocytes.

1.2. Chronic lymphocytic leukaemia.

CLL is always a disease of neoplastic B cells (Binet et al., 2006) and, although diagnosed by a panel of phenotypic markers, is characterised by the relentless accumulation of CD5+ B lymphocytes in the peripheral blood, BM and secondary lymphoid organs (lymph nodes and spleen) (Ghia et al., 2007a, Chiorazzi et al., 2005, Rozman and Montserrat, 1995). Until recent times CLL was often ignored by researchers being categorised as a less interesting disease (Delgado et al., 2009a), although the renowned haematologist Dameshek noted the disease as being of great interest in 1967 (Dameshek, 1967).

1.2.1. Incidence of CLL.

In the Western world, CLL is the most common form of adult leukaemia, accounting for 25-30% of all leukaemias in 1995 (Rozman and Montserrat, 1995), with 22-30/100,000 new cases worldwide (Eichhorst et al., 2009a). Statistics reveal: that only 25-30% of patients diagnosed with CLL are under 55 years of age, that the median age at diagnosis is 72 years (Delgado et al., 2009a), and that patients over the age of 65 represent the largest group of CLL sufferers. CLL has a male bias, with the ratio of female to male sufferers.
being around 1:2 (www.LRF.org.uk). It is rare in Asia and this is most notable in Japan and China (Boggs et al., 1987) and it remains rare in immigrants from these countries. There is also increasing evidence of familial predispositions to CLL (Houlston et al., 2003, Jonsson et al., 2005, Yuille et al., 1998) with 5-10% of CLL occurring in the offspring of CLL sufferers tending to manifest at a younger age. This, together with the low incidence in Asia, indicates that genetic factors are likely to play a role in the pathogenesis of CLL.

1.2.2. **Presentation of CLL.**

The majority of CLL patients are diagnosed as a consequence of routine blood tests. When patients do present with lymphocytosis and symptoms (such as anaemia (due to infiltration of the BM), fever without infection, night sweats and fatigue), they could be attributable to other lymphoproliferative diseases such as mantle cell lymphoma (MCL), follicular lymphoma, splenic lymphoma with villous lymphocytes, marginal zone lymphoma, or hairy cell leukaemia (Ghia et al., 2007a). To distinguish CLL for these other malignancies at least 2 different examinations should be performed to diagnose CLL; one on blood count and blood films and, immunophenotyping for the type of leukaemic cells present (Cheson et al., 1996).

The phenotype of CLL is highly distinctive. All mature B cells, normal and malignant, express the specialised adaptor protein CD19; therefore, immunostaining will reveal that the cells are CD19\(^{+}\). CLL cells can be distinguished from other B cells by their expression of low levels of CD20 and CD79b and the prevalence of high levels of dual CD5\(^{+}\)/CD23\(^{+}\). The latter population allows CLL to be distinguished from other CD5\(^{+}\) diseases, such MCL (Ghia et al., 2007b). CLL cells are also mono-typic with either kappa or lambda light chain restriction and are negative for the glycoprotein FMC7 (Matutes et
al., 1994, Delgado et al., 2003), which is found on other B cell malignancies. It is also characteristic for CLL B cells to have weak surface (S) immunoglobulin (Ig), usually IgM ± IgD but occasionally IgG or IgA (Ghia et al., 2007b). The Matutes scoring system of CLL diagnosis is based on the five parameters of CD23, CD5, FMC7, CD79b and weak SIg status (Matutes et al., 1994).

Thus, a diagnosis of CLL would be on the basis of an increase in the number of lymphocytes, in excess of \(5 \times 10^9/L\) for more than one month, together with the above morphologic and immunophenotypic characteristics of CD19+/CD5+ and with demonstration of clonality by light chain restriction (Binet et al., 2006). This is in accordance with the National Cancer Institute-Working group (NCI-WG) (Cheson et al., 1996). According to the CLL working group, for a blood count less than \(5 \times 10^9/L\), examination of the BM for infiltration of B lymphocytes is necessary, in which there must be an excess of 30% lymphocytes (Cheson et al., 1996). Examination of the BM is also a pre-requisite for patients to be considered in complete remission (CR) (Ghia et al., 2007b). However, the fourth and most recent edition of the world health organisation (WHO) guidelines are different and less specific. These guidelines state that, in the absence of ‘extramedullary infiltration’ (Ott et al., 2009), monoclonal B-lymphocytes must be present in the peripheral blood, in an excess of \(5 \times 10^9/L\) (Ott et al., 2009). No WHO guidelines are provided for a diagnosis in the event of a lymphocyte count less than \(5 \times 10^9/L\) (Ott et al., 2009), this is likely to be due to this status being classified as monoclonal B cell lymphocytosis (MBL).
1.3. MBL.

Advancements in flow cytometry (FC) and the inclusion of CLL like-MBL in the NCI-WG (Dagklis et al., 2009, Landgren et al., 2009), have led to an increase in research into MBL. MBL is characterised as the occurrence of a low number of monoclonal B-cell clones, i.e. less than $5 \times 10^9$/L in the peripheral blood of otherwise healthy subjects (Dagklis et al., 2009, Landgren et al., 2009). Based on FC analysis, MBL can be subcategorised into CD$5^{-ve}$ MBL, CD$5^{+ve}$/CD20$^{bright}$ atypical CLL-like and CD$5^{+ve}$/CD20$^{low}$ CLL-like MBL, which also express CD23, CD79 and low SIg (Dagklis et al., 2009). Considering the similarities between CLL and CLL-like MBL, the likelihood of MBL to progress to CLL is a consideration. In light of this, two recent studies into MBL have contrasting views on the likelihood of MBL preceding CLL. Langdren et al. (2009) studied stored, pre-diagnostic samples from 49 patients with CLL and discovered that 93% of the pre-diagnostic bloods had an abnormal kappa/lambda ratio and 96% immunoglobulin heavy chain variable (IgHV) rearrangements. In addition, 42% of the patients had the same light-chain restrictions at diagnosis of CLL as found in the pre-diagnostic sample (Landgren et al., 2009). Notwithstanding the stage of CLL, in almost all cases a pre-diagnostic clone was detected. Based on these findings the authors concluded that the majority of patients with CLL will have had a detectable precursor state of MBL from six months to six years prior to CLL diagnosis (Landgren et al., 2009). Conversely, Dagklis et al (2009) investigated a cohort of 1,725 healthy individuals, with no history of CLL, all from an isolated valley. In 89 participants (5.2% of the cohort) CLL-like MBL was detected, in which 93% showed abnormal kappa/lambda ratios and IgHV rearrangements. Like CLL, these CLL-like MBL cases were found to occur with increasing age and could be clustered in families.
However, the inter-family IgHV rearrangements were not the same (Dagklis et al., 2009). Additionally, the IgHV mutations found were different to those typically found in CLL patients. Therefore, the authors concluded that the development of CLL from MBL is extremely unlikely (Dagklis et al., 2009).

1.4. Staging systems for CLL.

There are two systems currently used for the staging of CLL: Rai and Binet. The systems are similar in their diagnostic criteria with the main difference being emphasis on the occurrence of lymphocytosis and lymphadenopathy in the Rai system and the number of sites involved in the Binet system. In the UK and Europe only the Binet system, developed in 1981, is used. It categorises CLL into 3 stages, and is summarised in table 1.
### Table 1. Binet staging of CLL

Adapted from (Binet et al., 1981)

<table>
<thead>
<tr>
<th>Group</th>
<th>Hb count</th>
<th>Platelet count</th>
<th>Number of sites involved</th>
<th>Cases within whole population of disease (%)</th>
<th>Expected survival (y)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Normal</td>
<td>Normal</td>
<td>&lt;3</td>
<td>55</td>
<td>In line with population of same age and distribution</td>
</tr>
<tr>
<td>B</td>
<td>Normal</td>
<td>Normal</td>
<td>&gt;3</td>
<td>30</td>
<td>Median 7</td>
</tr>
<tr>
<td>C</td>
<td>&lt;10g/dL</td>
<td>&lt;100,000/mm3</td>
<td>Not considered</td>
<td>&lt;15</td>
<td>Median 2</td>
</tr>
</tbody>
</table>
1.5. Genetic abnormalities in CLL.

As highlighted in table 1, for decades the variable progression of CLL has indicated it to be a heterogeneous disease (Galton, 1966). Recent developments in the field, with particular regard to genetic alterations, have begun to describe potential mechanisms behind this heterogeneity (Hamblin, 2002). The most frequent alterations are the presence of mutated IgHV gene(s) as discussed for MBL, deletions in chromosome 13q14 and/or 17p13, trisomy of chromosome 12 (Coll-Mulet and Gil, 2009) and deletions in 11q22-q23. The ataxia telangectasia mutated (ATM) tumour suppressor gene is located in 11q22-q23 (Stankovic et al., 1998), mutations of which are observed in around 15% of CLL patients. Although not always concurrent with 11q deletions (Dal-Bo et al., 2009), ATM is more common in patients with this deletion. At presentation, the tumour protein 53 (TP53) is mutated in around 10% of patients and is linked in many cases with a 17p deletion (Dal-Bo et al., 2009). A mutated TP53 status is currently considered to be the worst chromosomal prognostic indicator.

Any chromosomal alterations can be apparent at diagnosis or, in particular for 17p13, can be acquired following treatment. These chromosomal abnormalities can lead to the loss of sequences encoding short/micro RNA (miRNA). In particular, a cluster of miRNAs (mir-15a and mir-16-1) were discovered to be deleted in patients harbouring the 13q14 deletion (Ghia et al., 2007a). These miRNA deletions have been suggested to be relevant to the onset of CLL and more recently have been found to be down regulated in most CLL cases, with or without the 13q14 deletion (Calin et al., 2008). Additionally, mouse studies have revealed that reinstating the mir-15a and mir-16 deletions in New Zealand black (NZB) mice, re-sensitises CLL cells to chemotherapeutics (Salerno et al., 2009) and that inducing
a knock out of the DLEU2/miR-15a/16-1 cluster induces the CLL phenotype in mice observed in humans (Klein et al.).

1.5.1. Prediction of CLL Prognosis.

Prognosis of disease is assessed by classical factors, including lymphocyte count, \( \beta \)-2 microglobulin (B2M), which has currently fallen out of favour (Delgado et al., 2009b), and lymphocyte doubling time (LDT), as well as modern biological factors. CLLU1, the first gene reported to be specific in CLL is encoded in 12q22, and high CLLU1 levels have been associated with poor prognosis, although mainly in younger patients (Dal-Bo et al., 2009). The T cell specific zeta associated protein-70 (ZAP70) was first discovered in T cells as critical in tyrosine kinase signalling (Chu et al., 1998). More recently, ZAP70 has been linked with IgHV mutational status (Dal-Bo et al., 2009) but most currently has been shown to be an independent prognostic indicator, with high expression indicating poor prognosis (Ghia et al., 2007a). The 45kDa type II membrane glycoprotein CD38 is expressed by many haemopoietic tissues and its engagement signals cell activation and differentiation (Dal-Bo et al., 2009). High expression of CD38 is associated with poor patient prognosis (Ghia et al., 2003). Recently, studies into the significance of CD38 have increased with studies showing a correlation between CD38 and BCL2 proteins as poor prognostic indicators (Pepper et al., 2008). These factors, along with those in section 1.5, provide indications of the likelihood for disease progression, as summarised in table 2. These factors can change over time, i.e. progression through the Binet staging. Currently only the deletion of TP53 is used to direct therapy and, as yet, there is no clear role for other prognostic factors in directing therapy.
### Table 2. Prognostic indicators for CLL

<table>
<thead>
<tr>
<th>Factor</th>
<th>Output</th>
<th>Indication</th>
</tr>
</thead>
<tbody>
<tr>
<td>Binet staging</td>
<td>A</td>
<td>Good</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>Average-good</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>Poor</td>
</tr>
<tr>
<td>LDT</td>
<td>&lt;6 months</td>
<td>Disease stable</td>
</tr>
<tr>
<td></td>
<td>&gt;6 months</td>
<td>Disease progression</td>
</tr>
<tr>
<td>IgHV genes mutation</td>
<td>≥98% similarity to germ line (unmutated) sequence</td>
<td>Aggressive disease. median survival 8 y</td>
</tr>
<tr>
<td></td>
<td>&lt;98% homology</td>
<td>Milder disease: median survival 24 y</td>
</tr>
<tr>
<td>CD38 expression</td>
<td>Positive</td>
<td>Less time to progression</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lower survival</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>Good prognosis</td>
</tr>
<tr>
<td>CLLU.1</td>
<td>High</td>
<td>Poor prognosis</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>Good prognosis</td>
</tr>
<tr>
<td>Intracellular ZAP70</td>
<td>&gt;20% positive leukaemic cells</td>
<td>Poor prognosis</td>
</tr>
<tr>
<td></td>
<td>&lt;19% positive</td>
<td>Good prognosis</td>
</tr>
<tr>
<td>Chromosomal deletions</td>
<td>17p</td>
<td>Poor prognosis mean survival 32 months</td>
</tr>
<tr>
<td></td>
<td>TP53</td>
<td>Poor prognosis</td>
</tr>
<tr>
<td></td>
<td>11q</td>
<td>Poor prognosis mean survival 79 months</td>
</tr>
<tr>
<td></td>
<td>Trisomy of chromosome 12</td>
<td>No effect on outcome of disease</td>
</tr>
</tbody>
</table>

Table 2. Prognostic indicators for CLL. Modified from Ghia et al 2007 and Dal-Bo et al 2009.
1.6. The ontogeny of B lymphocytes.

Before discussing the biology that underpins the in vivo survival and accumulation of CLL cells it is useful to consider the ontogeny of normal B cell development. There are two types of recirculating B cell. The first are B1 cells, which are produced in the foetal liver and in the BM until the age of five, and are self-sustaining beyond that age. B1 cells are able to mount T cell independent immune response. The second type are conventional B cells, which are derived from haemopoietic stem cells (HSCs) in the BM (pro-T cells can be developed from the same HSC depending on the perceived stimulus). Unlike T cells that travel to the thymus to continue development, B cells remain in the BM, where the BM stromal cells provide the microenvironment for the pro-B cell to proliferate and differentiate into precursor (pre-) B cells (Hess et al., 1998). Cell adhesion molecules (CAM) (for example, integrins $\alpha 4\beta 1$; VLA-4) on the B cells bind to ligands, (in this example V (vascular) CAM-1) on the stromal cell (Barreiro et al., 2002). The stromal cells additionally express stem cell factor (SCF) on the outer membrane. The initial binding of CAMs, assists in binding of the pro-B cell c-Kit receptor to the stem cell factor (SCF) ligand of the stromal cell. This activation of c-Kit receptor drives the B cell into proliferation and differentiation, by heavy (H) chain gene rearrangement, into pre-B cells. The progeny then bind Interleukin-7 (IL-7), which is secreted from the stromal cells that further drives the maturation of the B cell (Hentges, 1994). The B cells are then detached from the stroma, following down-regulation of CAMs in the maturation process.

The pre-B cells must undergo further light chain rearrangement to develop into an immature B cell. The light chain rearrangement will commit an immature B cell to a particular antigenic specificity, related to the heavy chain VDJ and light chain VJ.
sequences. The immature B cell will also express membrane (m) bound IgM (mIgM), together with Ig-α and Ig-β. Collectively, the expression of these Igs form the B cell receptor (BCR) (MacLennan, 2005).

The immature B cell in the BM is not fully functional and engagement of the BCR at this stage should lead to anergy or cell death. Changes in the transcription of the heavy chain RNA, leads to full expression of IgD on the cell membrane. Thus the co-expression of IgM and IgD leads to the cell being fully mature. It was considered for many years that CLL cells were immature B cells (Dameshek, 1967). However, it is now accepted that these cells are of a more mature phenotype and are capable of some antibody secretion and B cell function (Ranheim and Kipps, 1993, Jurlander, 1998).

1.7. The lymph node.

Further stimulation of conventional B cells occur antigenically, once the cell is released from the BM. Although normal B cells are found in interstitial spaces as well as in the peripheral blood, the engagement of antigenically stimulated B-cells occurs in critical niches within secondary lymphoid organs such as lymph nodes (LN), which traps particles of antigen (Clark and Ledbetter, 1994, Natkunam, 2007). The LN can be divided into two zones: light (LZ) and dark (DZ). The LZ contains T cell zones and B cells cross the high endothelial venules (HEVs) and migrate to the edge of the T zone. If they encounter antigen here it is internalised by the B cell and the resulting peptides presented on the cell surface. This presentation of antigen and the BCR makes interaction with primed (alternatively termed activated/helper/CD4+/T_H) T cells highly efficient (Benson et al., 1997). This interaction induces rapid proliferation and clonal expansion of the B cell, isotype switching and production of both plasma cells, that secrete low affinity antibody.
or immunologic memory B cells (Natkunam, 2007). Cells that are released from the T zone will only have had fleeting interaction with primed T<sub>H</sub> cells (Stewart et al., 2007). However, if they do not differentiate into antibody secreting plasma cells, they can be re-recruited into proliferation if they re-enter the T-cell zone within the LN (MacLennan, 2005). If no antigen is encountered in the T zone they migrate to the cortex of the LN to the LZ and encounter primary B cell follicles (PBCF).

### 1.7.1. The role of the PBCF and the Germinal Centre.

In relation to the underlying biology of CLL, the PCBF is probably the most significant LN area to consider. PBCFs are formed within the LN following the first expansion of B cells (Natkunam, 2007, Clark and Ledbetter, 1994). T cells that have been activated in the T zone, migrate to the PBCFs. These cells drive a burst of the follicular B cell proliferation after which the central collection of follicular B cells differentiates into the germinal centre (GC) and the remaining blasts differentiate into centroblasts (reviewed in MacLennan, 1994). Hypermutation targeted Ig variable regions is switched on in the centroblasts, generating thousands of mutant IgHV<sub>s</sub>, with very little expression of protein Ig receptor. The centroblasts next exit cell cycle and migrate to the germinal centre area that is rich in follicular dendritic cells (FDCs) that have antigen trapped on their surface (Nossal et al., 1964). The centroblasts can take up antigen from the FDC and present to T<sub>H</sub> cells located in the FDC region. GC T<sub>H</sub> cells select only the B cells that will secrete antibodies of high affinity. The high rate of IgHV mutation within the GC allows the generation of specific antibodies and a high rate of B clonal cell ‘production’.
1.7.2. The biology of the B cell- T cell/ CD40-CD40L interaction.

The cognate interactions between B cells and T\(_H\) cells, inducing proliferation and survival, include signalling via CD40 expressed on the membrane of the B cell, which interacts with CD40L (also termed CD154) expressed on T\(_H\) cells (Willimott et al., 2007a) and IL-4. CD40L is a member of the tumour necrosis factor receptor (TNFR) family; with CD40 reciprocally belonging to the tumour necrosis factor (TNF) family. The cross linking that occurs when CD40 interacts with CD40L is critical to induce and sustain B-cell response, (Grdisa, 2003) and works by activation of protein kinases, phospholipase C and the activation and transcription of NF\(\kappa\)B. The activation of the B cell also causes increased expression of cytokine receptors including IL-5 and IL-7 and, importantly, IL-4.

1.8. The mechanisms underlying the accumulation of CLL cells.

For some time it was widely considered that G\(_0\)/G\(_1\) peripheral CLL cells were resistant to apoptotic stimuli and that this contributes to the accumulation of the malignant clone (Kitada et al., 1999). However, since CLL cells have been reported to rapidly undergo apoptosis \textit{in vitro}, if not provided with cytokine support, (Burger et al., 2000, Willimott et al., 2007b, Lanham et al., 2003) it has also been suggested that their apoptotic machinery must be intact. The peripheral accumulation of CLL cells was also thought to be due to an exceedingly slow proliferation rate of cells within the periphery, coupled with a defect in (or lack of) apoptosis (Chiorazzi, 2007, Dameshek, 1967). However, it is now widely recognised that CLL cells have undergone several rounds of proliferation compared to their normal counterparts, demonstrated by shortened telomeres and an appearance of being metabolically active (Chiorazzi, 2007, Dal-Bo et al., 2009). The \textit{in vivo} measurement of the proliferation rate of CLL clones demonstrated clonal expansion to be between 0.1-
1.75% per day (Messmer et al., 2005) or 1% and 10% per week (Chiorazzi, 2007, Messmer et al., 2005). Messmer et al calculated that these values translate to between $10^9$ and $10^{12}$ cells produced per day. However, the circulating lymphocyte count did not increase by the same degree (Chiorazzi, 2007, Messmer et al., 2005). This led Messmer et al to conclude that CLL cells must also be undergoing apoptosis and they calculated death rate to be up to 2% per day.

The observations that most CLL cells are out of cell cycle but have undergone significant numbers of divisions and that apoptosis rates are significant in the peripheral pool of cells, progressively suggests that CLL has two compartments: a circulating non-proliferative compartment and a tissue resident proliferating component. A review by Dameshek in 1967 supported the theory proposed by Gorman and Marmount that CLL cells occupy B cell niches in lymphoid tissues (Dameshek, 1967). Indeed, it has been suggested more recently that CLL cells may have a competitive advantage in proliferation and survival over normal B cells, allowing their occupation in the BM and LN and consequent accumulation in the periphery (Keating et al., 2003). This competitive advantage could be dependent on factors perceived in the microenvironments, in particular the LN (Ghia et al., 2008).

### 1.9. The role of the LN in CLL.

Considering the proliferation of B cells within the LN and the evidence of a proliferative compartment in CLL, it is certain the LNs play a vital role in the microenvironment of propagating CLL. Histological studies support the \textit{in vivo} interaction between T cells and CLL cells. CLL cells have been found to accumulate in the LN and, additionally, high numbers of primed T cells have been found intermingled with the B cells in the same
Chapter One - General Introduction

CLL involved tissues (Granziero et al., 2001, Patten et al., 2008). A histopathology study conducted in 1994 has confirmed the existence of B cell follicle like proliferative centres (PC) of CLL cells within the LN that contain higher numbers of T cells than in the surrounding tissue and a higher proliferative fraction of cells (Schmid and Isaacson, 1994). More recent histochemistry has demonstrated that within the PC CLL cells are proliferating, demonstrating a blast-like morphology, and that this is driven by CD40L signals (Luqman et al., 2008, Burger et al., 2009, Ghia et al., 2008). It is interesting to note, in light of prognostic indicators, that clonal expansion studies (similar to those discussed above) have postulated that, from within the proliferative tissue compartments, CD38^ve CLL cells generate approximately two-fold higher percentage of new CLL cells compared to their CD38^-ve counterparts (Calissano et al., 2009). In support of the Pepper et al study (2008) the CD38^ve cells were also found to have a reciprocal expression of the cell cycle protein Ki67 (Calissano et al., 2009) although Ki67 was also detected on the CD38^-ve fraction and telomere length in both groups was found to be the same (Calissano et al., 2009, Pepper et al., 2007). Therefore, it is possible that within the PC, CLL cells are CD38^ve and this plays a role in proliferation of CLL cells following engagement with CD40L but that on release of the cells from the PC into the periphery CD38 positivity is downregulated.

Evidently, CLL cells will perceive other stimuli, such as IL-2, IL-13, IL-7 and IL-15, all of which have been shown to increase DNA synthesis of CLL cells (reviewed in (Jurlander, 1998) and CD80-CD28 (another B-cell-T-cell interaction) (Clark and Ledbetter, 1994, Natkunam, 2007). However, no interaction has been shown to be as critically involved in CLL cell survival and proliferation as CD40-CD40L (Clark and Ledbetter, 1994), nor have
any been as well investigated. This mix of proliferating CLL cells in the presence of T cells has several terms: microenvironment, simply the LN, GC or the PC. It should be noted, however, that the BM also plays a role in the maintenance of CLL, and is likely to harbour different cell-cell interactions and provide divergent cell survival signals. Studies by Burger’s group in Austria have addressed the likelihood of nurse cells and stromal support networks within the BM providing survival signals to CLL cells. They, and others have identified the important role of stromal cell derived factor -1 (SDF-1) (Burger et al., 2000). SDF-1 is constitutively expressed by BM stromal cells. with its chemokine receptor CXCR4 (or CD184) widely expressed on both normal and tumour B cells (Meads et al., 2008). The expression of SDF-1 on the BM cells and the expression of CXCR4 on the B cells is thought to allow the more mature malignant CLL cells to home to the BM, where other factors such as interleukin-6 (IL-6) are thought to promote cell survival and reduce cell sensitivity to treatment (Meads et al., 2008). In vitro studies by the Burger group have identified that BM stromal cells from many sources can successfully promote cell survival and drug resistance of CLL cells, including the lines M210B4 (Kurtova et al., 2009, Burger et al., 2000), KUM4, ST-2 and KUSA H1, and that cell-cell interaction was essential to mediate this (Kurtova et al., 2009). Interestingly, however, primary stromal cells derived from human samples were less protective than any cell line or primary stromal BM cells derived from mice (Kurtova et al., 2009). Additionally others, using deuterated water in nine stable CLL patients, found that only the LN and not the BM contributed to CLL proliferation (van Gent et al., 2008). Thus, the microenvironment of CLL cells is potentially diverse and includes many involved tissues. The work presented in this thesis will focus upon the PCs within the LN microenvironment and not that of the less involved BM.
1.9.1. **In vitro recapitulation of the LN.**

Attempts to recapitulate the PCs *in vitro* have identified that CLL cells can be protected from spontaneous *in vitro* apoptosis by co-culture with autologous T cells (Ranheim and Kipps, 1993). In 1993 Crawford *et al.* demonstrated that use of a co-culture system of Fc gamma RII+ (CD32) L-cells together with an anti-CD40L antibody induced rapid proliferation of CLL cells taken from peripheral blood (Crawford and Catovsky, 1993). In addition they showed that whilst IL-4 induced very little DNA synthesis alone, when CLL cells were co-cultured in the same way, but with the addition of IL-4, proliferation was further enhanced (Crawford and Catovsky, 1993). Jacob *et al.* also observed that combined IL-4 and CD40L induced greater CLL cell proliferation than CD40L alone. However, their study additionally identified that CD32 used in Crawford and Catovsky’s original study was not required to stimulate proliferation (Jacob *et al.*, 1998). The significantly increased cell viability and proliferation upon culturing with both CD40L and IL-4 was proposed by Gridsa to indicate that each molecule induced alternative and complimentary pathways of signalling (Gridsa, 2003). It is interesting to note, however, that neither Gricks nor Gridsa reported an upregulation of cell cycle proteins (Gridsa, 2003) or genes (Gricks *et al.*, 2004) but they did report a significant increase in protection from apoptotic signals.
Figure 1. Schematic of culture conditions mimicking the culture conditions used for CLL in vitro experiments. **Top** shows the proliferative centre cells cultured with CD40L and **bottom** shows the peripheral CLL cells cultured with stroma without CD40L. Both systems have the addition of IL-4. This culture condition is based on that discussed by Willimot et al., 2007.
In this regard, *in vitro* cultures of CLL with CD40L delivered by stable stromal transfectants and IL-4 has been shown to reproduce the *in vivo* anti-apoptotic effect of Th-B cell interaction as depicted in figure 1. This interaction has also been demonstrated to induce an increase in anti-apoptotic proteins such as myeloid cell leukaemia-1 (MCL-1) and B-cell lymphoma-2 (BCL-2), further indicating the highly protective role of this cellular interaction (Willimott et al., 2007c) and these anti-apoptotic proteins are regarded as therapeutic targets in CLL (Pepper et al., 2008, Hallaert et al., 2007, Gandhi et al., 2008).

**1.10. The importance of the PC in CLL.**

Without evidence of a leukaemic stem cell, the occurrence of PCs in CLL is unique to this B cell malignancy. Only other chronic immune disorders such as rheumatoid arthritis have shown similar proliferative arrangements. Thus CLL can be regarded as a disease of constant immune stimulation (Burger et al., 2009, Ghia et al., 2008), with the PCs continually harbouring dividing leukaemic cells and has been considered an autoimmune disease by many since the 1960s (Dameshek, 1967). The interaction of CLL cells with CD40L provides the cells with both a pro-proliferative (Grdisa, 2003, Crawford and Catovsky, 1993, Luqman et al., 2008) and a pro-survival signal (Grdisa, 2003) that is presumably part-way responsible for engendering the reduced apoptotic index of the peripheral CLL cells. Although the microenvironment needs to be fully characterised for the disease to be fully understood (Motta et al., 2009) it is clear that the treatment and ultimately cure of CLL will require not only the removal of the peripheral B-cells but also more focus on the PC where the residing CLL clone can result in minimal residual disease (MRD).
1.10.1. Targeting MRD within the PC.

Treatment of CLL has for many years been based on alkylating agents and purine analogues but many patients are refractory to these treatments or relapse over time (Byrd et al., 2007). In addition, improved technology such as multi-colour flow cytometry, allows a more precise measure of CLL tumour load (Moreton et al., 2005). Therefore, therapies that may have appeared to achieve CR in the 1960s, would now possibly be classified as achieving partial response (PRS) owing to the more sensitive techniques employed (Moreton et al., 2005), that would detect MRD (even at exceedingly low levels).

It is becoming increasingly apparent that achieving CR, together with MRD negativity, can result in improved survival but at a cost of increasing toxicity (Moreton et al., 2005, Hillmen et al., 2007, Motta et al., 2009, Chanan-Khan et al., 2006). Recent studies have addressed this, utilising modern therapies including ofatumumab (Coiffier et al., 2008), lenalidomide (Chanan-Khan et al., 2006) and most notably alemtuzumab (Moreton et al., 2005, Hillmen et al., 2007). A comparison of alemtuzumab against the alkylating agent chlorambucil in 2007 concluded that alemtuzumab achieved a superior CR (Hillmen et al., 2007). This was an encouraging result, especially considering the claims that an MRD negative state is achievable with alemtuzumab therapy (Moreton et al., 2005). However, this was only achieved in patients with low (or no) enlarged LN. In patients with LNs in excess of 5cm, patients remained MRD positive (Moreton et al., 2005). This status was improved on the administration of additional therapies including fludarabine (F-ara-A) and autologous/allogeneic stem cell transplant, but this is unlikely to be available to the majority of elderly CLL sufferers. Together with the infection rate following alemtuzumab administration, the overall clinical benefit for the average patient remains to be determined.
An alternative way to tackle MRD is to eradicate the CD40-CD40 interaction or interfere with its downstream consequences, and many believe that a better understanding of this interaction and disruption of it would be a great step to curing CLL (Luqman et al., 2008, Jurlander, 1998, Willimott et al., 2007c). Treatment of CLL by targeting the CD40L stimulus has recently been investigated in many ways. A recent study utilised an anti-CD40 antibody, HCD122, to inhibit CD40-CD40L interactions and showed the ability of this antibody to do this and to induce cell lysis (Luqman et al., 2008). However, the number of patients studied was small (n=8), with most data shown for only one patient. Therefore, it remains to be proven whether this therapy would be clinically useful. Other approaches are the use of agents to sensitise cells within the PC to chemotherapeutics. An in vitro study, carried out by Hallaert et al (2008) utilised dasatinib (DSN) with chemotherapeutics such as F-ara-A and concluded that DSN increased cell death in response to chemotherapeutics (Hallaert et al., 2008). However, in these studies CD40L was not continually available to the CLL cells, therefore, it remains questionable as to whether this would truly translate in vivo within the PC (Hallaert et al., 2008). In chapter 5 experiments will be presented that address this possibility.

1.11. The decision to treat and current treatment strategies.

The treatment of CLL initially relies on the stage of disease at diagnosis. Patients in Binet stage A are left with a ‘wait and watch’ approach and are not currently treated until the disease progresses (Ghia et al., 2007b). This is due to the majority of patients (85% of stage A) having a good quality of life, unaffected by CLL, and dying of unrelated disorders. In addition, current common therapies are unable to halt the natural course or cure the disease (Dighiero et al., 1998) and a number of studies have failed to show any benefit for
treating early stage disease. Binet stage B will often be treated once the disease has started to progress, with stage C patients being given early treatment (Ghia et al., 2007b).

1.11.1. Established current therapies.

There are many established CLL therapies, many of which are covered in the following sections.

1.11.1.1. Alkylating agents.

Alkylating agents such as cyclophosphamide and chlorambucil were considered the ‘gold standard’ of therapy until the 1980s, with chlorambucil being the first line of therapy (Oscier et al., 2004). Alkylating agents have three described mechanisms of action: 1) to add alkyl groups to DNA bases causing the DNA to be fragmented during the repair processes, thus preventing DNA synthesis and RNA transcription; 2) cross linking DNA, thereby inhibiting transcription and 3) inducing DNA mutations by causing alkylated G nucleotides to pair with T nucleotides. Chlorambucil has a 10% CR rate and virtually all patients will be expected to relapse (Ghia et al., 2007b). The UK Medical clinical Research Council reported in 1988 that chlorambucil does not improve overall survival (OS) and that continuous administration is not beneficial (Catovsky et al., 1988). Additionally, to date, combining chlorambucil with other agents such as prednisolone (PRD) has no additional long term benefit (Jaksic and Brugiatelli, 1988). Recent studies are examining whether there is benefit in combining chlorambucil with rituximab or ofatumumab (Kay et al., 2006). Chlorambucil is well tolerated with manageable toxicities, although myelosuppression is common and related to duration of use.
1.11.1.2. **Purine analogues.**

Purine analogues such as cladribine and pentostatin overtook chlorambucil as first line therapy in the mid 1980s with F-ara-A becoming the most used in the developed world. These agents exert their activities by interfering with the function of multiple DNA polymerases, DNA primase, DNA ligase I and are therefore S phase-targeted. F-ara-A has a 38% CR rate and is considered to have a superior remission rate to chlorambucil (Rai et al., 2000). Nevertheless, studies continue to show that, as a single agent, use of F-ara-A does not improve OS (Leporrier et al., 2001, Abrisqueta et al., 2009, Eichhorst et al., 2009b). Attempts at combination therapy with agents such as cyclophosphamide have also showed that the OS rate is not improved (Eichhorst et al., 2006) but response rates are considerably better than with F-ara-A or chlorambucil alone (Catovsky et al., 2007). The new gold standard regimen for induction in CLL is a combination of F-ara-A, cyclophosphamide and rituximab (FCR) based on German studies (Michael Hallek ASH 2009) and MD Anderson data (Tam et al., 2008). The FCR regimen is associated with higher response rates but again there is a lack of clarity as to whether this leads to improved OS. Future studies are exploring whether the addition of an anthracyclin (mitozantrone) is beneficial and addressing the optimal dosing of rituximab (Dr Guy Pratt, personal communication).

1.11.1.3. **CAP, COP, CHOP.**

In the late 1980s and early 1990s new combination therapies were investigated including CAP (cyclophosphamide, doxorubicin and prednisone) (Keating et al., 1998), COP (cyclophosphamide, vincristine and prednisone) and Binet’s modified CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) regimen (Binet, 1993).
However results showed that overall survival rates using these therapies are not improved.

In addition to the lack of prolonged survival of CLL patients undertaking the above chemotherapeutic based therapies, they all have associated toxicities (Oscier et al., 2004) and have complications such as high rate of infections and anaemia (Steurer et al., 2006). Combining multiple chemotherapeutics together serves to increase the toxicity and reduce the benefits to older patients. This has led to studies advocating that, in the elderly, high dose chlorambucil maintains better patient benefit. (Jaksic and Brugiatelli, 1988, Jaksic et al., 1997).

1.11.1.4. *Methyl-prednisolone.*

Studies using a high dose of the glucocorticoid receptor (GR) antagonists, PRD and methyl-prednisolone (ML-PRD) in the late 1990s have shown that these agents can induce remission. Prednisolone is a useful agent with anti-tumour activity, particularly in the setting of CLL related marrow failure and is used as a palliative treatment. ML-PRD was more likely to be used in refractory patients, in whom other therapies had failed. ML-PRD combination therapy has recently gained renewed interest as a combination therapy with monoclonal antibodies (Castro et al., 2009). The combination of ML-PRD with rituximab (discussed in 1.12.1.1), has been shown to induce 19-50% CR (depending on the dose of rituximab used) with a 16.7% MRD negative state in the higher CR group. The associated rate of toxicity of this regimen was high, although most (80%) were classified as low grade toxicities (Eichhorst et al., 2009a).
1.12. Newly established approaches.

The therapies described in 1.11.1. are largely based on agents that were first established decades ago. The following sections will consider more recent approaches to treating CLL.

1.12.1. Monoclonal antibodies.

Studies using monoclonal antibody therapy have been ongoing since the late 1980s (Press et al., 1987) and include those raised against CD20 and CD52. Importantly, unlike chemotherapeutics, these novel agents do not rely on the ATM-p53 apoptotic pathway, which is often defective in relapsed and refractory CLL and is a major mechanism for chemo-resistance (Austen et al., 2005). These agents can potentially overcome chemo-resistant and poor risk disease states (Luqman et al., 2008).

1.12.1.1. CD20.

The most successful monoclonal antibody to date has been the use of CD20 antibody therapy. CD20 is a calcium channel that interacts with the B-cell Ig receptor complex on the surface of both normal and malignant B-cells. The first antibody therapy targeted against this phosphoprotein in B-cell malignancies was rituximab. The benefit of CD20 antibody therapy is that whilst normal mature B cells will also be affected; precursor B cells will remain untouched as they do not express CD20 (Reff et al., 1994). However, as B CLL cells express relatively little CD20, therapy using it alone has been disappointing and ineffective compared to F-ara-A (Hainsworth et al., 2003). Combination therapy using F-ara-A/cyclophosphamide/rituximab (FCR) has been shown to be much more effective (Wierda et al., 2005).
However, rituximab has its own associated side effects (Hainsworth et al., 2003) due it stimulating the release of cytokines. Combined with the toxicity of FC therapy, this means that this treatment is not well tolerated, especially in patients over the age of 70. The MD Anderson Cancer Centre, University of Texas reported FCR as first-line therapy in patients < 70 years of age and showed that 75% of patients experienced grade 3-4 myelosuppression, 11% experienced grade 3-4 infection and fewer than half were able to complete the six cycles of induction (Keating et al., 2005, Wierda et al., 2005).

Ofatumumab is a newly developed, alternative anti-CD20 antibody with a higher affinity for CLL cells, when tested in vitro (Coiffier et al., 2008) and an ability to activate complement. A phase I/II study of this agent did not produce any CRs, but lower lymphocyte numbers were reported in the BM following treatment and significant responses were seen (Coiffier et al., 2008). However, the trial additionally reported a 30% incidence of immunosuppression and a 57.6% rate of infection (Coiffier et al., 2008).

Further studies are examining ofatumumab in combination with chlorambucil and F-ara-A /cyclophosphamide combinations. Whilst these combinations may improve responses, it is likely that the immunosupression would be worse.

1.12.1.2. CD52.

Mature B cells, T cells, NK cells, macrophages all express CD52. Also, in contrast to CD20, all CLL cells also express this heavily glycosylated, membrane-anchored glycoprotein. Alemtuzumab is an antibody raised against CD52 and trial results from the use of this antibody used alone, (Rai et al., 2002) have shown that it is more effective than rituximab, especially in patients with more progressive CLL (Osterborg et al., 1997). Combination therapy of this antibody with rituximab with and without F-ara-A are currently underway.
(O'Brien, 2008). However, as previously discussed, there are known associated toxicities of alemtuzumab (Thursky et al., 2006) and on its own it is unlikely to treat MRD. Additional research and trials are currently underway, investigating the use of other monoclonal antibody therapies such as CD23, CD70 and different epitopes of CD20.

1.12.2. Flavopiridol.

Flavopiridol is a cyclin dependent kinase inhibitor that clearly has activity in CLL (Lin et al., 2009). Like antibody therapies, it does not rely on the ATM-p53 apoptotic pathway (Pepper et al., 2003). Trials into flavopiridol demonstrated that it can have activity in relapsed high risk disease (Byrd et al., 2007) but only in younger patients. As it is difficult to deliver and it has associated significant toxicity (Lin et al., 2009) its therapeutic use, especially in the over 65s, is questionable.

Thus, although there have been improvements in responses in CLL, it remains incurable for the vast majority of patients and there is a need for novel treatments with acceptable toxicity. Also, given the heterogeneity of disease progression, eradicating MRD may be a reasonable aim for fitter patients or patients with high risk disease (Nabhan et al., 2007) whereas palliation may be the best approach for a significant proportion of CLL patients. It is clear that new treatment strategies for CLL are desirable. In order to achieve these different strategies should be applied - such as drug redeployment.

1.13. History of drug redeployment.

The theory of utilising existing drugs in new settings is a research area that has recently gained interest (Chong and Sullivan, 2007). It is well known that therapeutic agents designed many years ago probably have targets other than those for which they have been prescribed (Keiser et al., 2009). In the acute leukaemia setting this has led to the
successful use of retinoic acid (RA) (Fenaux et al., 2007) and arsenic trioxide (Chen et al., 2007) in APL, valproic acid in AML (Kuendgen and Gattermann, 2007) and thalidomide in multiple myeloma (MM) (Prince et al., 2007, Breitkreutz and Anderson, 2008, Breitkreutz et al., 2007) as individual agents. In the case of B cell malignancies, bendamustine, a cytostatic alkylating agent, has been redeployed for the treatment of Hodgkin’s lymphoma and CLL (Cheson and Rummel, 2009, Kalaycio, 2009, Kath et al., 2001, Knauf et al., 2009), although not without associated toxicities (Kath et al., 2001, Knauf et al., 2009) including tumour lysis syndrome (Hummel et al., 2005). Investigations have also highlighted the potential use of medroxyprogesterone acetate (MPA), a female sex steroid, in canine CLL (Florio et al., 2003).

In 2003, this group published a study on the use of ‘old drugs’ in a new setting of Burkitt’s Lymphoma (BL) (Fenton et al., 2003). The agents utilised in this study were members of the fibrate family, clofibrac acid (CA) or bezafibrate (BEZ), and MPA. This study showed that combining these 2 classes of agents, induced apoptosis, reduced proliferation and caused mitochondrial damage to BL cells and cell lines.


Fibrac acid derivatives, including Bezafibrate (BEZ), are lipid regulating agents that reduce triglycerides by 30-50% and moderately reduce (by 15-20%) low-density lipoprotein cholesterol (LDL-C) (Goldenberg et al., 2008b). They are synthetic peroxisome proliferator–activated receptor (PPAR) ligands with highest specificity for PPARα (Tenenbaum et al., 2003), with BEZ being a pan-PPAR ligand. The PPARα mediated effects are both lipid and non-lipid related. These include increased gene expression for lipoprotein lipase (LpL), a fatty acid binding protein, and a decrease
in expression for cyclooxygenase 2 (COX-2), VCAM-I and interleukin-6 (IL-6) (Goldenberg et al., 2008b). In addition to its regulation of triglycerides, BEZ has also been shown to have beneficial cardio-protective effects that are long-lived after therapy has ceased (Goldenberg et al., 2008a) and significantly reduce the incidence of new onset type 2 diabetes in patients with impaired fasting glucose (Tenenbaum et al., 2004).

BEZ has also been demonstrated to induce oxidative stress. It has been shown that, when used against AML cell lines, it induces differentiation in a dose dependant manner that was reduced in the presence of anti-oxidants (Scatena et al., 1999). Additionally, BEZ is known to induce mitochondrial depolarisation and mitochondrial specific ROS in rodent hepatocytes (Qu et al., 2001). Scatena et al (2003) have also shown that BEZ inhibits mitochondrial respiratory chain at the level of NADH cytochrome C reductase activity in AML cell lines. Both Qu et al (2001) and Scatena et al (2003) have suggested that, whilst the generation of ROS and the mitochondrial effects may be due to PPARα mediated effects, it is most likely that these are independent of PPAR.

1.15. MPA and steroidal activity.

The synthetic progesterone family of progestins is large with all members having effects against the progesterone receptor (PR). MPA belongs to the medrogestone class and unlike some of the other progestins, is not metabolised by the liver and therefore does not have a first pass effect (Schindler et al., 2003). As the family of progestins is large and structurally diverse all progestin members are known to exert additional activities. An example of this is the binding capacity of MPA to other steroid receptors. Relative to their known natural ligands the binding affinity of MPA to the PR is 115%, with affinities of 29% for the glucocorticoid receptor (GR), 5% for androgen receptor (AR) and 160% for
mineralocorticoid receptor (MR). It is thought that MPA has no binding affinity for oestrogen receptor (ER) (Schindler et al., 2003).

1.15.1. Non-steroidal activities of MPA.

Whilst Fenton et al (2003) did not investigate the mechanisms of action of MPA in BL cells, a recent study from this group has also investigated the mechanism of MPA against AML cell lines (Khanim et al., 2009). It was demonstrated that, in AML cells, MPA inhibits the enzyme aldoketoreductase (AKR) 1C3 (AKR1C3). AKR1C3 is a member of the AKR1 enzyme superfamily. In humans, four members of the AKR1 family have been detected: AKR1C1, AKR1C2, AKR1C3 and AKR1C4 (Mindnich and Penning, 2009). In vitro the enzymes have high substrate promiscuity. Penning et al demonstrated that all of the AKR1C1 family members are capable of reducing 5α-dihydrotestosterone (5α-DHT), oestrone and progesterone. However, AKR1C3 is thought to be unique in its metabolism of prostaglandin (PG) D₂ (PGD₂), described as 11β-PGD₂ ketoreductase activity. It has been shown in vitro that AKR1C3 will reduce PGD₂ to 9α, 11β-prostaglandin F₂ (11-epi PGF₂α) (Suzuki-Yamamoto et al., 1999). The metabolism of PGD₂ with and without AKR1C3 is shown in figure 2.

In the AML study from this group (Khanim et al., 2009) it was demonstrated that the anti-leukaemic activity of MPA was mediated, at least in part, via the inhibition of the 11β-PGD₂ ketoreductase activity of AKR1C3 leading to accumulation of PGD₂ and its bioactive metabolite 15dΔ^{12,14}PGJ₂.
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Figure 2. Schematic of the conversion of PGD$_2$ via both the non-enzymatic pathway to 15d$\Delta^{12,14}$PGJ$_2$ and AKR1C3 pathway to 11$\beta$-PGF$_{2\alpha}$. AKR1C3 is shown as its crystal structure as determined by Lovering et al (2004).
1.16. The generation and signalling of PGD₂.

PGDs are naturally occurring cyclic 20 carbon fatty acids, synthesised mainly from arachidonic acid (AA) (Straus and Glass, 2001). The synthesis of most PGs is COX dependent. In the first step, AA is transformed to an unstable endoperoxide by COX, which is converted to several related PGs, including PGD₂, by their specific PG synthases. PGD₂ has a cyclopentane ring (Straus and Glass, 2001) that undergoes dehydration to produce the J series PGs.

Although relatively unstable, PGD₂ is found in bodily fluids in the nanomolar range, increasing to micromolar during highly allergenic responses (Offenbacher et al., 1986). It has been shown to exert wide ranging biological activities including regulation of body temperature, regulation of the sleep cycle, inhibition of platelet aggregation, relaxation of smooth muscle and chemotactic recruitment of inflammatory cells (Herlong and Scott, 2006). Its best known activity is pro-inflammatory, although it has also been demonstrated that it has the potential to inhibit inflammation as it can induce T cell apoptosis (Nencioni et al., 2003).

PGD₂ has two known mechanisms of signalling by two distinct seven transmembrane G-protein coupled receptors. The role of these receptors in leucocytes has been reviewed by Sandig et al in 2006. The first receptor, DP1, was discovered first in mice and then in humans by Boie et al in 1995. This receptor has been reported to be expressed by T lymphocytes (Tanaka et al., 2004) cells in the immune system as well as other cells. DP1 has been suggested to mainly have a pro-inflammatory role, although some reports have been published of the anti-inflammatory response mediated by DP1 (reviewed in (Sandig
et al., 2007)). The second receptor for PGD$_2$, is chemoattractant-receptor homologous molecule expressed on T$_H$ (CRTH2)/DP2 that was independently discovered by three different groups in the years 1999-2001 (reviewed in (Sandig et al., 2007)).

The DP1 receptor is widely regarded to be the classical PG receptor as it shares similarity to other PG receptors. The reported $K_i$ value for PGD$_2$ to this receptor is 1.7nM, whereas the $K_i$ for PGD$_2$ to DP2 receptor is 2.4nM (Herlong and Scott, 2006). In addition, DP2 is known to bind many other agonists with high affinity. These include 15d$\Delta^{12,14}$PGJ$_2$, the nonsteroidal anti-inflammatory drug (NSAID) indomethacin (IDM) and metabolites of thromboxane (Herlong and Scott, 2006). Reviews on PGD$_2$ and its signalling also concede that the precise action of PGD$_2$ and its metabolites are not fully understood and there remains the possibility that they act on many other receptors (Herlong and Scott, 2006, Sandig et al., 2007)

1.16.1. The anticancer activities of 15d$\Delta^{12,14}$PGJ$_2$.

As mentioned in 1.6, PGD$_2$ is short lived and either is metabolised by AKR1C3 to produce 11-epi PGF$_2\alpha$ or spontaneously dehydrates to form prostaglandin J$_2$ (PGJ$_2$). In turn PGJ$_2$ undergoes a series of non-enzymatic reductions until it becomes the terminal derivative 15d$\Delta^{12,14}$PGJ$_2$ that has a cyclopentenone structure with unsaturated $\alpha,\beta$ ketone moieties (Piva et al., 2005). This, in particular of all the J series prostanoids, has been reported to have wide ranging anticancer activities, including inhibition of proliferation and induction of apoptosis (Piva et al., 2005). Pertinently, 15d$\Delta^{12,14}$PGJ$_2$ has also been shown to have antineoplastic properties against haematological malignancies (Laurora et al., 2003), including lymphoid (Padilla et al., 2002, Nencioni et al., 2003). Some of the activity of 15d$\Delta^{12,14}$PGJ$_2$ is exerted through it being a ligand for PPAR$\gamma$, which was demonstrated by
Kleiver et al in 1995 by the use of 15dΔ^{12,14}PGJ_2 to induce adipocyte differentiation. Whilst the evidence that 15dΔ^{12,14}PGJ_2 acts as a ligand for PPARγ was demonstrated, this is likely to be cell type specific. In haematological malignancies 15dΔ^{12,14}PGJ_2 has been demonstrated to reduce proliferation and induce apoptosis independently of PPARγ (Laurora et al., 2003, Piva et al., 2005).

1.17. The Isoprostane pathway.

In addition to the synthesis of PGs by the COX pathway, in the 1990s the formation of PGs was shown to occur *in vivo* via the isoprostane pathway (IoPP). Subsequently, the Morrow and Roberts’s group have demonstrated that PGD_2 is formed non-enzymatically from the free-radical-catalysed peroxidation of AA (Gao et al., 2003). Briefly, after AA peroxidation, three arachidonyl radicals are produced and undergo endocyclisation to form four PGH_2-like bicyclic endoperoxide intermediate regioisomers. These then undergo a reduction reaction to form, four F-ring regioisomers, each consisting of eight racemic diastereoisomers. Isoprostanes (IoPs) are initially formed esterified on phospholipids (PL) and are then released in free form by phospholipase(s) (Morrow et al., 1992b, Morrow et al., 1992a). However, the IoP endoperoxides may undergo rearrangement *in vivo* to form an E- or D-ring and thromboxane-ring compounds (Morrow and Roberts, 1997). Subsequently, E_2- and D_2-IoPs can form reactive cyclopentenone A_2 and J_2-IoPs after undergoing dehydration reactions.

Therefore, *in vivo*, PGD_2 can still be formed independently of COX, the inhibition of which would not stop the PGD_2 being formed via the IoPP. This has been shown by the Morrow group to occur significantly *in vivo* when cells are subjected to oxidative stress (Morrow et al., 1992a, Morrow et al., 1992b, Morrow and Roberts, 1997).
1.17.1. *Oxidative stress and Reactive oxygen species.*

Free radicals are mainly derived from oxygen and are commonly termed reactive oxygen species (ROS). They can be generated from various sources including, environmental toxins. The ROS family includes superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), hydroxyl radicals (HO) and peroxynitrite (ONOO$^-$). ROS are continually found within cells but they can cause direct damage to DNA, lipids and proteins. Antioxidants, including glutathione, vitamins C and E and antioxidant enzymes such as superoxide dismutases (SOD) and catalase, actively scavenge free radicals and are able to attenuate their effects (Gutteridge and Mitchell, 1999). However, if an imbalance arises between the generation and scavenging of ROS or there is an excessive production in ROS, this can lead to oxidative stress.

1.17.2. *The link between ROS and lipid peroxidation.*

The effects of oxidative stress and lipid peroxidation (LPO) have been comprehensively reviewed by Montuschi *et al* (Montuschi et al., 2007, Montuschi et al., 2004). Free radical attack on lipids is known to lead to LPO. This, in turn, is damaging to the cells by a chain of events. First, the biophysical properties of the membrane are altered leading to inactivation of membrane bound receptors and/or enzymes. Finally, this may impair normal cellular function.

Whilst lipoxygenase products (IoPs) are not a major product of LPO, the fact they are chemically stable and readily detectable in all major tissues and fluids (Morrow and Roberts, 1997, Morrow et al., 1992b, Pratico et al., 2001) means that they are currently considered to be the best measure of oxidative stress-mediated-LPO (Montuschi et al., 2004).
PGD₂-like IoPs have been shown to readily react with thiols forming glutathione and protein adducts in the same way as PGD₂ (Chen et al., 1999). However, the receptors by which IoPs exert their effects are currently unknown. It has been demonstrated that they can activate prostanoid receptors (Montuschi et al., 2007) and a novel IoP receptor has been reported to exist in vascular smooth muscle of the rat (Morrow and Roberts, 1997).

As well as effects on LP, ROS also have a role in signalling. This so-called ‘redox cell signalling’ has been demonstrated to be important at many levels of cell processes and can lead to the activation of pathways that control cell differentiation and apoptosis (Droge, 2002).

1.18. The significance of ROS in CLL.

The total levels of ROS and concentration gradients within a cell have been reported to be critical for basic cell signalling processes and not all ROS will be produced in equal amounts. Therefore, the presence of ROS within cells may not be detrimental to their survival. Indeed, a study on ROS generation in CLL cells showed that, post-chemotherapy, the surviving CLL cells showed mutations in mitochondrial DNA (mtDNA) associated with increased ROS (Carew et al., 2003), perhaps indicating a survival advantage in these cells. However, a sudden and sustained increase in ROS has been hypothesised to lead to apoptosis in the lymphoma cell line Ramos (Ryan et al., 2008). Additionally, it has been proposed that the treatment of CLL with 2-Methoxyestradiol (2-ME) is successful at inducing apoptosis in vitro due to its induction of ROS (Zhou et al., 2003). As well as finding ROS within the cytoplasm of the cell, it is found compartmentalised within organelles. This compartmentalisation of ROS,
especially in the mitochondria, has been suggested to play a major role in the redox state of the cell (Droge, 2002)

1.19. Mitochondrially mediated ROS.

Mitochondria are key organelles in conversion of energy, regulation of cellular signalling and amplification of programmed cell death. Synthesis of ATP by mitochondria depends on energy derived from the flow of electrons from metabolic intermediates through the electron transport chain to molecular oxygen. Although the electron transport chain converts about 85%–90% of the oxygen utilized by cells to water, 1%–2% of the oxygen is converted to ROS (Richter et al., 1995). A review conducted in 1995 concluded that mitochondria are strongly linked to apoptosis, either as endpoint organelles of cell death or as an environmental sensor, but highlighted that the mechanisms by which this occurred were unclear (Cavalli and Liang, 1998).

Mitochondrial ROS including superoxide (MSO) can cause damage to mitochondrial proteins, lipids and DNA, thereby disrupting mitochondrial function. It is thought that mtDNA is particularly susceptible to MSO owing to it being located in close proximity to the site of MSO production. Mitochondrial damage owing to MSO has been reported in clinically relevant situations such as cardiovascular disease (Victor and Rocha, 2007), although the importance of MSO in vivo is not currently understood.

Normally mitochondrial ROS causes little oxidative damage because mitochondria possess an efficient antioxidant defence system composed of manganese superoxide dismutase (MnSOD/SODII), glutathione peroxidase, NADPH transhydrogenase, reduced glutathione, NADPH, and vitamins E and C (Raymond and Johnson, 2004). It has also
been hypothesised that there is a range of mnSOD activity: high enough to remove excess MSO and low enough to allow the controlled release of H$_2$O$_2$ (Kim et al., 2004).

MnSOD is the key free radical scavenger in the mitochondria. It converts MSO to O$_2$ and H$_2$O$_2$, which is either enzymatically detoxified within the mitochondria or diffuses into the cytoplasm (Victor and Rocha, 2007). However, MnSOD has also been shown to have pro-oxidant activity by the production of H$_2$O$_2$. These anti- and pro-oxidant activities of mnSOD are thought to be a key regulator of the redox state of the cell (Kim et al., 2004).

The described evidence of mitochondrial redox states in cell death suggests this could be an exploitable target for the induction of apoptosis in malignant cells, including CLL. Indeed, many agents, including plant derivatives, have been shown to be anti-leukaemic by causing mitochondrial damage.

1.20. The history of the potential use of plant derived compounds in leukaemia therapy.

Plants have been used medicinally by mankind for centuries. Indeed, extracts from the *Narcissus* genus of *Amaryllidaceae* plants were documented to have been used as cancer therapy by Hippocrates in the 4th century BC (Evidente et al., 2009). In recent decades, the therapeutic use of many plant derived compounds has been investigated by the scientific community. From such studies many plant derived compounds have been implicated for their use as anti-leukaemia therapies. These include: PEP005, derived from the plant *Euphorbia peplus* in AML (Hampson et al., 2005); jasmonates, found in all plants, in AML, CLL and B cell lymphoma (Davies et al., 2009, Flescher, 2005, Flescher, 2007, Heyfets and Flescher, 2007, Rotem et al., 2005); and pancratistatin and lycorine from the *Amaryllidaceae* plant family in AML and APL (Liu et al., 2004, Liu et al., 2007, Liu et al., 2009).
1.20.1. Investigations into the use of Jasmonates.

The small hydrophobic plant stress hormones, jasmonates, have been investigated for their anti-neoplastic therapy in recent years. Jasmonate family members include jasmonic acid (JA) and methyl jasmonate (MeJ). MeJ in particular has been found to be selectively toxic towards leukaemic cells in the ALL cell line MOLT-4 (Flescher, 2005). The jasmonate family have been proven in animal models to prolong survival (Fingrut and Flescher, 2002) and in human B cell lymphoma line, carrying the p53 mutation, to be pro-apoptotic. Jasmonates have additionally been investigated in non-proliferating CLL cells (Rotem et al., 2005). In this setting, MeJ induced high levels of Δψm depolarisation and, therefore, Rotem et al (2005) have postulated that this direct damage to the mitochondria is the target of MeJ. Rotem et al have investigated this further with analysis of hexokinase, an enzyme bound to the external mitochondrial membrane, essential for the ATP-dependant phosphorylation of glucose to glucose-6-phosphate in glycolysis. Hexokinase is typically overexpressed in cancer cells and it has been shown that MeJ is able to detach mitochondrial bound hexokinase, thereby dramatically reducing the glycolytic rate of the cell. This was demonstrated in primary murine colon cancer, melanoma and B cell lymphoma and human Molt-4 cell lines (Goldin et al., 2008). However, JA was unable to exert the same activity and, therefore, it has been suggested that the jasmonate family exert their actives using another, yet unknown, mitochondrial mechanism (Goldin et al., 2008).

1.20.2. The induction of apoptosis by lycorine.

Lycorine is the most abundant alkaloid of the Amaryllidaceae family and has wide ranging biological activities. Studies this century have indicated that lycorine interferes with
replication of the polio, small pox and SARS viruses (Hwang et al., 2008, Deng et al., 2007, Li et al., 2005), has anti-fungal (Mackey et al., 2006) and anti-parasitic activities, including against malaria (Sener et al., 2003). In the last decade research has begun to investigate the potential use of this compound to treat leukaemia (Evidente et al., 2009, Li et al., 2005, Liu et al., 2004, Liu et al., 2007, Liu et al., 2009) although, to date, no studies have investigated its use in the treatment of CLL.

**In vitro** studies of lycorine against APL and AML cells have shown it to induce apoptosis. This has been shown to be attributable to the rapid down regulation of the anti-apoptotic protein MCL-1 (Liu et al., 2009) and subsequent caspase activation and cell cycle arrest (Li et al., 2007). Additionally, lycorine has been shown to be specifically targeted to malignant cells. In an HL-60 xenograft model (Liu et al., 2007) lycorine successfully reduced the tumour load without exerting any reported adverse effects. It should be noted, however, that published results of this study only just reach significance.

**1.2.0.3. Structural significance of Lycorine.**

A recent publication in 2009 has shown that for lycorine to induce apoptosis, the free ring-C 1,2-diol is required (McNulty et al., 2009); selective mono-silylation of the C-2 hydroxyl group, rendered lycorine biologically inactive. It has been hypothesised that owing to the structural similarity of lycorine (shown in section 2.7; table 6) to pancratistatin, another Amaryllidaceae alkaloid, they may exert their activities via the same receptor (McNulty et al., 2009). Certainly studies have shown that both alkaloids destabilise mitochondria (McLachlan et al., 2005, Evidente et al., 2009). Nevertheless, further studies are required to firmly establish the mechanism of action of lycorine.
1.20.4. **Lycorine and the mitochondria.**

Investigations in *Saccharomyces cerevisiae* have demonstrated that cells lacking mtDNA (Davey et al., 1998) are unaffected by lycorine, whilst wild type (WT) cells are sensitive to the compound. *S. cerevisiae* are able to survive in the absence of mtDNA by retrograde (RTG) signalling, in which some nuclear genes are altered to allow the cells to compensate for the resultant deficiency in respiration. This signalling is mediated via RTG genes. In 2005 it was shown that *S. Cerevisiae*, expressing null alleles of RTG genes, recapitulated the inhibition of growth observed in the lycorine treated WT cells. It is possible that, as mtDNA is easily damaged by ROS, which in turn renders the Krebs cycle inoperable (Skulachev, 2009), this is a mechanism of lycorine that would explain its lack of activity in cells without mtDNA. In order to establish the mechanism of lycorine signalling, eukaryotic models should be investigated further.

Despite the mechanistic action of lycorine being unknown, interest is growing in the use of this compound as therapy. In 2009, Evidente et al. (2009) identified lycorine as a novel lead for anticancer drug design and Jones et al. (2009) have published the first successful study of its synthesis.

1.21. **Aims of this study.**

The central concept of this study was to determine whether the anti-tumour activities of BEZ and MPA observed by this group against BL (Fenton et al., 2003) can be successfully recapitulated in CLL cells. In addition to investigating the effects of BEZ and MPA against peripheral CLL cells, the study also investigated their ability to overcome the protective effects of CD40L on CLL cells. In the studies of these agents against BL, apoptosis was readily observed. In addition to investigating BEZ and MPA, this study
also examined the anti-tumour activities of other promising agents *in vitro* and focused on DSN (based on recent publications) and lycorine.

A central component of the studies herein has been the assessment of variable apoptosis between patient samples in response to the drugs alone, in combination and in the presence of current and potential therapeutics. Apoptosis can be measured *in vitro* by several techniques such as the Δψm marker 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1), fragmented DNA and the binding of annexin V (AV). Since the measurement of apoptosis by AV has been met with some controversy it is worth considering here. AV is a PL binding protein that has a high affinity for phosphatidyl serine (PS) that becomes exposed on the outer membrane during the early stages of apoptosis. However, it has been noted that B cells that bind AV do not always go on to die (Holder et al., 2006). Despite this, a study by Pepper *et al* into flow cytometric analysis of CLL cell apoptosis identified that, providing events are gated with caution, AV could sensitively distinguish between live and apoptotic cells (Pepper *et al.*, 1998). In accord with this, many studies of CLL apoptosis utilise AV binding. In this thesis, AV binding together with the uptake of propidium iodide (PI) has been extensively used, although the other markers were also used to confirm whether AV binding correlated with cell death.

In the studies herein it is also demonstrated that CLL cells engaged with CD40L, using an *in vitro* system, undergo proliferation and protection. The ability of BEZ and MPA to induce apoptosis in this system and reduce proliferation, by measuring the incorporation of tritiated (³H) thymidine (³H- thymidine) is investigated. Further, the claims that DSN can overcome CD40L promoted survival are investigated and compared with treatment of
cells with BEZ+MPA+lycorine. It is shown that the induced apoptotic cell death of BEZ and MPA of non-CD40L treated CLL cells is associated with the generation of MSO. In contrast, the failure of these drugs to induce apoptosis in CD40L-protected cells was reciprocally associated with a failure to induce MSO accumulation. The ability of lycorine to reinstate this MSO response is investigated.
2. Materials & Methods
Unless stated otherwise, all chemicals were purchased from Sigma (Poole, U.K.) and all FC analyses were conducted using a FACSCalibur and Cell QuestPro software (BD, Oxford, U.K.).

2.1. Patients’ and donors’ cells.

Unselected patients with B-cell CLL attending the outpatient clinic at Birmingham Heartlands Hospital were recruited to the study. The patients had been diagnosed according to standard morphologic, immunophenotypic and clinical criteria (Oscier et al., 2004) and had provided informed written consent for the study which had received local ethical approval. Normal donors were recruited following informed consent.

2.1.1. Primary Cell purification.

Blood samples were diluted 1 to 1 in RPMI (Invitrogen Gibco, Paisley, U.K.) supplemented 100U/ml penicillin, 100µg/ml streptomycin (Invitrogen Gibco). Leucosep tubes (Greiner Bio-one, Gloucester, U.K.), which have an inbuilt filter, were loaded with 15ml ficoll paque plus (G.E Healthcare, Amersham, U.K.) and centrifuged at 1000xg for 1 minute, to force the liquid below the filter. The diluted blood was poured onto the leucosep filter prior to centrifugation at 1000xg for 10 minutes without the brake. The mononuclear cells (MNCs) were visible as a band above the filter and were carefully pipetted, diluted 1:3 in RPMI (as above) and centrifuged at 374 x g for 10 minutes. The MNCs were subsequently washed twice more in RPMI prior to counting using a haemocytometer.

2.1.2. Isolation of CD19+ve cells.

For experiments using CD19+ve cells, MNCs were isolated as above (section 2.1.2) and washed three times in RPMI medium. 2x10⁷ cells were washed in cold MACS buffer (see
buffers and recipes appendix) by centrifugation at 374 x g for 5 minutes. The cell pellet was resuspended in 320µl cold MACS buffer and 80µl anti-CD19 MACS beads (Miltenyi Biotec, Surrey, U.K), prior to incubation for 15 minutes at 4ºC. The cell suspension was then centrifuged as previous and the pellet resuspended in 0.5ml MACS buffer. The cells were positively selected using a MACS column LS (Miltenyi Biotec) that was placed in the magnetic field of a MACS separator magnet (Miltenyi Biotec) and washed using 2ml MACS buffer. The cell suspension was then added to the column and the negative fraction discarded. The column was washed three times with 1ml MACS buffer, prior to removal from the magnet. 5ml MACS buffer was added to the column, the plunger firmly applied and the positive fraction collected in a 15ml tube. The positive fraction was counted and the purity assessed by staining an aliquot with anti-CD19 antibodies and analysis using flow cytometry (see section 2.2.). The remaining cells were pelleted, prior to resuspension at an appropriate density for experimentation.

2.2. **Analysis of cell phenotype using flow cytometry (FC)**

Following positive selection of CD19 cells, or where noted in the text, cell expression of phenotypic markers was carried out, using individual markers or a panel of markers from shown in table 3. In each case, appropriate fluorophore isotypes were used to account for non-specific binding (BD, Oxford, U.K.).

Staining was carried out on 3x10^5 cells made up to 200 µl final volume in phosphate buffered saline (PBS) (Invitrogen Gibco) and incubation with 2µl the appropriate antibodies at room temperature (RT) in the dark. Cells were subsequently washed in 2ml PBS by centrifugation at 374 x g for 5 minutes, the supernatant was removed and the cells
resuspended in 300µl FACS fix (see buffers and recipes appendix). This staining was analysed within two weeks.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Flurophore</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-CD19</td>
<td>PerCP</td>
<td>BD</td>
</tr>
<tr>
<td>Anti-CD7</td>
<td>FITC</td>
<td>BD</td>
</tr>
<tr>
<td>Anti-CD5</td>
<td>PE</td>
<td>BD</td>
</tr>
<tr>
<td>Anti-CD40</td>
<td>FITC</td>
<td>BD</td>
</tr>
<tr>
<td>Anti-CD154</td>
<td>PE</td>
<td>BD</td>
</tr>
<tr>
<td>Anti-CD11b</td>
<td>FITC</td>
<td>AbD SeroTec, Oxford, U.K.</td>
</tr>
<tr>
<td>Anti-CD34</td>
<td>APC</td>
<td>BD</td>
</tr>
</tbody>
</table>

Table 3. Details of antibodies used for phenotyping.
2.3. Maintenance of leukaemia cell lines.

The ALL cell lines REH and Nalm6 were a kind gift from Dr Pamela Kearns (University of Birmingham). The AML cell lines HL-60, KG1a and K562 were obtained from the Health Protection Agency cell bank. All the cell lines were maintained in: RPMI 1640 (Invitrogen, Gibco) supplemented with 100U/ml penicillin, 100µg/ml streptomycin and 10% v/v foetal bovine serum (FBS)(all Invitrogen Gibco), from this point termed compete medium. Cultures were maintained at 37°C with 5% CO₂. Cells were diluted 1 part in 3 parts when appropriate to 2.54x10⁵ cells/ml, to maintain cells in exponential growth.

2.4. L3-L-cells (stromal cells).

2.4.1. Maintenance of L3-L cells.

Murine L3- L cells, stably transfected with plasmids encoding CD40吕 (CD40吕 -L cells), (Gagro et al., 2000) as well as non-transfected L cells were a kind gift from Prof. John Gordon (University of Birmingham, U.K.) and were maintained at 37°C with 5% CO₂ in complete medium. When 80-90% confluent, as assessed by microscopy, the media was removed, the cell monolayer washed with 10ml warm PBS and 2ml Trypsin EDTA added (Invitrogen Gibco). Following incubation for 5 minutes, the now detached cells were repeatedly pipetted to produce a single cell suspension and 8 ml of media added. 1ml of this cell suspension was left behind with the addition of 10ml fresh RPMI. The cells were routinely analysed for the expression of CD40吕, according to section 2.2. A representative plot of non-transfected L-cells and CD40吕-transfected L cells is shown in figure 3.
Figure 3. Example of analysis of expression of CD40L by L-control and CD40L transfected stromal cells. 3X10^5 cells were incubated with 2μL anti-CD40L (PE) and anti-CD40 (FITC) for 15 minutes in the dark, prior to analysis by FC. Plots shown are representative. a) dot plots for L-control (right) and L-40 (left) stroma, CD40L is shown in FL-2 and CD40 in FL-1. b) histogram overlay of expression of CD40L by L-control (purple) and L-40 (pink) stroma.
2.4.2. Storage and recovery of cell lines in liquid nitrogen.

Unless described differently in the text, 1x10^7 cells were harvested by centrifugation at 374 x g for 5 minutes. The supernatant was discarded, the cell pellet resuspended in 1ml freezing mix (see buffers and recipes appendix) and the mix transferred to a cryovial. Cells were slowly frozen by placing the vials in a sealed polystyrene container and incubating this at -20ºC for 60 minutes and -80ºC overnight and stored in liquid nitrogen thereafter.

To re-establish a culture, a frozen vial was thawed rapidly in hot water for 2-3 minutes. Cells were transferred to a 25ml universal and 0.5ml of cold complete medium added drop wise. The cell suspension was allowed to stand for 1 minute before repeating this with 1, 2, 5 and 10ml. This method gradually dilutes the freezing mix and prevents cellular apoptotic shock. Subsequently, the cell suspension was centrifuged at 374 x g for 5 minutes and the supernatant discarded. The cell pellet was resuspended as described above for L-cells or for leukaemia cell lines in 2ml cold media, transferred to a 24 well plate and incubated at 37ºC with 5% CO₂ until the cells had recovered sufficiently to be transferred into a 25cm² flask and maintained as before.

2.4.3. Mitomycin C treatment of L-cells.

Complete medium, containing 0.02M Mitomycin C (MC) (Sigma, Poole, U.K.) was prepared. When L cells were 100% confluent, assessed by microscopy, the media was removed and replaced with the 0.02M MC media. Cells were incubated for 3 hours, the monolayer washed 3 times in warm PBS (as before), trypsinised (as before) as above and counted. Aliquots of 5x10^6 cells were frozen as described in section 2.4.2, to ensure that any remaining MC was destroyed.
2.5. Co-culture of primary cells.

2.5.1. Co-culture of L-cells with CLL cells or CD19 sorted cells.

L-cells either with or without CD40L were thawed from -80°C as described previously. Cells were counted and resuspended at 1x10^5 cells/ml in complete medium. L-cells were seeded in plates according to table 4. For 96 well plates, only the inner wells were used, leaving the outer edges empty. These wells were filled with 200µl PBS as a humidity barrier. Cells were allowed to attach over night at 37°C with 5% CO₂.

MNC, CLL cells or CD19⁺ cells were diluted to 1x10⁶cells/ml in complete medium with the addition of 4x1ng/ml IL-4 (R&D systems, Oxford, U.K.) and seeded onto the stromal L-cells, according to table 5 and were allowed to settle over night at 37°C with 5% CO₂.

<table>
<thead>
<tr>
<th>Number of wells/plate</th>
<th>Cell volume (ml)</th>
<th>Cell number</th>
</tr>
</thead>
<tbody>
<tr>
<td>96</td>
<td>0.1</td>
<td>1x10⁴</td>
</tr>
<tr>
<td>12</td>
<td>1</td>
<td>1x10⁵</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>2x10⁵</td>
</tr>
</tbody>
</table>

Table 4. Seeding density of L-cells in different plate sizes.

<table>
<thead>
<tr>
<th>Number of wells/plate</th>
<th>Cell volume (ml)</th>
<th>Cell number</th>
</tr>
</thead>
<tbody>
<tr>
<td>96</td>
<td>0.05</td>
<td>1x10⁵</td>
</tr>
<tr>
<td>12</td>
<td>0.5</td>
<td>1x10⁶</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>2x10⁶</td>
</tr>
</tbody>
</table>

Table 5. Seeding density of CLL cells in different plate sizes
2.5.2. Removal of cells from stromal support.

Prior to analysis, the cells were removed from the stromal cells by repeated pipetting, creating a vortex using a P200 for 96 wells and a P5000 for larger wells. To ensure that all the patient cells had been recovered, that the stroma was still intact, and that no CLL cells were under the stromal surface, all plates were assessed using phase-contrast microscopy.

2.6. Cell line seeding for treatments.

REH, Nalm6, HL-60, KG1a and K562 were maintained as in section 2.5. For experimentation, cells were counted and REH and Nalm6 cells diluted to 1x10⁶/ml, HL-60, KG1a and K562 diluted to 2.5x10⁵/ml, in supplemented RPMI. REH and Nalm6 were and seeded at 100µl (1x10⁵) cells/well. HL-60, KG1a and K562 were seeded in flasks at a minimum of 4ml/flask for Fe cylinder experiments and 10ml/flask for ³H-PGD₂ experiments. Cells were treated as described below (section 2.7) and were analysed as denoted in the text.

2.7. Treatments.

2.7.1. Agent stocks.

Concentrated stocks of each agent were made according to table 6 and stored at -20°C.
<table>
<thead>
<tr>
<th>Agent</th>
<th>Stock concentration</th>
<th>Solvent</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>BEZ</td>
<td>0.5M (1000X)</td>
<td>DMSO</td>
<td>[Image]</td>
</tr>
<tr>
<td>MPA</td>
<td>5mM (1000X)</td>
<td>Ethanol</td>
<td>[Image]</td>
</tr>
<tr>
<td>Chlorambucil</td>
<td>10mM (10000X)</td>
<td>Ethanol</td>
<td>[Image]</td>
</tr>
<tr>
<td>15dΔ\textsuperscript{12,14}PGJ\textsubscript{2}</td>
<td>35mM (1000X)</td>
<td>DMSO</td>
<td>[Image]</td>
</tr>
<tr>
<td>PGD\textsubscript{2}</td>
<td>50mM (1000X)</td>
<td>DMSO</td>
<td>[Image]</td>
</tr>
<tr>
<td>GW 9662 (Cayman Chemicals, Michigan, U.S.A)</td>
<td>1mM (1000X)</td>
<td>DMSO</td>
<td>N/A-</td>
</tr>
<tr>
<td>BW 868C (Cayman Chemicals)</td>
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<td>Ethanol</td>
<td>N/A-</td>
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<tr>
<td>MFE</td>
<td>1mM</td>
<td>Ethanol</td>
<td>[Image]</td>
</tr>
<tr>
<td>PRD</td>
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<td>[Image]</td>
</tr>
<tr>
<td>ML-PRD</td>
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<tr>
<td>Hydrocortisone</td>
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<td>Ethanol</td>
<td>[Image]</td>
</tr>
<tr>
<td>Compound</td>
<td>Concentration</td>
<td>Carrier Solvent</td>
<td>Chemical Structure</td>
</tr>
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<td>---------------</td>
<td>----------------</td>
<td>-------------------</td>
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<tr>
<td>IDM</td>
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<td>DMSO</td>
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<tr>
<td>FLA</td>
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<td>Ethanol</td>
<td><img src="image2.png" alt="Image" /></td>
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<tr>
<td>Fe Cylinder (University of Birmingham)</td>
<td>25mM</td>
<td>RPMI</td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
<tr>
<td>JA</td>
<td>1M (1000x)</td>
<td>Ethanol</td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
<tr>
<td>MeJ</td>
<td>1M (1000x)</td>
<td>Ethanol</td>
<td><img src="image5.png" alt="Image" /></td>
</tr>
<tr>
<td>Lycorine</td>
<td>10mM (10000X)</td>
<td>Ethanol</td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>zVADfmk (Southampton, U.K.)</td>
<td>50mM</td>
<td>DMSO</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table 6. **Compounds used experimentally to treat CLL cells.** Table shows concentration of stock, carrier solvent and chemical structure where appropriate.
2.7.2. **Cell treatments.**

For 96-well plate experiments stocks were diluted to a working concentration of 4x in complete medium for CLL and purified CD19 cultures and 2 x for ALL cell lines. The working stocks were added to the wells in 50µL and 100µl volumes for CLL/purified CD19 cultures and CLL/ALL cell lines respectively. Following this final addition to CLL cell cultures, the IL-4 concentration was now 1ng/ml. For larger plate or flask experiments, the appropriate drug treatments were added directly. For PGD$_2$ receptor antagonist experiments, cells were pre-treated overnight with 100nM BW 868C. All treatments were set in triplicate. Controls in every case were set up and denotes solvent control, containing the highest volume of carrier solvent used in the corresponding treatments.

2.8. **Assessment of cell proliferation and viability.**

2.8.1. **Thymidine incorporation assay.**

$^3$H-thymidine (G.E. Healthcare) was diluted to 20µCi/ml in RPMI supplemented as above and stored at 4°C. Cells were incubated in 96-well plates with treatments for 60 hours prior to the addition of 20µl (0.4µCi ) and culture for a final 12-16 hours. Subsequently, the plate was stored at -20°C. Samples were harvested to a filter mat (Wallac, Massachusetts, USA) using a Skatron cell harvester (Skatron Instruments, Bath, U.K.) and readings taken using a Beta-Plate scintillation counter (Skatron Instruments). The mean thymidine incorporation of three treatments and the standard error of the mean (s.e.m.) calculated.
2.8.2. Cellularity/cell titre blue assay.

At day 5, 20 µL cell titre blue cell viability assay reagent (Promega) was added to each well of a 96-well plate. Following mixing, the plate was incubated for 4 hours at 37ºC, and subsequently, fluorescence was measured at an excitation of 530/25 nm and emission of 595/35 nm on a KC4 fluorometer. Fluorescence bears a direct relationship to cell viability in cultured cells. Background readings were assessed using wells containing medium alone, and these measurements were subtracted from readings obtained for samples. To account for differences in readings due to the different feeding regimens on days 2 and/or 4, results were adjusted accordingly, to account for cells that were removed from the cultures.

2.8.3. Cell cycle analysis.

An aliquot of 3x10⁵ cells were removed from the cell culture and made up to 1 ml in PBS (as before). Following centrifugation at 374 x g for 5 minutes the supernatant was removed and 500µL cell cycle buffer (see buffers and recipes appendix) added. Cell suspension was vortexed and stored, in the dark at 4ºC for 24 hours prior to analysis.

2.9. Analysis of cell death.

2.9.1. AV/PI staining.

Single AV and co-AV/PI positivity was assessed by FC following staining using an AV FITC kit (BD) on pooled triplicate wells, according to manufacturer’s instructions. Briefly, the cells were harvested and pooled in a FACS tube and 1ml cold PBS, containing Mg and Ca (Invitrogen, Gibco) added. Cells were centrifuged for 5 minutes at 374 x g. The supernatant was removed and cells resuspended in 100µl 1x AV binding buffer and 5µl
each of AV FITC antibody and PI stain, prior to incubation at RT in the dark for 15 minutes. Staining was analysed within one hour.

2.9.2. **Assessment of Δψm and Apoptosis by JC-1.**

The Δψm was measured by JC-1 (Sigma) and FC. The JC-1 was dissolved at 1mg/ml in DMSO (Sigma) and stored at -20°C. Following treatment, CLL cells from triplicate wells were transferred from the stroma, pooled and washed in 1ml warm PBS. Cells were centrifuged, supernatant removed and the cell pellet resuspended in 500 µl warm PBS containing 2.5µg/ml JC-1 and incubated for 30 minutes at RT. Staining was analysed within 1 hour.

2.9.3. **Assessment of Caspase cleavage.**

As a positive control, 1µM staurosporin (Sigma) treatment was carried out for 12 hours for each patient sample. Caspase cleavage was analysed using CaspaTag assay Kit (Millipore, Watford, U.K.), a pan-caspase *in situ* assay kit on pooled wells according to manufacturer’s instructions. Briefly, the methodology is based on cell permeable and non-cytotoxic, fluorochrome inhibitors of caspases (FLICA) and a sulforhodamine-labelled fluoromethyl ketone peptide inhibitor of caspase (SR-VAD-FMK), which produces a red fluorescence. On entering the cell, the FLICA binds covalently to the active caspase. The SR-VAD-FMK probe is then added and covalently binds to a reactive cysteine residue that resides on the large subunit of the active caspase heterodimer, thereby inhibiting further enzymatic activity. The bound labelled reagent is retained within the cell, while any unbound reagent will diffuse out of the cell and is washed away. The red fluorescent signal measured by FC is a measure of the amount of active caspase present in the cell at the time the reagent was added.
2.10. Investigations into oxidative stress.

2.10.1. Assessment of accumulation of Reactive Oxygen Species (ROS).
Carboxy-H$_2$DCFDA (H2DCFDA) (Invitrogen Molecular Probes, Paisley, U.K) binds to all ROS and was dissolved in DMSO to yield a 2000x stock, of 10µM. This was aliquoted into 10µl volumes and was stored under nitrogen at -20°C. Immediately prior to use, a 5µM working dilution was made in warm PBS (Invitrogen Gibco). The media in each well was carefully removed, without disturbing the cells and was replaced with 200µl of the working stock and incubated at 37°C for 40 minutes. Following incubation, appropriate wells were pooled into FACS tubes and immediately analysed.

2.10.2. Assessment of accumulation of Mitochondrial Superoxide (MSO).
MitoSOX Red (Invitrogen Molecular Probes, Paisley, U.K) was used to assess the presence of MSO on pooled triplicate wells, according to manufacturer’s instructions. Briefly, triplicate wells were pooled, 1ml of warm PBS added and centrifuged at 374 x g for 5 minutes. The supernatant was removed and a vial of MitoSOX Red was diluted in 13µl of DMSO to yield a 10mM stock. A working stock of 10µl MitoSOX Red was prepared in warm PBS (Invitrogen Gibco) and this was added to the cells, prior to incubation at 37°C for 10 minutes.

2.11. mRNA analysis: Quantitative real-time polymerase chain reaction (QRT-PCR).

2.11.1. RNA extraction.
RNA was extracted from a pellet of 1x10^6 cells using a Qiagen RNeasy mini kit (Qiagen, Crawley, U.K.) according to the manufacturer’s instructions. Briefly, cells were resuspended in 350µl buffer RLT (plus β-mercaptoethanol; Sigma). The sample was then homogenised using a QIAshredder spin column. One volume of 70% ethanol (Fischer
Loughborough, U.K.) was added to the homogenised sample and 700µl of this mixture added to an RNeasy mini column. The column was centrifuged for 15 seconds at 14,000 rpm in a microfuge. Further DNA removal was carried out using the RNase-free DNase set. 350µl buffer RW1 was added to the column and the column centrifuged for 15 seconds at 14,000 rpm in a microfuge. The DNase I stock solution was added to buffer RDD according to manufacturer’s instructions and 80µl of this mix added to the column and incubated for 15 minutes at room temperature. 350µl buffer RW1 was added to the column and the column centrifuged for 15 seconds at 14,000 rpm in a microfuge. Buffer RPE (500µl) was added to the column and centrifuged for 15 seconds at 14,000 rpm in a microfuge. Another 500µl RPE was applied to the column prior to centrifugation for 2 minutes at 14,000 rpm in a microfuge. The RNeasy column was transferred to a 1.5ml collection tube, RNA eluted with the addition of 30µl RNase-free water and centrifugation for 1 minute at 14,000 rpm in a microfuge, and the RNA stored at -20°C.

2.11.2. RNA quantification.

RNA samples were diluted 1 part to 50 parts RNase-free water (Invitrogen Gibco) in a total volume of 100µl. The absorbance at 260nm was measured at OD 260 and the RNA concentration calculated using the following equation:

RNA concentration (µg/µl) = (OD$_{260}$ x 40 x dilution factor)/1000.

2.11.3. Reverse transcription.

cDNA was produced from 100ng RNA using reverse transcription. Unless stated otherwise, all constituents were obtained from Invitrogen (Paisley, U.K.) and the procedure carried out as follows: 1µl of both random primers (Promega) and dNTPs (Bioline, London, U.K.) were added to 100ng RNA, the volume made up to 12µl with
DNase RNase-free water (Invitrogen Gibco), and the mix heated to 65°C for 5 mins, transferred to ice and centrifuged for 15 seconds at 14,000 rpm in a microfuge. A master mix was made as 1 x mix of the following: 1x buffer, 0.1M DTT, RNase Out (Promega) and Superscript: After centrifugation, 8 µl of master mix was added to the RNA, primer, dNTP mix. The mix was incubated at 25°C for 10 minutes, 42°C for 90 minutes and 70°C for 15 minutes in a thermocycler.

2.11.4. β-actin PCR.
To assess that the RT reaction had worked, PCR reactions for β-actin were performed.
The sequence of these primers is as follows:
Forward 5’ GTCACCAACTGGGACGACA  3’
Reverse 5’ TGGCCATCTCTTGCTCGAA  3’
The 1x reaction mix was set up as follows: Taq buffer, primers (33 µM), dNTP’s (10 mM), MgCl₂ (50 mM), Taq polymerase, cDNA and DNase RNase-free water to 50 µL. The PCR cycle included an initial denaturation step (95°C for 2 minutes) followed by 38 cycles of 94°C for 20 seconds, 55°C for 30 seconds and 72°C for 60 seconds, followed by a final incubation at 72°C for 5 minutes. Subsequently, 6 µl was mixed with 2 µl 10x DNA gel loading buffer (Bioline), loaded onto a 1% agarose gel and electrophoresed in 1xTBE (see Appendix A1) at 60 V for 45 minutes. β-actin products were visualised under UV transillumination.

2.11.5. Real-time PCR.

2.11.5.1. Measurement of AKR1C gene expression.
Reactions were performed using an ABI Prism 7700 sequence detector (Applied Biosystems) using an 18S ribosomal RNA internal standard kit. Gene specific primers
were synthesized by Alta Bioscience (University of Birmingham) for AKR1C3 or Sigma Genosys (Sigma, Dorset, U.K.) for AKR1C1, 1C2 and 1C4, and all probes were synthesized by Eurogentec (Southampton, U.K.). Thermocycler conditions were 50°C for 2 minutes, 95°C for 10 minutes, followed by 44 cycles of 95°C for 15 seconds and 60°C for 1 minute. Each AKR1C reaction contained 900 nM gene specific 5' and 3' primers, 1X MasterMix (containing pre-optimized dNTPs, MgCl₂ and buffer concentrations; (Applied Biosystems, Warrington, U.K.), 125nM gene specific probe (5’-6-FAM, 3’-TAMRA labelled) and cDNA (1 µl) in a total volume of 20 µl with dH₂O.

Control 18 S reactions contained 50nM 18S 5’ and 3’ primers, 200nM 18S probe (5’-VIC, 3’-TAMRA labelled) (Applied Biosystems) and cDNA in a total volume of 20 µl. Reactions were performed in triplicate.

AKR1C3 primers and probes were designed and optimised for use by Dr. Farhat Khanim and are shown in table 7.
<table>
<thead>
<tr>
<th>AKR1C</th>
<th>Forward sequence 5’</th>
<th>Reverse sequence 5’</th>
<th>Probe. 5’-FAM</th>
<th>Product (base pairs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CCTAAAAGTAAAG</td>
<td>GAAAATGAATAA</td>
<td>CGGAAGCCAGCTT</td>
<td>268</td>
</tr>
<tr>
<td></td>
<td>CTTTAGAGGCCACC</td>
<td>GGTAGAGGTCAACATAAT</td>
<td>CAATTGCC-TAMRA</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>CCTAAAAGTAAAG</td>
<td>GAAAATGAATAA</td>
<td>GGCAATAGAAGCC</td>
<td>258</td>
</tr>
<tr>
<td></td>
<td>CTCTAGAGGCCGT</td>
<td>GATAGAGGTCAACATAG;</td>
<td>GGGTCCAC-TAMRA</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>GGGATCTCAACGA</td>
<td>AAAGGACTGGGT</td>
<td>TGGACCCGAACCTC</td>
<td>268</td>
</tr>
<tr>
<td></td>
<td>GACAAACG</td>
<td>CCTCCAAGA</td>
<td>CCCCGGTG-TAMRA</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>CGAGGAAACAGAGC</td>
<td>GAGAGCCATTGG</td>
<td>CGGAAGCCAGCTT</td>
<td>277</td>
</tr>
<tr>
<td></td>
<td>TGTAGAGGTCAC</td>
<td>GAAATGAAGA</td>
<td>CTATTGCTAATTTLG-TAMRA</td>
<td></td>
</tr>
</tbody>
</table>

Table 7. Sequences of primers and probes used for QRT-PCR.
2.11.5.2. **Q-PCR data analysis.**

Q-PCR data was first analysed using ABI Prism 7000 software (Applied Biosystems) according to manufacturer’s guidelines. Briefly, cycle threshold (CT) values were determined for both 18S internal control and gene of interest in each sample by placing a threshold line over the exponential phase of the PCR cycle profiles. The average CT values were calculated from the duplicates. The 18S internal control value was then subtracted from the value for the gene of interest to give ΔCT values. K562 was analysed in parallel as a positive control. The K562 ΔCT value was subtracted from sample ΔCT values to give ΔΔCT values. This value was converted to fold change in gene expression relative to control using the equation: fold change=2^{-ΔΔCT}, and fold change converted to percentage expression relative to control via multiplication by 100. The average and standard error of the mean of samples was calculated.

2.12. **Prostaglandin D\(_2\) Enzyme-linked immunosorbent assay (ELISA).**

Cells were cultured as described in section 2.5. and treated in 6 well plates as described in section 2.7. for 6 hours. Subsequently, cells were harvested along with 1ml of supernatant removed prior to centrifugation. Cell pellets were resuspended in appropriate retained medium followed by homogenisation using a Precellys 24 ceramic bead based homogenisation system (Omni International). Cell homogenates were incubated on ice for 5 minutes with 1ml ice cold acetone prior to centrifugation at 3000xg for 20 minutes. Supernatant was removed and kept on ice. A further 0.5ml ice cold acetone was added to homogenised pellet followed by further centrifugation. The subsequent supernatant was pooled with that from the first centrifugation step and kept at - 80ºC until use. PGs were extracted using C18 reverse phase extraction columns (Chromabond) and all assay
specific reagents for PG D₂-MOX EIA kit (Cayman Chemicals) were prepared and PG levels determined according to manufacturer’s instructions.

2.13. Analysis of PGD₂ and DHT metabolism by thin layer chromatography (TLC).

If pre-treatment was required, as denoted in the text, cells were seeded as described in section 2.5, and treated as described in section 2.7, overnight. Subsequently, cells were cultured as 200µl cultures in 96 well plates at 2x10⁶ cells/well (1x10⁷ for experiments analysing cells only) in PBS (Invitrogen Gibco), with 0.2µCi of ³H-PGD₂ (Amersham) for 9-18 hours. Following incubation, the suspension was centrifuged at 14,000 rpm using a microfuge. The cell pellet and supernatant were stored separately at -20ºC.

2.13.2. Extraction and visualisation of prostanoids.
Prostanoids were extracted from the supernatant by: the addition of 0.5ml methanol (Fischer) and 2.5µL 10%(v/v) formic acid (Sigma) and vortexing, before the addition of 1ml of chloroform and 50µl 4.2% (w/v) KCL (Sigma) and from cell pellets by: resuspension in 1 ml 50% methanol and vigorous vortexing prior to the addition of 2.5µL 10%(v/v) and vortexing for a further 2 minutes. The cell suspension was pelleted at 14,000 rpm in a microfuge, for 2 minutes and the supernatant transferred to borosilicate glass culture tubes. To either the supernatant or the cellular extract mix, 2ml dichloromethane (Fischer) and 100µl 4.2% (v/v) KCL (Sigma) was added and vortexed for 3 minutes. The mixed suspensions were then centrifuged at 374xg for 15 minutes. The proteinaceous and aqueous layers were removed by aspiration and discarded. The remaining sterol-containing fraction was evaporated to dryness under a stream of nitrogen prior to being re-dissolved in 70µl methanol and applied to Silica gel TLC plates (Fluka). A solvent
mixture of 90:8:1:0.8 chloroform, methanol, glacial acetic acid (all Sigma) and distilled water was prepared and left to equilibrate in TLC tanks. The prostanoids were separated by incubation in the tank for 3 hours and, once dry, were read on a Bioscan AR-2000 plate reader. The migration of prostanoids was identified by their co-migration with known standards as revealed by incubation in iodine vapour.

2.13.3. Cell incubation with $^{3}$H-5α-DHT.
To measure steroid activity, cells were seeded in 24 well plates, according to table 5 with the addition of 1µCi $^{3}$H- 5α-DHT (Amersham) and incubation of 18 hours. Following incubation, cell supernatants were harvested and stored at -20ºC.

2.13.4. Extraction and visualisation of sterols.
To extract sterols, the supernatants were thawed, transferred to borosilicate glass culture tubes and 5ml of dichloromethane (Fisher Scientific) added. The mixture was vortexed for 5 minutes prior to centrifugation at 374g for 15 minutes. The resulting aqueous and proteinaceous layers were removed by aspiration and discarded. The remaining, sterol containing chloroform fraction was evaporated to dryness under a stream of air at 55ºC. The sterols were re-dissolved in 80µl of dichloromethane and applied to Silica gel TLC plates (Fluka). A solvent mixture of 1:4, n-hezane:hexanol (both Fischer) was prepared and left to equilibrate in sealed TLC tanks. The sterols were separated by incubation in the tank for 3 hours, prior to being read on a Bioscan AR-2000 plate reader.


2.14.1. Protein extraction and quantification.
For protein extraction, 1x10⁶ cells were resuspended in 50 µl RIPA buffer (see Appendix A1), supplemented with 1X protease inhibitor (Sigma), and incubated for 30 minutes on
ice prior to centrifugation at 14,000rpm at 4°C for 10 minutes in a microfuge. The supernatant was transferred to a 1.5ml centrifuge tube and frozen at -20°C.

For protein quantification, the D\textsubscript{c} protein assay protocol (Bio-Rad, Hemel Hempstead, U.K.) was followed according to manufacturer’s instructions. Briefly, 5µl BSA standards (0, 0.625, 1.25, 2.5, 5 and 10mg/ml) were added to duplicate wells of a 96 well plate and 2µl of each protein sample was added to 3µl distilled water in replicate wells. 20µl reagent S was added to each ml of reagent A required to make solution ‘A’, and 25µl A’ added to each well. 200µl reagent B was then added to each well, the wells mixed, and the reaction allowed to develop for 15 minutes before the optical density was measured at 645nm using a plate reader.


Protein samples (20-40µg) were mixed in a 3:1 ratio with 4x SDS gel loading buffer (see buffer and recipes appendix), heated to 100°C for 10 minutes in boiling water. A 10% resolving gel mix was prepared (see Appendix A1) and allowed to fully polymerise at RT for 40 minutes prior to the addition of the stacking gel; prepared according to the Appendix A1.

Protein samples, and 5µl pre-stained precision dual stained markers (Bio-Rad) were loaded onto the gel and electrophoresed at 150V with 1x SDS gel running buffer for 90 minutes.

2.14.3. Protein transfer.

PVDF membrane (Millipore, Watford, U.K.) was soaked in methanol (Fischer) for 15 seconds, dH\textsubscript{2}O for 2 minutes and equilibrated in transfer buffer (see Appendix A1) for 10 minutes. Semi-dry transfer was carried out using four layers of pre-transfer buffer-soaked
3mm paper (Fischer) on the cathode and anode and the transfer was carried out using a Mini-Protean transfer tank (Bio-Rad) at 25V for 1 hour.


The membrane was rinsed in TBS-T (see buffer and recipes appendix) and then blocked for 45 minutes in 5% blocking solution (see Appendix A1). Primary mouse anti-human AKR1C3 antibody (Sigma) was diluted 1 in 1000 in 5% blocking solution and the membrane incubated overnight at 4°C with rocking. The membrane was washed three times for 5 minutes in TBS-T, the secondary antibody (horseradish peroxidase (HRP)) conjugated rabbit anti-mouse, (Sigma) diluted 1 in 1000 in 5% blocking solution and the membrane incubated for 45 minutes in this solution at RT, with rocking. The membrane was washed as above and signal developed using Supersignal West Pico Chemiluminescent substrate (Pierce, Northumberland, U.K.) and signal detected by exposure to Xomat scientific imaging film (Kodak, Sigma) for 5 minutes. Films were developed using an AGFA CURIX 60 (Agfa, Mortsel, Belgium). Equal loading was checked using mouse anti-human β-actin antibody (Sigma) and secondary rabbit anti-mouse following the same protocol (dilutions 1 in 10,000 for each).

2.15. Statistical analysis.

Data are reported as mean values and their standard deviation (s.d.) or standard error of the mean (s.e.m.) as stated.

2.15.1. Statistical analysis of patients’ drug responses.

These statistics were carried out by Dr J Delgado at the University of Barcelona. Briefly, one-factor within subjects’ analysis of variance was used to assess drug responses. Homogeneity of variance was assessed by the Mauchly sphericity test. When data sets
significantly violated this requirement, the Greenhouse-Geissler test was used to calculate a more conservative P value for each F ratio. Partial eta-squared ($\eta^2_p$) was used to measure the size of the statistical association, and pair-wise comparisons were adjusted according to the Bonferroni method.

Several CLL prognostic factors were analysed for their association with \textit{in vitro} drug responses using the Mann-Whitney test. Drug responses were measured as drug/control ratios. CLL prognostic factors included Binet stage (A vs B/C), lymphocyte doubling time ($< vs >$ 12 months), ZAP-70 expression (negative vs positive), CD38 expression (negative vs positive), IgVH mutation status (mutated vs unmutated) and cytogenetics (normal vs abnormal). Statistical analyses were performed using SPSS, version 14.

2.15.2. \textit{Statistical analysis of experiments}.

Unless stated otherwise in the text, statistical analyses shown were calculated using the McNemar and Wilcoxon tests in SPSS, version 14.
3. Effects of BEZ & MPA on Peripheral & ‘PC’ CLL Cells
3.1. Introduction

This chapter describes the effects of BEZ, MPA and BEZ+MPA on both the quiescent peripheral and the proliferating CLL cells. The proliferative signal was generated using a stroma of L-cells stably transfected with CD40\(_L\), to mimic the PCs. For the quiescent counterparts, the CLL cells were also co-cultured with stromal L-cells but without expression of CD40\(_L\), providing a support but not driving proliferation. In both systems IL-4 was added (to a concentration of 1ng/ml). A schematic of this system was shown in figure 1.

The effects of BEZ and MPA, both individually and in combination, were assessed for the induction of apoptosis in both the absence and presence of CD40\(_L\), and for anti-proliferative activity when CD40\(_L\) was present. Having established the effectiveness of these agents against unpurified CLL cells, their ability to target malignant B cells, their effects on normal B-cells and their use, compared to the commonly used chemotherapeutic, chlorambucil, was investigated (Oscier et al., 2004).

All of the work presented in this chapter was published in 2009 by Macmillan Publishers Ltd. The full details of this manuscript can be found in Appendix A2.
3.2. The assessment of BEZ and MPA on quiescent CLL cells

3.2.1. BEZ and MPA induce apoptosis in non-proliferating CLL cells.

The previous study using BEZ and MPA, against BL cells (Fenton et al. 2003) utilised doses of 0.5mM BEZ and 5µM MPA. Therefore, these doses were chosen for initial experiments to test their effectiveness against CLL cells. Single agents and the combination of BEZ+MPA were used against CLL cells on non-CD40L expressing stroma and subsequently tested for the induction of apoptosis at 24 hours as measured by AV and PI co-incubation. Cells in the early stages of apoptosis will lose membrane asymmetry, which causes PS to be exposed on the cell surface to which AV can then bind (Koopman et al., 1994). In vivo this process signals for macrophages to remove the apoptosing cell. At later stages of cell death, the cells lose plasma membrane integrity, allowing dyes such as PI to accumulate (Ormerod et al., 1992). Although this would not normally occur in vivo until the cell has been phagocytised by a macrophage, in vitro cells incur membrane damage causing the accumulation of PI (Pepper et al., 1998). The response of a single CLL sample to BEZ, MPA and the combination, of BEZ+MPA, as measured by this assay are shown in figure 4. Both BEZ and MPA induced apoptosis but the combination generated greater apoptosis than either agent alone (figure 4). This data is commensurate with that of the BL study (Fento et al., 2002).
Figure 4. BEZ, MPA and BEZ+MPA treated cells have reduced viability, bind AV and accumulate PI in the absence of CD40. CLL cells were cultured on non-CD40 expressing stroma in a ratio of 10:1 in 96 well plates. Treatments of either solvent control, 0.5mM BEZ, 5µM MPA or BEZ+MPA were performed in triplicate for 24 hours, prior to harvesting and staining with AV and PI and analysis by FC. a) Lower left (LL) quadrant shows viable cells, lower right (LR) indicates cells in early stages of apoptosis, upper right (UR) is indicative of late apoptosis. Total AV staining was calculated by adding the events in the LR with those in the UR in further experiments. b) FSC/SSC analysis: gate indicates viable events. The plots are representative of n=>50 experiments.
However, as it has been reported that AV positive B cells do not always go on to die (Holder et al., 2006) further measures of apoptosis were employed. As cells become apoptotic they reduce in size and increase in granularity. This change can be detected by FC, by a shift from right to left in forward scatter (FSC) and bottom to top in side scatter (SSC). Such a shift is shown in figure 4; as the cells lose viability with treatment, the number of events visible in the viable drawn gate decrease. As a further measure, the Δψm marker, JC-1, was used as an additional marker of apoptosis in a subset of patients. Viable cells have high Δψm, in the presence of which JC-1 spontaneously forms aggregates detected by red fluorescence. In apoptotic cells, Δψm lowers; and the JC-1, stays as a monomer which is detected by green fluorescence (Green and Reed, 1998).

Figure 5 shows a clear induction of apoptosis by either agent but overall the combination of BEZ+MPA gives greater Δψm depolarisation. This data is in accordance with the PI and AV positivity and the remaining cell viability by FSC/SSC shown in figure 4, therefore, it was concluded that AV positivity reflects true apoptosis. Consequently, this assay was chosen as the main measure of apoptosis for further experiments. Total percentage of AV positivity was calculated by the addition of the AV positive PI negative events in the LR to those with dual AV and PI positivity in the UR (figure 4). This analysis portrays cells in both early and late stages of apoptosis.
Figure 5. Cells treated with BEZ, MPA and BEZ+MPA show Δψm depolarisation in the absence of CD40L. CLL cells were cultured on non-CD40L-expressing stroma in a ratio of 10:1 in 96 well plates. Treatments of either solvent control, 0.5mM BEZ, 5µM MPA or BEZ+MPA, were performed in triplicate for 24 hours, prior to harvesting and staining with JC-1 and analysis by FC. Plots were gated on FSC and SSC to show viable (R1) and non-viable (R2) regions. The regions are translated onto the FL-1/FL-2 plots. Events captured in the FL-2 region (red dots) indicate Δψm is preserved whereas those captured in the FL-1 channel (green dots) highlight aggregation of JC-1, indicative of a loss of Δψm. The plots shown are representative of n=10 patients.
3.2.2. **BEZ and MPA exert pro-apoptotic effects in a dose dependent manner.**

Whilst the doses shown in figures 4 and 5 show similarity with those of the BL study, a two-fold dose titration of the single agents was carried out to establish their effectiveness at lower and higher concentrations. Total AV positivity measured at 24 hours (figure 6) showed similar results to those observed in BL; apoptosis induced by MPA essentially plateaued beyond 5µM and a similar level of apoptosis was observed in response to 0.5mM BEZ. Subsequent experiments used 5µM MPA and 0.5mM BEZ either as single agents or in combination. The pertinence of these doses for potential in vivo treatment of CLL is addressed in chapter 6.

3.2.3. **Induction of CLL cell apoptosis by BEZ and MPA is time-dependent.**

The experiments described to this point were all analysed at 24 hours. To determine the time dependency of drug induced apoptosis, CLL cells from 4 patients were treated with solvent control or BEZ+MPA and analysed for total AV positivity and Δψm depolarisation at 7, 24, 48 and 72 hours (figure 7). It is important to note that this set of experiments was conducted using CLL cells that had previously been frozen and stored at -80°C. Upon thawing, the cells were plated immediately on to stroma and allowed to settle for a few hours before treatment. Analysis at 7 hours, showed a high level of apparent apoptosis in the solvent control treated cells, as measured by AV (figure 7, top graph, black circles). This level of apoptosis was not observed with the JC-1 staining. Furthermore, at 24 hours the levels of total AV positivity was reduced from a mean of 60% to 22%.
Figure 6. Dose titration of BEZ and MPA as single agents show induction of apoptosis at doses commensurate with those seen against BL cell lines. CLL cells were cultured on non-CD40 expressing stroma in a ratio of 10:1 in 96 well plates and were treated with solvent control and either MPA (top) or BEZ (bottom) in a 2-fold dose escalation (1.25µM-10 µM MPA and 0.125-1 mM BEZ) for 24 hours. Samples were analysed for the binding of AV and uptake of PI using FC. Data are the mean ± s.e.m. of n=7 patients.
Figure 7. The BEZ+MPA effect peaks at 24-48 hours. CLL cells thawed from -80°C were cultured on non-CD40L expressing stroma in a ratio of 10:1 in 96 well plates. Treatments of either solvent control or BEZ+MPA were performed in triplicate and were harvested and pooled at 7, 24, 48 and 72 hours and assessed for a) the binding of AV and uptake of PI and b) Δψm depolarisation using JC-1, and analysis by FC. Plots shown are the mean ± s.e.m. from n=4 patients.
Overall, this suggests that, at seven hours, the majority of freshly thawed CLL cells had PS exposed on the surface membrane but that they recovered and did not go on to die. The 24 hour time point, with the reduced total AV positivity, is more in line with the overall control apoptosis levels seen throughout the rest of the experiments and for this reason all further experiments did not utilise frozen stored cells. Compared to controls, at 24 hours, BEZ+MPA showed a high induction of apoptosis (mean values, 22% to 72% for total AV and 19% to 43% Δψm depolarisation). This level of apoptosis was not significantly different at 48 hours (mean values 19% to 81% total AV and 16% to 58% for Δψm depolarisation). These data (figure 7) therefore indicate that BEZ+MPA treatment induces apoptosis that peaks between 24 and 48 hours. For subsequent experiments, a time point of 24 hours post treatment was chosen for analysis of apoptosis.

3.2.4. **Analysis of 28 consecutively acquired and treated CLL cells show diverse responses.**

Figure 8 depicts the responses to BEZ, MPA and BEZ+MPA in the presence of control L-cells of 28 consecutively acquired CLL samples, the patient characteristic for which are shown in table 8. In the case of induced apoptosis, the box plot (figure 8) indicates significant increases caused by both drugs when used alone. However, the stronger response is the BEZ+MPA drug combination, commensurate with the plots shown in figures 4 and 5.
Figure 8. Patients have a variable response to BEZ, MPA and BEZ+MPA. Box plots are shown for n=28 consecutively acquired patients cells cultured on non-CD40L expressing stroma in a 10:1 ratio, and treated with solvent control, 5µM MPA, 0.5mM BEZ or BEZ+MPA for 24 hours and analysed for the binding of AV and uptake of PI using FC. Line in box indicates median value; bottom line and top line of each box show 25th and 75th centile respectively, the bottom and top whiskers correspond to 10th and 90th centile; circles represent outliers.
Table 8. Patient characteristics of 28 consecutively acquired samples. The factors shown are for patients’ samples shown in figures 8 and 19.
Detailed statistics were carried out on these results by statistician Dr J Delgado. These statistical tests showed that the drug effects were real and also genuinely large. Pair-wise comparisons identified that BEZ, MPA and BEZ+MPA increased apoptosis compared to controls ($P = 0.001$, $P = 0.002$ & $P < 0.001$ respectively). There was no overall difference in apoptosis induction either by BEZ or MPA ($P = 1$) but the combination was more effective than either drug alone (both $P < 0.001$). It is important to note that within these samples the responses displayed by individuals showed high variability (figure 9). Whilst some samples displayed responses that typified the summated response, depicted by the box plot, others seemed to show apparent synergy; with the individual drugs having minimal effects and the combination giving a large effect. In a few samples neither the single drugs nor the combination had much effect on apoptosis (figure 9).

3.2.5. Treatment of BEZ+MPA can induce caspase activation but when inhibited this does not prevent the cells from apoptosis.

Induction of apoptosis is tightly regulated. Caspases are proteolytic enzymes, involved in classical apoptosis. These enzymes participate in a series of reactions that result in the cleavage of protein substrates, causing the disassembly of the cell. Following the observations of BEZ+MPA inducing apoptosis in CLL cells, eight patient samples were investigated for drug induced activation of caspase cleavage.
Figure 9. Based on the patient’s response to BEZ and MPA, the BEZ+MPA response can be divided into four categories. Chronological plot of 17 patients cells cultured on non-CD40L expressing stroma in a 10:1 ratio and treated with solvent control, 5µM MPA, 0.5mM BEZ or BEZ+MPA, for 24 hours and analysed for the binding of AV and uptake of PI using FC. Coloured ovals highlight variability of response into 4 categories.
Staurosporin, a known inducer of caspase activation (Giuliano et al., 2004), was used at 1µM as a positive control in four of the patients analysed. CLL cells were treated as described in the preceding experiments and, subsequently, stained with CaspaTag that utilises SR-VAD-FMK, which recognises all activated caspases intracellularly as described in detail in section 2.9.3. Figure 10 shows four patients treated with solvent control, staurosporin or BEZ+MPA. Each patient showed variation in both staurosporin and BEZ+MPA induced caspase cleavage. In one sample, the cleaved caspase percentage was similar upon both staurosporin and BEZ+MPA treatment whilst in the others, staurosporin tended to be the stronger inducer of caspase activation. Overall, some degree of BEZ+MPA induced caspase was observed in 5 out of the 8 samples (figure 10).

In an attempt to demonstrate that the apoptosis observed as a result of BEZ+MPA treatment was due to caspase-dependent apoptosis, the pan-caspase inhibitor N-benzyloxycabonyl-Val-Ala-Asp-fluoromethylketone (zVADfmk) (King et al., 1998) was used, at a dose of 50µM. CLL cells were pre-treated with zVADfmk for 1 hour prior to treatment with BEZ, MPA or BEZ+MPA. Total AV positivity at 24 hours (figure 11) identified identical levels of apoptosis between cells treated with or without the caspase inhibitor.

Together, these data indicate that although caspase activation can be seen, overall the apoptosis induced by BEZ, MPA or BEZ+MPA may be independent of caspases.
Chapter Three - Effects of BEZ & MPA on Peripheral and ‘PC’ CLL Cells

Figure 10. Treatment with BEZ+MPA induces caspase cleavage in some but not all patient samples. CLL cells were cultured on non-CD40-L expressing stroma in a ratio of 10:1 in 96 well plates. Triplicate treatments of either solvent control (left; black fill), 1µM staurosporin (middle; green line) or BEZ+MPA (right; purple line) were harvested and pooled at 24 hours and incubated with CaspaTag Pan-Caspase In Situ Assay Kit (using SR-VAD-FMK), prior to being analysed by FC. The plots shown are representative of n=8 and show the observed variation in caspase cleavage.
Figure 11. Apoptosis induced by BEZ+MPA may be caspase independent. CLL cells were cultured on non-CD40L expressing stroma in a ratio of 10:1 in 96 well plates. Triplicate treatments of either solvent control, 0.5mM BEZ, 5µM MPA or BEZ+MPA, in the presence (purple fill) and absence (blue fill) of the caspase inhibitor 50µM zVADfmk were harvested and pooled at 24 hours and analysed for the binding of AV and uptake of PI using FC. Data are the mean ± s.e.m. from n=4 patients.
3.2.6. **BEZ+MPA pro-apoptotic effects are targeted at CLL cells.**

The data shown so far was generated using non-purified MNCs. However, other cells including non-malignant B cells, T cells, myeloid cells and natural killer (NK) cells will have been present. As one of the problems associated with established therapy for CLL is the reduction in healthy as well as malignant cells (Oscier et al., 2004) more selective therapies are desirable. In order to assess the selectivity of BEZ+MPA against the malignant cells, the effects of BEZ+MPA against both purified CLL cells and normal B cells were investigated.

First, CLL cells were positively selected for the B cell marker CD19. These CD19+ve CLL cells displayed basal levels of total AV positivity greater than in their unpurified counterparts (figure 12) (mean values, 52% compared to 24%). This may have been due to cellular stress during the purification process. This considered the apoptotic responses of CD19+ve cells to BEZ, MPA and BEZ+MPA was similar to that of non-purified cells. In the combination, the percentage of total AV was increased from 52% to 83% compared to 63% in the non-purified.
Figure 12. The effects of BEZ and MPA are recapitulated against purified CLL cells. Five patient samples were either unsorted or sorted for CD19 expression using indirect anti-CD19 IgG1 antibody and anti-IgG1 microbeads and positive selection in a magnetised ferrous LS column. The cells were subsequently cultured on non-CD40 expressing stroma in a 10:1 ratio and treated with solvent control, 5µM MPA, 0.5mM BEZ or BEZ+MPA, for 24 hours and analysed for the binding of AV and uptake of PI using FC. Data are the mean ± s.e.m. from n=5 patients.
Next, the effects of BEZ, MPA and, BEZ+MPA were investigated on B cells derived from the blood of healthy volunteers. The B cells from such donors express CD19, like CLL cells, but have low CD5. The B cells were purified by CD19 selection and were treated and analysed at 24 hours for total AV positivity as shown in figure 13. Compared to the levels of apoptosis seen previously in the CLL solvent controls, the background apoptosis seen in the normal donors was high (mean of 43%). However, there was no induced apoptosis by either the single agent treatments or the combination of BEZ+MPA. These data on the CD19^+ve purified CLL cells and peripheral blood cells from healthy donors together strongly suggest that the effects of BEZ, MPA and, most importantly, the combination are targeted towards the malignant CLL cells.
Figure 13. BEZ, MPA and BEZ+MPA do not induce apoptosis of normal B cells. CD19<sup>++</sup> positive B cells were isolated from healthy donors by indirect anti-CD19 IgG1 antibody and anti-IgG1 microbeads and positive selection in a magnetised ferrous LS column. The cells were subsequently cultured on non-CD40<sup>L</sup> expressing stroma in a 10:1 ratio and treated with solvent control, 5µM MPA, 0.5mM BEZ or BEZ+MPA in triplicate. At 24 hours the appropriate wells were harvested, pooled and analysed for binding of AV and uptake of PI using FC. Data are the mean ± s.e.m from n=3 donors.
3.2.7. *BEZ+MPA show effects similar to those of chlorambucil.*

In order to assess the potential value of BEZ+MPA as anti-CLL therapy, it is pertinent to compare the responses of CLL cells with those to treatments that reflect current therapies. One of the most common first line therapies for the treatment of CLL, particularly in the elderly, remains the alkylating agent chlorambucil (Oscier et al., 2004). Therefore, the actions of BEZ and MPA, as assessed in this *in vitro* system, were compared with that of this agent. Strikingly, as shown in figure 14, the pro-apoptotic actions, in the absence of CD40L, of BEZ or MPA alone, were similar to that of chlorambucil (*P*= 0.99 for both). Notably, apoptosis was enhanced when chlorambucil was combined with BEZ or MPA although this did not reach significance. The incorporation of BEZ+MPA with chlorambucil did not generate significantly more apoptosis than BEZ+MPA alone, however, this combination was significant over chlorambucil alone (*P*=0.013). The implications of these findings for future trials in CLL are returned to in chapter 6.
Figure 14. BEZ+MPA emulate the pro-apoptotic actions of chlorambucil with the combination exerting still greater effects. CLL cells were cultured on non-CD40L expressing stroma in a ratio of 10:1 in 96 well plates. Triplicate treatments of either solvent control, 0.5mM BEZ, 5µM MPA or BEZ+MPA, in the presence (purple fill) and absence (blue fill) of 1µM chlorambucil were harvested and pooled at 24 hours, and analysed for the binding of AV and uptake of PI using FC. Data are the mean ± s.e.m. from n=4 patients; P values were calculated using ANOVA.
3.3. The assessment of BEZ and MPA on ‘PC’ CLL cells.

3.3.1. CLL cells cultured with CD40L–L cells and IL-4 show a proliferative response.

As described in section 3.1., the isolated MNC cells from patient blood samples were cultured on stroma expressing CD40L with the presence of 1ng/ml IL-4 to recapitulate the proliferation of CLL cells in the PCs. Unless stated otherwise, all samples were analysed in parallel with non-CD40L stimulated cultures discussed in figures 4-14. Proliferation was assessed using the incorporation of ³H-thymidine at 0.4µCi/well. Cells were cultured for 72 hours in total, with the addition of ³H-thymidine for the final 12-16 hours. The counts/min ³H-thymidine incorporated in cells cultured without CD40L ranged between ~150 and 400 cpm, which is essentially that seen in the stroma alone. However, in the majority of CLL MNC preparations cultured with CD40L in the presence of IL-4, thymidine incorporation was greatly enhanced (ranging from ~1,000-16,000 cpm).

3.3.2. In the presence of CD40L: the effects of BEZ and MPA are dose dependent.

Individually, BEZ and MPA caused a reduction in CD40L mediated proliferation of CLL cells that was dose dependent (figure 15). As observed in the absence of CD40L, the effects of MPA effectively plateaued beyond 5µM. BEZ continued to increase its anti-proliferative effects beyond 0.5mM. As shown for the absence of CD40L (figure 6), the doses of 5µM MPA and 0.5mM BEZ either as single agents or in combination were chosen for further investigation.
Figure 15. BEZ and MPA reduce the CD40L proliferative response of CLL cells in a dose dependant manner. CLL cells were cultured on CD40L expressing stroma in a ratio of 10:1 in 96 well plates and were treated with solvent control and either MPA (top) or BEZ (bottom) in a 2-fold dose escalation (1.25µM-10 µM MPA and 0.125-1 mM BEZ) for 72 hours. Cells were pulsed with ³H-thymidine for the final 16 hours of culture and ³H-thymidine incorporation was calculated, relative to control. Data are the mean ± s.e.m. of n=7 patients.
3.3.3. Analysis of apoptosis in the presence of CD40L.

The apoptosis data obtained from CLL cells cultured without CD40L has shown BEZ+MPA were effective at inducing apoptosis (figure 8). The same analyses were conducted on CLL cells cultured in the presence of CD40L. In this culture system, neither the individual agents of BEZ and MPA nor the combination of BEZ+MPA displayed induction of AV/PI positivity (figure 16). Similarly, neither of the individual agents nor, most importantly, the combination was effective at inducing Δψm depolarisation (figure 17).

This data demonstrates that BEZ, MPA and BEZ+MPA do not induce apoptosis of CLL cells when CD40L is present. This observation agrees with those of others that, in addition to the pro-proliferative activity of CD40L, this protein also has a protective role. This is highlighted by it effectively shielding the CLL cells from BEZ and MPA mediated apoptotic signals.
Figure 16. In the presence of CD40L, BEZ, MPA and BEZ+MPA treated CLL cells do not bind AV or accumulate PI. CLL cells were cultured on CD40L expressing stroma in a ratio of 10:1 in 96 well plates. Treatments of either solvent control, 0.5mM BEZ, 5µM MPA or BEZ+MPA were performed in triplicate for 24 hours, prior to harvesting and staining with AV and PI and analysis by FC. a) plots show AV/PI positivity gated as in figure 4 b) analysis of FSC and SSC, gate shows viable population. Note the difference in cell size compared to figure 4. The plots shown are representative of n=15 patients and were set in parallel to those shown in figure 4.
Figure 17. In the presence of CD40L, BEZ, MPA and BEZ+MPA do not cause Δψm depolarisation. CLL cells were cultured on CD40L expressing stroma in a ratio of 10:1 in 96 well plates. Treatments of either solvent control, 0.5mM BEZ, 5µM MPA or BEZ+MPA, were performed in triplicate for 24 hours, prior to harvesting and staining with JC-1 and analysis by FC. Events captured in the FL-2 region (red dots) indicate Δψm is preserved, whereas those captured in the FL-1 channel (green dots) highlight aggregation of JC-1 indicative of a loss of Δψm. The plots shown are representative of n=6 patients and were set in parallel to those shown in figure 5.
3.3.4. *The lack of BEZ+MPA induced apoptosis is not time dependant.*

Although BEZ, MPA and BEZ+MPA failed to induce apoptosis at 24 hours, it was considered that this apparent protective action of CD40\(_L\) may be overcome with longer treatment. The total AV positivity (figure 18a) and Δψ\(_m\) depolarisation (figure 18b) was assessed at 7, 24, 48 and 72 hours. It is important to note that these samples are the same as those described in figure 6 that had previously been frozen. As discussed in relation to figure 6, the control cells analysed at 7 hours by AV/PI showed a higher level of apoptosis than at 24 hours, which was not observed in the analysis by JC-1. This is indicative of cell stress post-thawing but that is overcome overnight and then plateaus. BEZ+MPA did not induce apoptosis as measured by AV/PI, JC-1 (figure 18) and a change in FSC/SSC at any time point analysed. It was found that the cells could not be treated and analysed for longer than 72 hours because, beyond this point, the majority of samples tended to be over-stimulated by CD40\(_L\) resulting in apoptosis of the control cells.

3.3.5. *BEZ, MPA and BEZ+MPA are effective at reducing the proliferative response of CLL cells to CD40\(_L\).*

Thus far it has been shown that CD40\(_L\) induces both CLL cell proliferation, as shown by others (Grdisa, 2003, Jacob et al., 1998), and protection of CLL cells from BEZ+MPA mediated apoptosis. However, whilst BEZ and MPA were unable to induce apoptosis in the presence of CD40\(_L\), both agents substantially reduced the proliferative capability of CLL cells. The reproducibility of this reduction of proliferation was investigated in a panel of consecutively acquired CLL cells.
Figure 18. Prolonged exposure to BEZ+MPA does not induce apoptosis in the presence of CD40L. CLL cells thawed from -80°C were cultured on CD40L-expressing stroma in a ratio of 10:1 in 96 well plates. Treatments of either solvent control or BEZ+MPA were performed in triplicate and were harvested at 7, 24, 48 and 72 hours and assessed for (a) the binding of AV and uptake of PI and (b) $\Delta \psi_m$ using JC-1 and analysis by FC. Data are the mean ± s.e.m. from n=4 patients and were set in parallel to those shown in figure 6.
Figure 19 shows a similar box plot for the inhibition of cell proliferation of CD40L-stimulated CLL cells from 27/28 of the same samples analysed in figure 8 (one sample yielded too few cells for both types of analysis). The patient characteristics are detailed in table 8. The same statistical analyses were carried out by Dr J Delgado, as described previously in relation to figure 8. Despite the range in CD40L-stimulated proliferation, the overall responses to both the individual agents and the combination was strongly significant.

Pair-wise comparisons identified that proliferation following BEZ, MPA and BEZ+MPA treatments was significantly less than in untreated controls ($P = 0.011$, $P=0.042$ and $P = 0.003$ respectively). The anti-proliferative effect of BEZ was not statistically different from that of MPA ($P = 0.199$) but the combined treatment was significantly more anti-proliferative than either BEZ or MPA alone ($P = 0.007$ and $P = 0.003$ respectively).

Therefore, whilst BEZ+MPA may not be effective at inducing apoptosis in the presence of CD40L, the combination reduces the proliferative capabilities of CLL cells in vitro. As a result, it is possible that the agents would be effective at reducing proliferation within the PC.
Figure 19. BEZ, MPA AND BEZ+MPA arrest CD40L induced proliferation of CLL cells. CLL cells were cultured on CD40L expressing stroma in a ratio of 10:1 in 96 well plates. Triplicate treatments of either solvent control, 0.5mM BEZ, 5µM MPA or BEZ+MPA were incubated for 72 hours with the addition of 0.4µCi of ³H-thymidine for the final 12-16 hours of culture. Plot shows ³H-thymidine incorporation after 72 hours of treatment in n=27 patients and are the same as those shown in figure 8 without CD40L. Each box shows variation in initial proliferation and its subsequent reduction. Line in box indicates median value; bottom line and top line of each box show 25th and 75th centile respectively, with the bottom and top whiskers corresponding to 10th and 90th centile, open circles represent outliers.
3.3.6. **BEZ, MPA and BEZ+MPA exert greater anti-proliferative activities against purified CLL cells than their normal counterparts.**

The anti-proliferative actions of BEZ+MPA were encouraging, but it was important to investigate the extent to which this was a targeted action. This was assessed in two ways. First, the effects of BEZ, MPA and BEZ+MPA were analysed on CD19\(^{+}\) cells purified from CLL MNCs. Notably, the CD19\(^{+}\) cells displayed a slightly reduced proliferative response to CD40\(L\) (figure 20). However, as the purified cells were isolated by engagement of CD19 on the cells’ surface, this is to be expected. It is known that engagement of CD19 reduces the cells’ ability to proliferate (J Gordon, personal communication). Despite this dampened CD40\(L\) proliferative response of the purified CLL cells, both BEZ and MPA reduced this overall proliferation (figure 20). BEZ+MPA proved to be most significant; reducing cpm incorporated to background 130-252 (mean 172.4± 14.5 SEM; P =<0.0001; n=5).

Second, the anti-proliferative actions of BEZ, MPA and BEZ+MPA were investigated against CD19\(^{+}\) B-cells from healthy donors (figure 21). The proliferation of normal B cells was attenuated by BEZ and MPA, however; not to the same degree as in the CLL cells. Therefore, some proliferation was maintained even in the presence of both drugs (figure 21). This data indicates that, whilst BEZ+MPA may reduce the proliferative capacity of normal B cells, some normal proliferation of these cells would be maintained *in vivo.*
Figure 20. The anti-proliferative effects of BEZ and MPA are targeted against the CLL cells. Five patient samples were either unsorted or sorted for CD19 cells using indirect anti-CD19 IgG1 antibody and anti-IgG1 microbeads and positive selection in a magnetised ferrous LS column. The cells were subsequently cultured on CD40L expressing stroma in a 10:1 ratio, and treated with solvent control, 5µM MPA, 0.5mM BEZ or BEZ+MPA for 72 hours. Cells were pulsed with ³H-thymidine for the final 16 hours of culture and ³H-thymidine incorporation was calculated. Data are the mean ± s.e.m. from n=5 patients.
Figure 21. BEZ and MPA exert milder anti-proliferative effects against normal B cells. CD19 positive B cells were isolated from healthy donors by indirect anti-CD19 IgG1 antibody and anti-IgG1 microbeads and positive selection in a magnetised ferrous LS column. The cells were subsequently cultured on CD40L expressing stroma in a 10:1 ratio, and treated with carrier control, 5µM MPA, 0.5mM BEZ or BEZ+MPA in triplicate. Cells were pulsed with \(^{3}\text{H}\)-thymidine for the final 16 hours of culture and \(^{3}\text{H}\)-thymidine incorporation was calculated. Data are the mean ± s.e.m. from n=3 donors.
3.3.7. Activity of BEZ and MPA in combination with chlorambucil in the presence of CD40L.

Following the encouraging results from the combination of BEZ+MPA with chlorambucil in the absence of CD40L (figure 14), the effects of this combination in this culture system were investigated.

Chlorambucil treatment was markedly anti-proliferative against CD40L-stimulated CLL cells alone (figure 22a). The addition of BEZ, MPA or BEZ+MPA further reduced the proliferation significantly (P<0.001 for all). As chlorambucil is used therapeutically, it was anticipated that this strong reduction in thymidine incorporation in chlorambucil alone treatment would be associated with cell death and that BEZ+MPA may enhance the level of apoptosis observed. However, treatment of CLL cells with chlorambucil did not induce apoptosis (figure 22b) and the addition of BEZ+MPA did not potentiate this.
Figure 22. Together BEZ+MPA and chlorambucil cause greater growth arrest in the presence of CD40L than by chlorambucil alone. CLL cells were cultured on CD40L expressing stroma in a ratio of 10:1 in 96 well plates. Triplicate treatments of either solvent control, 5mM BEZ, 5µM MPA or BEZ+MPA, in the presence (purple) and absence (blue) of 1µM chlorambucil were harvested at a) 72 hours after the addition of 0.4µCi of $^3$H-thymidine for the final 12-16 hours of culture and b) at 48 hours analysed for the
binding of AV and uptake of PI using FC. Data are the mean ± s.e.m. from n=4 patients; P values calculated using ANOVA.

3.4. Discussion.

This chapter has demonstrated that the lipid lowering agent BEZ and the contraceptive MPA induced significant apoptosis of CLL cells cultured in the absence of CD40L and that the combination induced greater apoptosis than either drug alone. Data using caspase inhibitors indicated that this apoptosis may be caspase independent. This is not surprising given that caspase independent apoptosis has been previously described in CLL (Mone et al., 2006) and other cell types (Igney and Krammer, 2002).

BEZ and MPA each inhibited the CD40L driven proliferation of CLL cells with the combination of BEZ+MPA having more activity than either drug alone. Additionally, the drugs had little effect on normal CD19^+ve peripheral blood B-cells, indicating that these agents have a tumour cell specific effect.

Although not normally considered as anti-cancer therapy, BEZ+MPA was as potent in vitro as the established CLL agent chlorambucil. This, combined with the relatively low toxicity profiles of BEZ and MPA, renders it plausible to consider their use in the treatment of CLL patients. As the combination shows effects in both the absence of CD40L (by the induction of apoptosis) and in presence of CD40L (by the reduction in proliferation), this combination could be considered for the use indolent disease. However, reducing the proliferative capacity of the PC alone may not be sufficient and thus resistant cells in the PCs may be problematic and the addition of additional agent(s) may be necessary to target this disease as a whole.
4. Investigating the Mechanisms of BEZ & MPA
4.1. Introduction.

In the previous chapter it was demonstrated that BEZ, MPA and BEZ+MPA successfully reduce the proliferation and selectively induce apoptosis of non-CD40\textsuperscript{L} protected CLL cells. Following these findings, the mechanisms by which these agents elicited their effects were investigated.

As discussed in section 1.13., BEZ and MPA have been investigated for their use in a parallel AML study. There it was demonstrated that BEZ+MPA are effective in inducing differentiation and apoptosis of AML cells (Khanim et al., 2009). Studies from this group, have shown that AML cells have high levels of AKR1C3 (Birtwistle et al., 2009), which exhibits PGD\textsubscript{2} ketoreductase activity. Khanim et al (2009) further demonstrated that this activity is inhibited by MPA, thereby increasing the level of PGD\textsubscript{2} and its bioactive dehydration product 15d\Delta\textsuperscript{12,14}PGJ\textsubscript{2}.

BEZ is one of the only members of the fibrate family to have been shown to have pan-PPAR activity (Tenenbaum et al., 2005). In 2000, PPAR\textgamma was shown to be expressed by B cells (Padilla et al., 2000b) and in 2003 it was demonstrated that the use of PPAR\textgamma agonists in B lineage cells can induce apoptosis (Padilla et al., 2002). Work by Kleiwer et al in 2000 showed that 15d\Delta\textsuperscript{12,14}PGJ\textsubscript{2} is a PPAR\textgamma ligand and studies by Padilla et al and Ray et al have shown that the treatment of B cells with 15d\Delta\textsuperscript{12,14}PGJ\textsubscript{2} induces apoptosis (Padilla et al., 2002, Ray et al., 2006). However, this was demonstrated to be PPAR\textgamma independent.

An alternative action of BEZ is to induce elevated ROS which, in turn, leads to induction of oxidative stress (Scatena et al., 2004, Scatena et al., 2003). As presented in chapter 1, oxidative stress can lead to cellular damage, including to membrane lipids. In turn this
has been shown to induce LP, a component of which is the production of PGs from the IoPP (Gao et al., 2003).

The use of specific ELISAs demonstrated that both BEZ and MPA increased PGD\(_2\) levels in AML cell lines and that the increase was even greater when the agents were combined. The combined activity against AML cells exerted by BEZ+MPA was, therefore, attributed to the increases in PGD\(_2\) caused by the inhibition of AKR1C3 by MPA and IoPP PGD\(_2\) synthesis following BEZ treatment. The increased PGD\(_2\) allowed the subsequent accumulation of PGD\(_2\)’s metabolite 15d\(\Delta^{12,14}\)PGJ\(_2\), which was responsible for the cellular responses.

This chapter investigates whether the activity of BEZ, MPA and BEZ+MPA against CLL cells is similar to that described for AML cells.

Much of the work presented in this chapter was published in 2009 by Macmillan Publishers Ltd. The full details of this manuscript can be found in Appendix A2.
4.2. The actions of MPA against CLL cells.

4.2.1. Only AKR1C3 of the AKR1C family is readily detected in CLL cells.

Analysis of AKR1C1, 2, 3 and 4 expression was carried out by QRT-PCR. Western blot was also used to determine protein levels of AKR1C3. The myeloid cell line K562, has been demonstrated to have moderate expression levels of AKR1C3 (Birtwistle et al., 2009). This was, therefore, used as a positive control. AKR1C1 and 2 were observed (figure 23) in very few samples and overall were not present. AKR1C4 could not be detected owing to it being liver specific (data not shown). AKR1C3 was readily detected in all 18 samples (figure 23). Expression was variable but overall levels were largely in line with those observed in K562. Western blot analysis (figure 24) of AKR1C3 also showed that protein levels of AKR1C3 were similar to those seen in K562. Thus, these data were consistent with a model that MPA may act against CLL cells by virtue of inhibiting AKR1C3.
Figure 23. CLL cells express AKR1C3 almost exclusively and to varying levels. Uncultured CLL cells from 18 patients were analysed by quantitative real-time PCR for the expression of AKR1C1, AKR1C2 and AKR1C3 and the message detected displayed relative to K562, used as a positive control.
Figure 24. Protein levels of AKR1C3 in CLL cells are variable.
Western blot was carried out on 40μg of protein extracted from 5x10^6 cells from the same 18 samples shown in figure 21, for the expression of AKR1C3. The protein β-actin was used as a loading control.
4.2.2. Investigation into the ability of BEZ and MPA to induce the production of PGD$_2$ in CLL cells.

An ELISA was employed to measure the levels of PGD$_2$ within $5 \times 10^6$ CLL cells and 1ml of their supernatant following treatment with and without MPA, BEZ and combined BEZ+MPA. Three patient samples were studied in triplicate (figure 25). As there was inter-patient variability in PGD$_2$ levels, each patient was plotted separately. In the control cells of both CD40L deprived (figure 25 top) and CD40L stimulated (figure 25 bottom), the levels of PGD$_2$ varied between 10 – 200 pg/ml. In every patient and in both stromal culture systems, BEZ caused PGD$_2$ accumulation, with patient three showing the highest accumulation (200pg/ml to 950pg/ml without CD40L and 10pg/ml to 600pg/ml with CD40L).

Overall, there was a lack of enhanced PGD$_2$ accumulation when MPA was used alone or when added to BEZ. In the non-CD40L CLL cells, only patient two showed enhanced PGD$_2$ accumulation in BEZ+MPA over that seen in BEZ alone. In patients one and three, the overall level of PGD$_2$ was reduced in BEZ+MPA when compared to BEZ alone, although it should be noted that this was only a significant decrease in patient three. When CD40L was present, BEZ+MPA induced an increase in PGD$_2$ accumulation over that of BEZ alone in patients one and three but it was observed to only reach significance in patient three.
Figure 25. Treatment of CLL cells with BEZ and BEZ+MPA causes the production of PGD₂ in both the absence and presence of CD40. CLL cells from three patients were cultured in the absence (top) and presence (bottom) of CD40 in a ratio of 10:1 in 6 well plates and treated with solvent control, 0.5mM BEZ, 5µM MPA or BEZ+MPA for 6 hours prior to harvesting of the cells, together with 1ml of supernatant, extracting the prostanoids and analysis by ELISA. Data are shown for the three individual patients, analysed in triplicate and shown as mean ± s.e.m.
The above data demonstrate that, as in AML cells, BEZ induces an increase of PGD$_2$ in CLL cells. Conversely, it was conclusive that MPA did not cause an increase in PGD$_2$. To investigate this disparity further it was investigated whether CLL cells exert 11β-PGD$_2$ ketoreductase activity. Birtwistle et al. (2009) have shown that the exogenously added $^3$H-PD$_2$ and both its enzymatic and non-enzymatic metabolites should be detected in the supernatant of K562 cells post-incubation. Using the K562 line as a positive control, cells were cultured with 0.2µCi $^3$H-PGD$_2$ for 16 hours and the prostanoids in the supernatant extracted and analysed by TLC. For these experiments, CLL cells were cultured without stromal support to avoid any metabolism of PGD$_2$ by these cells.

The representative trace of K562 cells (figure 26) clearly shows 3 peaks, corresponding to remaining (unconverted) PGD$_2$, non-enzymatically produced 15d$^{12,14}$PGJ$_2$ and AKR1C3 synthesised 11-epi-PGF$_{2\alpha}$. Surprisingly, when the traces from CLL cells (figure 26) were obtained, no 11-epi-PGF$_{2\alpha}$ could be detected.

### 4.2.3. CLL cells do not appear to take up PGD$_2$

The above observation was generated from analyses of the cell supernatants. Further analysis of the prostanoids extracted from the cells was carried out. Following incubation of CLL cells with $^3$H-PGD$_2$, prostanoids were extracted and compared to extracts from the myeloid cell line KG1a that has been shown to have high levels of AKR1C3 (Birtwistle et al., 2009). Figure 27 shows traces from the extracts from 3 CLL samples, compared to a trace obtained from KG1a. In this trace, the KG1a cells no longer had any PGD$_2$ detectable. This would be expected owing to the incubation time and the high level of AKR1C3 expressed by these cells.
Figure 26. CLL cells do not appear to have 11β-PGD2 ketoreductase activity. 5x10⁶ CLL or K562 cells were exposed to ³H-PGD2 in PBS for 18 hours and the prostanoids extracted, and analysed by TLC. Top trace is representative of samples analysed from 8 patients, (each in duplicate) with K652 (bottom trace) used as a positive control each time. The migration of cold standard PGD2, PGF2α and 15d[14,12]PGJ2 were revealed by incubation in iodine vapour.
Figure 27. When cellular extracts from the myeloid cell line KG1a are compared to CLL cells, PGs cannot be detected in CLL. 2$x10^7$ CLL cells or the cell line KG1a were exposed to $^3$H-PGD$_2$ in PBS for 18 hours. Prostanoids were extracted from the and were analysed by TLC. Top trace, of KG1a, shows two peaks, probably of the J series prostanoids. Traces 2-4 are samples from three CLL patients. Note the differences in scale of counts for KG1a and the CLL samples.
Additionally, there was only a very small detectable PGF$_{2\alpha}$ peak in the KG1a cells. This is not surprising, as it has been shown that PGF$_{2\alpha}$ is exported by this cell line and readily detected in the supernatant (as shown in figure 26). Despite the lack of these 2 prostanoids, there was a readily detected peak relating to 15dΔ$^{12,14}$PGJ$_2$. The observation of this $^3$H-labelled peak serves to prove that the exogenously added PGD$_2$ had entered the myeloid cells either by diffusion or active uptake and been converted non-enzymatically to 15dΔ$^{12,14}$PGJ$_2$ (some of which is remaining in the cell) and enzymatically to PGF$_{2\alpha}$ (which is then secreted). In contrast, the CLL cells show none of these prostanoid related peaks (figure 27). Indeed, the traces contained no clearly determinable peaks and the CPM detected was essentially background. Taken together, these data would indicate that CLL cells are unable to take up PGD$_2$. Alternatively, PGD$_2$ may be taken up by the cell but then be rapidly exported, thus protecting the cell from any exogenous PGD$_2$.

4.2.4. Analysis of steroid dehydrogenase activity of CLL cells.

The above data indicate that PGD$_2$ is not a likely substrate of AKR1C3 in CLL cells. AKR1C3 also has steroid dehydrogenase activity. As CLL cells have been shown in figure 23 to only typically express AKR1C3 and not the other AKR1Cs, it was hypothesised that MPA was exerting its effects by inhibition of this activity. This was investigated using the steroid substrate 5α-DHT and looking for its product of 3α-ASD. K652 cells were used as a positive control.
Figure 28. Comparison of CLL cells to K562 indicates that CLL cells do not have steroid dehydrogenase activity. $5\times 10^6$ of cell line K562 (top) or CLL cells (middle and bottom) were treated with solvent control (left panel) or 5µM MPA (right panel) overnight prior to incubation with 5α-DHT in supplemented media for 24 hours. Sterols were extracted from the supernatant and analysed by TLC. 5α-DHT and 3α–ASD peaks were identified following co-migration of cold standard and incubation in iodine vapour.
Traces from K562 cells (figure 28) clearly showed 2 peaks: 5α-DHT and 3α-ASD. Furthermore, in the absence of MPA the product was greater than the substrate, whereas in the presence of MPA metabolism was very much diminished. Conversely, the traces from CLL cells (figure 28), only display a large 5α-DHT peak. No detectable 3α-ASD peak was observed in one patient and only a very small peak in the other. This small 3α-ASD peak was not commensurate with that seen in the K562 sample and, given the 18 hour incubation time, is exceptionally small. The fact that 3α-ASD is either undetected or only moderately detected in these samples could be explained by the findings of Birtwistle et al (2009) who demonstrated that cellular steroid dehydrogenase activity is mainly attributable to AKR1C2. Therefore, as CLL cells have very little of this enzyme (as shown in figure 23), the inability of these cells to convert 5 α-DHT to 3α –ASD would be consistent with this model. Consequently, this negates the possibility of MPA working via inhibition of this activity.

4.3. Investigation of MPA exerting effects via known receptors.

The above data indicate that MPA may not exert its affect against CLL cells via the well-known activities of AKR1C3. Therefore, additional experiments were performed to consider other possible activities of MPA that might explain its actions against CLL cells. It is known (and was discussed in section 1.15.) that besides being a ligand of PR, MPA also binds to GR and MR (Schindler et al., 2003, Schindler et al., 2008) but it is not thought to have any AR activity (Schindler et al., 2003, Schindler et al., 2008). It was therefore hypothesised that MPA could be working in CLL by exerting agonistic or antagonistic affects on one or several steroid receptors.
4.3.1. **Comparison of MPA with mifepristone.**

The effects of MPA were first compared with mifepristone (MFE), a corticosteroid, predominantly used as a PR antagonist. It is also known as RU146 and is most commonly used in combination with PGF$_{2\alpha}$ during pregnancy terminations or to induce labour (Gottlieb and Bygdeman, 1991). At a dose of 100µM, MFE was seen to exert a small pro-apoptotic effect against CLL cells, similar to that seen by MPA (figure 29). When combined with BEZ+MPA (figure 29), MFE did not increase the apoptosis seen by BEZ+MPA. Together, these observations suggest that MPA and MFE could be exerting their effects via the same target. However, at high enough doses, MFE also has GR antagonist effects and if MFE were affecting CLL via the PR, a much lower dose than 100µM would be expected to induce the effects described for figure 29. At the lower doses of 100ng - 1µM, MFE did not exert any activity against CLL (data not shown). Therefore, if MPA and MFE were acting via a common mechanism it was possible that it was GR rather than PR.

4.3.2. **The non-methylated GR agonist, prednisolone, exerts no effect against CLL cells whilst its methylated form is commensurate with MPA.**

To further investigate the possible role of GR, PRD, a GR agonist that notably has been used in the treatment CLL (Hamblin, 2001) was used. ML-PRD, has a methyl group at the 6α position (see section 2.7; table 6.), was developed in the 1950s and has taken preference, clinically, over PRD.
Figure 29. MFE induces a similar level of apoptosis to MPA. CLL cells were cultured on non-CD40-expressing stroma in a ratio of 10:1 in 96 well plates. Triplicate treatments of either solvent control, 0.5mM BEZ, 5µM MPA or BEZ+MPA in the presence (purple) and absence (blue) of 100µM MFE were harvested and pooled at 24 hours and analysed for the binding of AV and uptake of PI using FC. Data are the mean ± s.e.m. from n=4 patients.
ML-PRD has increased potency, first postulated to be due to an increased half life but now thought attributable to improved interaction with the cellular membrane via the methyl group (Waddell et al., 1977). HD ML-PRD combined with Rituximab has also been advocated for the treatment of CLL in patients with the 17p deletion (Bowen et al., 2007).

A dose titration of PRD (figure 30a) against CLL cells showed that PRD exerted no pro-apoptotic activity and that, when combined with MPA, there was no additive or reductive effect on that observed with MPA alone (figure 30a). However, in a dose titration of ML-PRD (figure 30b) a pro-apoptotic effect was observed at a concentration of 20µM and this effect was commensurate with that seen exerted by MPA alone. Further, the combination of 20µM ML-PRD and MPA was not additive to that of either agent used alone.

Following these data, further experimental analysis of 20µM ML-PRD was conducted. When compared to BEZ+MPA, BEZ+20µM ML-PRD showed similar apoptotic effects against CLL (figure 31). Once again 20µM ML-PRD+BEZ+MPA had no additive apoptosis to BEZ+MPA alone. Together these data indicate that the similar levels of apoptosis induced by ML-PRD and MPA could be via the same target. If this target were the GR then it seemed plausible that a more specific GR agonist should also exert similar outcomes and that a GR antagonist would reduce the pro-apoptotic effects exerted by ML-PRD and MPA.
Figure 30. ML-PRD but not PRD recapitulates but does not potentiate the actions of MPA. CLL cells were cultured on non-CD40L expressing stroma in a ratio of 10:1 in 96 well plates. Triplicate treatments of solvent control, 1µM, 5µM and a) 20µM PRD or b) ML-PRD, either in the presence (purple) or absence (blue) of 5µM MPA were harvested and pooled at 24 hours and analysed for the binding of AV and uptake of PI using FC. Data are the mean ± s.e.m. from n=4 patients.
Figure 31. Combining ML-PRD with BEZ recapitulates the apoptotic effects of BEZ+MPA but all 3 agents combined are not additive. CLL cells were cultured on non-CD40L expressing stroma in a ratio of 10:1 in 96 well plates. Triplicate treatments of solvent control, 5µM MPA, 20µM ML-PRD and ML-PRD+MPA, either in the presence (purple) or absence (blue) of 0.5mM BEZ, were harvested and pooled at 24 hours and analysed for the binding of AV and uptake of PI using FC. Data are the mean ± s.e.m. from n=4 patients.
Dexamethasone (DEX) was selected as a highly specific GR agonist. The effects of 1µM DEX on CLL cells in the absence of CD40 were compared with the level of apoptosis induced by MPA and ML-PRD (figure 32). As previously discussed in section 4.3.1, MFE is a GR antagonist and this was therefore combined with MPA, ML-PRD or DEX in an attempt to antagonise any apoptosis observed. DEX induced very little apoptosis compared to that exerted by MPA and ML-PRD. Furthermore, whereas the effects of MPA and ML-PRD have been shown to be variable between samples the small, lesser effect of DEX was consistent. In addition, MFE was unsuccessful at inhibiting the MPA or ML-PRD effects and the small effect by DEX (figure 32). Taken together these data would suggest that, while MPA and ML-PRD may share a common target, it is not likely to be GR.
Figure 32. The effects exerted by MPA and ML-PRD are not via GR. CLL cells were cultured on non-CD40L expressing stroma in a ratio of 10:1 in 96 well plates. Triplicate treatments of 5µM MPA, 20µM ML-PRD and 1µM DEX, either in the presence (purple) or absence (blue) of 1µM MFE, were harvested and pooled at 24 hours and analysed for the binding of AV and uptake of PI using FC. Data are the mean ± s.e.m. from n=3 patients.
4.3.3. Mineralocorticoid investigations reveal that HD antagonists emulate the MPA apoptotic effect.

Whereas DEX is a highly specific GR agonist, PRD has been shown to also have relatively high affinity for the MR (Juruena et al., 2006). PRD has been shown to have similar binding affinity for the MR as its natural ligand, cortisol, whilst DEX has a 70% lower binding affinity (Lan et al., 1981). It is almost certain, that as ML-PRD is only different to PRD by its methyl group, the binding efficiencies of these agents will be the same. Therefore, as ML-PRD induced apoptosis to a similar level to MPA (and DEX induced none), it is possible that this is via the MR. Thus, it was hypothesised that an MR antagonist may inhibit MPA and ML-PRD induced apoptosis. A dose titration of the MR antagonist spironolactone (SPN) alone (figure 33) indicated that at the active inhibitor dose of 100nM, the agent had no pro-apoptotic activity. However, at the 10-fold higher dose of 1µM, SPN induced apoptosis to a similar level of that of MPA observed in previous experiments. Following these results, HD (1µM) and LD (100nM) SPN were investigated, in the presence and absence of BEZ and MPA.

When compared to the pro-apoptotic effects of MPA and BEZ, HD SPN induced similar levels of apoptosis to both of these agents (figure 34a). Moreover, HD SPN when combined with BEZ+MPA did not potentiate the BEZ+MPA alone response (figure 34a).
Figure 33. HD SPN exerts pro-apoptotic effects. CLL cells were cultured on non-CD40 expressing stroma in a ratio of 10:1 in 96 well plates. Cells were treated in triplicate with the doses shown of SPN. After 24 hours, triplicate wells were harvested, pooled and analysed for the binding of AV and uptake of PI using FC. Data are the mean ± s.e.m. from n=4 patients.
Figure 34. HD SPN exerts similar effects to both MPA and BEZ without potentiating BEZ+MPA, whilst LD SPN does not antagonise their effects. CLL cells were cultured on non-CD40L expressing stroma in a ratio of 10:1 in 96 well plates. Triplicate treatments of solvent control, 5µM MPA, 0.5mM BEZ, BEZ+MPA either in the presence (purple) or absence (blue) of a) 1µM SPN or b) 0.125µM SPN were harvested and pooled at 24 hours and analysed for the binding of AV and uptake of PI using FC. Data are the mean ± s.e.m. from n=5 patients.
As LD SPN did not yield apoptosis when used alone, it is possible that when combined with MPA it would antagonise the MPA effect. When used in combination with BEZ+MPA, a mild reduction in apoptosis could be seen but this did not reach significance. These data tend to suggest that the effect of MPA against CLL cells is not as an MR agonist. This statement was supported by the fact that hydrocortisone (a synthetic corticosteroid that, reciprocally to ML-PRD, can bind both the MR and the GR) showed no effect on CLL cells (figure 35).

Overall, this body of data, using corticosteroids as agonists and antagonists of the PR, GR and MR showed that 20µM ML-PRD and 1µM SPN induce similar levels of apoptosis to MPA when used alone. Additionally, combining these agents with BEZ+MPA did not result in additive apoptosis. It therefore remains possible that all three agents are effective against a common target. However, it is unlikely that any of these agents, including MPA, are exerting their activities by their known receptors. Additionally, the target is unlikely to be AKR1C3; whilst MPA has been shown to inhibit AKR1C3, neither ML-PRD nor SPN are able to do so at the concentrations tested here (Dr Nick Davies, personal communication).
Figure 35. Hydrocortisone does not induce apoptosis of CLL cells. CLL cells were cultured on non-CD40L expressing stroma in a ratio of 10:1 in 96 well plates. Cells were treated in triplicate with a 2-fold titration of hydrocortisone (from 1mM to 62.5µM). After 24 hours triplicate wells were harvested, pooled and analysed for the binding of AV and uptake of PI using FC. Data are the mean ± s.e.m. from n=5 patients.
4.3.4. AKR1C3 inhibitors other than MPA do not induce apoptosis of CLL cells.

The data so far have shown that MPA is unlikely to be working via its known steroid targets, or indeed MR. Additionally, MPA cannot be affecting 11β-PGD: ketoreductase activity as CLL cells do not appear to be able to perform this activity. However, it remained possible that MPA is effecting an alternative mechanism of AKR1C3. Other known inhibitors of AKR1C3 were used to address this. Lovering et al (2004) showed that the anti-inflammatory agents flufenamic acid (FLA) and IMN bind the active sites of AKR1C3 at doses of around 20µM. Similarly, this group have demonstrated that the plant derived hormone MeJ inhibits cellular AKR1C3 activity (Davies et al., 2009). It was hypothesised, therefore, that if AKR1C3 was the target of MPA then FLA, IMN and MeJ should have similar effects on CLL cells to that observed by MPA. However, comparing each of these agents against MPA and ML-PRD showed that none of the agents caused apoptosis of CLL cells (figure 36).
Figure 36. Alternative inhibitors of AKR1C3 do not induce apoptosis of CLL cells. CLL cells were cultured on non-CD40L expressing stroma in a ratio of 10:1 in 96 well plates. Triplicate treatments of solvent control, 5µM MPA, 20µM ML-PRD, 20µM FLA, 20µM IDM and 1mM MeJ were harvested and pooled at 24 hours and analysed for the binding of AV and uptake of PI using FC. Data are the mean ± s.e.m. from n=4 patients.
4.3.5. **The potential use of ALL cell lines to investigate the importance of AKR1C3 for MPA activity.**

In order to definitively establish or refute AKR1C3 as the target of MPA interfering RNA experiments should be undertaken. Disappointingly, CLL cells are difficult to manipulate and attempts at Sh RNA experiments failed to yield a high enough efficiency for this to be possible. CLL cell lines are rare as it has been shown that the normal method of generating a cell line, i.e. transfection with Epstein Barr virus (EBV), does not induce the cells into cell cycle, nor prolong their lifespan in culture (Crawford and Catovsky, 1993). Therefore, it was investigated whether cell lines from an alternative B-cell malignancy could be used as a model to study the role of AKR1C3 in lymphoid malignancy.

There are many ALL cell lines both from T cell and B cell lineages. Gene expression of AKR1C1, 2 and 3 was analysed in a panel of these lines using QRT-PCR, as well as protein levels of AKR1C3, as determined by western blot. Additionally, the $11\beta$-PGD$_2$ ketoreductase activity of these lines was analysed. From this, two cells lines, Nalm6 and REH, were chosen to investigate further. The reasoning behind this was two-fold; first because both lines are of pre-B cell origin and, second, because they differed in expression of AKR1C3 and $11\beta$-PGD$_2$ ketoreductase activity. REH expressed high levels of AKR1C3 (ranging from 800 to 1552% relative to K562) (figure 37) and displayed $11\beta$-PGD$_2$ ketoreductase activity that was inhibited by MPA (figure 38). In contrast, Nalm6 had no, or very little, expression of the enzyme (ranging from 0.06 to 1.6%, relative to K562) (figure 37) and, as would be anticipated, did not have $11\beta$-PGD$_2$ ketoreductase activity.
Figure 37. Analysis of expression of AKR1, 2 and 3 of the ALL cell lines REH and Nalm6. a) QRT-PCR for AKR1C1, AKR1C2 and AKR1C3 was performed on cDNA from 100ng RNA extracted from 2x10^6 REH (blue) and Nalm6 (purple). Message levels are plotted as percentage relative to K562 shown as mean ± s.e.m from n=3. b) Western blot analysis was performed on 40µg protein extracted from 5x10^6 cells. K562 is displayed as a positive control.
Figure 38. REH display 11β-PGD₂ ketoreductase activity. 2x10⁶ cells were treated with solvent control or 5μM MPA overnight, prior to incubation with ³H-PGD₂ in PBS for a further 18 hours. Prostanoids were extracted from the cells’ supernatant using methanol and chloroform and analysed by TLC. Conversion of PGD₂ to PGF₂α was calculated using the equation (product/(product + substrate))x100. Graph shown is mean ± s.e.m from n=3.
Importantly, like the majority of CLL samples tested, neither of these lines displayed expression of AKR1C1 and only Nalm6 had a very small (around 0.5%, relative to K562) of AKR1C2 (figure 37). Using all these data and, in particular, the disparate levels of AKR1C3 it was proposed that these two cell lines could provide evidence as to whether AKR1C3 is likely to be the target of MPA in B cell malignancies.

4.3.6.  The AKR1C3-ve ALL cell line, Nalm6, is most sensitive to MPA and BEZ+MPA.

REH and Nalm6 were treated with MPA, BEZ and BEZ+MPA, and the effects analysed by calculation of cellular activity or ‘cellularity’ by cell titre blue and total AV positivity. Although REH was demonstrated to have high levels of AKR1C3, which MPA was able to inhibit, MPA alone did not induce any apoptosis (figure 39a). Whilst BEZ alone exerted a moderate amount of total AV, the combination of BEZ+MPA proved to be most effective, with the two agents showing apparent synergy. This result was also observed in the cellularity assay, cell titre blue (figure 39b), with MPA having no effect on cell number and BEZ+MPA being the most effective treatment.

Interestingly, Nalm6 that was demonstrated to have only traces or no AKR1C3, showed a significant level of apoptosis to MPA alone (figure 40a). BEZ alone was also able to induce apoptosis. Most striking was the additive level of cell death induced by BEZ+MPA. The total mean AV measured was 79% compared to 15% observed in the controls. This level of apoptosis was far greater than seen in any CLL sample and was much higher than the BEZ+MPA effect observed in REH.
Figure 39. MPA exerts little activity against REH cells. Triplicate wells of $5 \times 10^4$ REH cells were treated with solvent control, 0.5mM BEZ, 5µM MPA or BEZ+MPA for 5 days, prior to a) harvesting, pooling and analysis for the binding of AV and uptake of PI using FC; or b) incubating with cell titre blue and determining cellular activity by analysing OD at 530/565. Plots shown are mean ± s.e.m. from n=5 experiments.
Figure 40. MPA and BEZ+MPA are significantly pro-apoptotic against the AKR1C3 negative ALL cell line Nalm6. Triplicate wells of 5x10^4 Nalm6 cells were treated with solvent control, 0.5mM BEZ, 5µM MPA or BEZ+MPA for 5 days, prior to a) harvesting, pooling and analysis for the binding of AV and uptake of PI using FC or b) incubating with cell titre blue and determining cellular activity by analysing OD at 530/565. Plots shown are mean ± s.e.m. from n=5 experiments.
When the effects of the agents were observed in the cellularity assay of cell titre blue, both MPA and BEZ reduced the cellularity to a similar amount (figure 40b). The combination proved to reduce the cellularity the most; yet the combinatorial effect was less striking than by the AV/PI assay (figure 40b), though it was still significantly additive.

Overall, these data on ALL cell lines, with contrasting levels of AKR1C3 expression, serves to suggest that the ability of MPA to promote the apoptosis of malignant B-cells is not exerted via AKR1C3 either by inhibition of the 11β-PGD$_2$ ketoreductase activity or by another described or undescribed activity of this enzyme.

### 4.3.7. $15d\Delta^{12,14}$PGJ$_2$ and PGD$_2$ induce apoptosis of CLL.

Although the above experiments collectively refute a role for AKR1C3 as either the target of MPA in CLL cells or in mediating PGD$_2$, the observation that BEZ treatment does elevate PGD$_2$ remained (figure 25). Therefore, to investigate the possible actions of BEZ, CLL cells were treated with exogenously added PGD$_2$ and its dehydration product, $15d\Delta^{12,14}$PGJ$_2$ and tested for the recapitulation of the effects seen by BEZ, either alone or in combination with MPA. Two-fold dose titrations of PGD$_2$ and $15d\Delta^{12,14}$PGJ$_2$ from 50µM to 6.25µM were conducted. In the case of PGD$_2$ (figure 41a), a 50µM dose induced apoptosis at levels similar to those exerted by BEZ+MPA. Lower doses had minimal effects. $15d\Delta^{12,14}$PGJ$_2$ exerted powerful pro-apoptotic effects at 50µM and induced around 50% apoptosis at the-fold lower doses of 25µM (figure 41b). Therefore, for further studies, doses of 50 µM PGD$_2$ and 35 µM $15d\Delta^{12,14}$PGJ$_2$ were selected.
Figure 41. PGD$_2$ and 15d$\Delta^{12,14}$PGJ$_2$ induce apoptosis in the absence of CD40L. CLL cells were cultured on non-CD40L expressing stroma in a ratio of 10:1 in 96 well plates. Triplicate treatments of either solvent control, or a dose titration of a) PGD$_2$ or b) 15d$\Delta^{12,14}$PGJ$_2$ (from 50 $\mu$M to 6.25 $\mu$M). At 24 hours, triplicate wells were harvested, pooled and analysed for the binding of AV and uptake of PI using FC. Plots shown are the mean ± s.e.m. from n=4 patients.
However, it should be noted that these doses are much higher than those seen to exert activity in other B cells (Padilla et al., 2000a, Padilla et al., 2000b) and in the AML study (Khanim et al., 2009).

4.3.8. **PGD₂ effects against CLL cells are not DP1 receptor mediated or PPARγ dependent.**

Many PGD₂ effects are mediated via cell surface PGD₂ receptors (DP1/DP2). In this study, it is unlikely that PGD₂ is working via the DP2 receptor due to it being T cell specific. Therefore, only the DP1 receptor mediated effects were assessed. The quiescent and proliferating CLL cells were treated with BEZ, MPA, BEZ+MPA or PGD₂ with and without the DP1 receptor antagonist BW 868C. In the absence of CD40L (figure 42a), the antagonist exerted no effect alone and was not successful in antagonising the effects of PGD₂. Similarly, BW 868C did not remove the pro-apoptotic activities of BEZ, MPA or BEZ+MPA. Equally, BW 868C did not reinstate proliferation of CD40L cells after treatment with PGD₂ or BEZ, MPA or BEZ+MPA (figure 42b). Consequently, it is unlikely that the effects of PGD₂ or BEZ are mediated via this receptor.
Figure 42. The activities of BEZ and MPA against CLL cells are not mediated via PGD$_2$ DP1 receptor. CLL cells were pre-incubated for 1 hour with solvent control or the PGD$_2$ receptor antagonist BW868C in the a) absence or b) presence of CD40L prior to treatment with solvent control, 0.5mM BEZ, 5 µM MPA, BEZ+MPA or 50 µM PGD$_2$, with (purple) or without (blue) BW868C. a) Cells cultured on the non-CD40L-expressing stroma were analysed for total AV positivity by FC after 24 hours treatment. b) CLL cells on CD40L-
expressing stroma were treated for 72 hours with the addition of 0.4μCi ³H-thymidine for the final 16 hours of culture. Data shown are the mean ± s.e.m from n=3 patients. A second indirect form of PGD₂ signalling is via its non-enzymatic dehydration to the PPARγ ligand, 15dΔ¹²,¹⁴PGJ₂. Therefore, BEZ, MPA, BEZ+MPA and 15dΔ¹²,¹⁴PGJ₂ treatments were combined with the PPARγ antagonist GW 9662. Again, this antagonist did not affect the induction of apoptosis of non-CD40L-stimulated CLL cells by 15dΔ¹²,¹⁴PGJ₂ or BEZ, MPA or BEZ+MPA (Figure 43a). The CD40L-stimulated proliferation was slightly promoted by GW 9662 and this effect was also reflected in the treatment groups (figure 43b). Nevertheless, this pro-proliferative effect of GW 9662 did not reach significance.

Collectively these data imply that the activities of BEZ and MPA against CLL cells are not significantly mediated via either PGD₂ DP1 receptor or PPARγ.
Figure 43. The activities of BEZ and MPA against CLL cells are not mediated via PPARγ. CLL cells cultured in the a) absence or b) presence of CD40L and treated with solvent control, 0.5 mM BEZ, 5 µM MPA, BEZ+MPA or 50 µM PGD2, with (purple) or without (blue) GW 9662. a) Cells cultured on the non-CD40L-expressing stroma were analysed for total AV positivity by FC after 24 hours treatment. b) CLL cells on CD40L-expressing stroma were treated for 72 hours with the addition of 0.4 µCi 3H-thymidine for the final 16 hours of culture. Data are the mean ± s.e.m from n=3 patients.
4.3.9. The activities of BEZ and MPA against CLL cells are associated with the generation of ROS.

In 2006, Ray et al demonstrated that 15dΔ12,14PGJ2 induces apoptosis in Ramos, the B-cell lymphoma cell line, via PPARγ-independent mechanisms (Ray et al., 2006). Furthermore, they demonstrated that 15dΔ12,14PGJ2 induced Ramos cell apoptosis was associated with induction of ROS and MSO. However, they did not investigate whether PGD2 was also effective. As shown, and discussed for figure 41, PGD2 and 15dΔ12,14PGJ2 exerted similar effects on CLL cells as those demonstrated for BEZ, MPA and BEZ+MPA. In light of these findings, and as an attempt to establish a mechanism for these agents and their use as a combination, ROS generation was analysed following treatment both in the presence and absence of CD40L. The agent H2DCFDA recognises all ROS. Incubation with this, following treatment, showed that BEZ, MPA and BEZ+MPA induced ROS both in the absence and presence of CD40L (figure 44). In the absence of CD40L the percentage of ROS produced was extremely variable between patient samples, especially in regard to MPA treatment. Overall, without CD40L, BEZ induced the production of more ROS than MPA and the combination of BEZ+MPA was not substantially more than with BEZ alone. Nevertheless, the average ROS produced in the presence of CD40L was more with BEZ+MPA than with either agent alone.
Figure 44. BEZ, MPA and BEZ+MPA induce ROS in both the absence and presence of CD40. 

a) Cells were cultured in the absence (left) and presence (right) of CD40L and treated in triplicate with solvent control (black fill), 0.5mM BEZ (pink line), 5µM MPA (blue line) or BEZ+MPA (purple line) for 24 hours prior to harvesting, pooling and incubating with H2DCFDA for 40 minutes, and analysis by FC. 

b) Data are the mean ± s.e.m. from n=3 patients, in the presence (purple) and absence (blue) of CD40L.
Figure 45. BEZ, MPA and BEZ+MPA induce MSO only in the absence of CD40L.

a) Cells were cultured in the absence (left) and presence (right) of CD40L and treated with solvent control (black fill), 0.5mM BEZ (pink line), 0.5µM MPA (blue line) or BEZ+MPA (purple line) for 24 hours prior to harvesting, pooling and incubating with MitoSOX Red for 10 minutes and analysis by FC. b) Data are the mean ± s.e.m. from n=3 patients, in the presence (purple) and absence (blue) of CD40L.
4.3.10. Treatment with BEZ+MPA induces MSO only in the absence of CD40L.

MitoSOX Red was used to detect the production of MSO following treatment. In the absence of CD40L (figure 45) BEZ and MPA, as individual agents, both induced MSO production to similar levels. Nevertheless, as with many CLL cell apoptotic responses, the combination of BEZ+MPA was additive. More strikingly, though, was the lack of MSO produced by BEZ, MPA or BEZ+MPA when CD40L was present (figure 45a right plot and b purple fill). When treated with PGD2 or 15dΔ12,14PGJ2 (figure 46) ROS was produced to a similar level as that seen by BEZ+MPA in the absence of CD40L. Notably, 15dΔ12,14PGJ2 and PGD2 also generated some increased MSO in CD40L-stimulated cells, possibly indicating an alternative mechanism of action to those exhibited by BEZ+MPA. Nonetheless, the percentage of MSO produced was minor, in comparison to non CD40L-stimulated cells. Overall the data suggests that BEZ elicits similar cellular effects on CLL cells to 15dΔ12,14PGJ2 and PGD2 and that this action is not interfered with by MPA. It could, therefore, be postulated that the ROS and MSO generation in response to BEZ and BEZ+MPA, in the absence of CD40L, may be the consequence of PGD2 and thus 15dΔ12,14PGJ2 elevation. This is likely to be the case for BEZ but, in light of the data previously shown, MPA is not causing the same induction of prostanoids.
Figure 46. PGD2 and 15dΔ^{12,14}PGJ2 induce ROS and MSO to a comparable level to BEZ+MPA. Cells were cultured in the absence (left) and presence (right) of CD40L and treated with solvent control (black fill), 50µM PGD2 (green line) or 35µM 15dΔ^{12,14}PGJ2 (red line) for 24 hours prior to harvesting, pooling and incubating with a) H2DCFDA for 40 minutes or b) MitoSox Red for 10 minutes and analysis by FC. Plots are representative of n=3 experiments.
4.3.11. **Chlorambucil does not induce the production of MSO.**

In chapter 3, the effects of BEZ+MPA and BEZ+MPA+chlorambucil were compared to the *in vitro* levels of apoptosis induced by chlorambucil alone. It was shown that BEZ+MPA induced apoptosis, in the absence of CD40L, to similar levels as chlorambucil and that chlorambucil, like BEZ+MPA, did not induce apoptosis in the CD40L-stimulated CLL cells. This led to the analysis of MSO produced following chlorambucil treatment, compared to BEZ+MPA and BEZ+MPA+ chlorambucil. It was surprising to note that chlorambucil did not induce MSO without CD40L stimulation (figure 47). However, the lack of MSO by chlorambucil, in the presence of CD40L, was less surprising in light of the result that, like BEZ+MPA, chlorambucil also did not induce apoptosis of CD40L-stimulated CLL cells. As might be expected, these data indicate that the mechanisms of action of BEZ+MPA and chlorambucil are different and may explain why more apoptosis was observed in the combination of chlorambucil with BEZ+MPA. The differential targets of three agents combined may lead to a better *in vivo* response than the use of these regimens individually.
Figure 47. Unlike BEZ+MPA, chlorambucil treatment does not induce MSO. CLL cells were cultured on non-CD40L expressing stroma in a ratio of 10:1 in 96 well plates. Triplicate treatments of either solvent control (left; black fill), 1mM chlorambucil (middle; grey line) or BEZ+MPA (right; purple line) were harvested and pooled at 24 hours and incubated with MitoSOX Red for 10 minutes, prior to analysis by FC. Plots shown are representative of variability of response observed from n=4 patients.
4.4. Discussion

Unlike in the parallel study of MPA treatment of AML cells, the 11β-PGD₂-ketoreductase-activity of AKR1C3 could not be detected in CLL cells and no strong evidence was obtained that AKR1C3 represents the CLL target of MPA. Investigations into the alternative activities of MPA revealed that ML-PRD and SPN exerted similar effects to those of MPA, but these were not associated with their known receptors or other receptors of the corticosteroid family. However, it remains possible that these agents are exerting their effects via an unknown, common target.

BEZ was able to induce and increase in PGD₂ and when added exogenously, PGD₂ and 15dΔ₁₂,₁₄PGJ₂ were able to induce apoptosis in a dose dependent manner, recapitulating the effects of BEZ and BEZ+MPA.

It has previously been reported that 15Δ₁₂,₁₄PGJ₂ is associated with the generation of ROS and MSO. Additionally BEZ has been associated with oxidative stress. In the absence of CD40L, BEZ, MPA and moreover the combination induced ROS and MSO. A proposed mechanism for the induction of ROS and/or MSO by BEZ and thus the induction of apoptosis in this setting is shown in figure 62 at the end of chapter 6.

Conversely, in the presence of CD40L, only the presence of ROS was observed. These data imply that CD40-ligation protects CLL cells against drug induced MSO production and that this generation of MSO within the PC may be the key to causing apoptosis in this protected niche.
CHAPTER

FIVE

5. Targeting the PC
5.1. Introduction.

For a therapy to be successful in CLL, the tumour load, both in the PC as well as in the periphery, needs to be eradicated. Chapter 3 demonstrated that BEZ+MPA were effective at inducing apoptosis of the quiescent peripheral CLL cells and reducing the proliferation of the CD40L-stimulated cells. Conversely, like most therapies tested in vitro, they were unsuccessful at causing death of cells cultured in the presence of CD40L. This regimen would be beneficial to patients as it would reduce or stop the production of the malignant cells from the PCs. Whilst this would potentially improve a patient’s life and reduce the tumour burden, once the therapy is ceased it is probable that, without a suppressive signal, the PCs would be re-established and the patient relapse.

Chapter 4 showed that whilst BEZ+MPA induced the production of MSO and ROS in the absence of CD40L, only ROS was produced in the presence of CD40L. Therefore, CD40L is likely to be protecting against MSO production and thus, potentially, preventing cell death. It is apparent, therefore, that this protective signal needs to be overcome in order to induce cell death within the PCs. Targeting mitochondria within tumour cells has recently been cited as a potential way to overcome apoptotic blocks (Goldin et al., 2007).

In this chapter it is proposed that, in the PC CLL setting, this could be executed by inducing a high enough level of MSO that CD40L cannot counteract, or by a third mechanism, different to that of BEZ+MPA, that leads to the diminution of this protection. Hallaert et al (2008) reported that the protective effects of CD40L could be overcome by the use of the c-Abl kinase inhibitor DSN. It was demonstrated that this was due to a reduction in anti-apoptotic proteins such as BimEL and Mcl-1. However, the CD40L signal
was removed for 24-48 hours prior to analysis of the patient samples. Therefore, it cannot be concluded that DSN can overcome CD40L when the signal is continually present.

In this chapter, several agents were investigated for the potential of inducing apoptosis in this system and any successful agents compared to the effectiveness of DSN. These compounds are different to BEZ and MPA in that they are not currently available drugs. Each of them is a novel compound that could be developed as a therapeutic agent if effective.

Parts of sections 5.2 and 5.3.1 were published in 2008/9 by Elsevier and AACR Journals respectively. Full details of these manuscripts can be found in Appendix A2.
5.2. Use of an intercalating DNA agent.

A tetracationic supramolecular cylinder, [Fe2L3]4 (Fe cylinder), was developed in 2001 (Hannon et al., 2001) and has since been proven to intercalate in the major groove and affect DNA coiling (Khalid et al., 2006). It has been suggested that this agent could be used as an alternative to chemotherapeutics such as cisplatin. Collaborative studies led to the investigation of the cellular effects of this agent and were first tested in AML. The ability of Fe cylinder to induce apoptosis and inhibit proliferation in the AML cell line HL-60 was analysed.

At a dose of 10µM Fe cylinder, inhibition of cell cycle was seen with an accumulation of G1/G0 at 24 hours (figure 48a). The percentage of events in G1/G0 in the controls was observed at 40.0% ± 3.5% compared to 64.7% ± 4.5% in treated cultures. Additionally, a reduction in the percentage of cells in S phase (22.8% ± 6% compared to 43.8% ± 7%) was seen. The higher dose of 25 µM did not induce the same G1/G0 arrest but the reduction in S phase was maintained (figure 48b; left panel). Additionally, at this dose, sub-G1 events were higher compared to the controls. This is indicative of cell death and, as cell counts of the cultures revealed 50% less cell in those treated (data not shown) compared to controls, it was hypothesised that Fe cylinder was inducing apoptosis. This was investigated further using the AV/PI analysis used for the CLL experiments. At 24 hours, very little AV binding or PI accumulation could be detected (figure 48b). This was slightly enhanced to 17% at 48 hours but was not sufficient enough to account for the reduction in cell number. Further, analysis of the cell cycle profile also revealed a reduction in the PI signal in Fe cylinder treated cultures.
Figure 48. Fe cylinder causes cell cycle arrest, reduces the fluorescence intensity of PI and induces apoptosis in HL-60 cells. HL-60 cells were seeded at a density of 2.5x10^5/ml and treated with Fe cylinder. a) Induction of G1 cell-cycle arrest at 24 hours, following treatment of 10 μM as assessed by PI staining of DNA and FC analysis. Plots are representative of n=3 experiments. b) Induction of apoptosis following 25μM treatment as assessed by (left panel) PI staining and FC and (right panel) binding of AV and uptake of PI and FC at 0 hours (top) 24 hours (middle) and 48 hours (bottom). c) The associated table shows the percentage of events in each stage of the cell cycle and the percentage of total AV positive cells ± s.d. from n=3 experiments.
Compared to the untreated controls this indicated that the PI used to stain DNA, was unable to bind with the same efficiency. As it has been previously shown that Fe cylinder can displace ethidium bromide from calf thymus DNA (Hannon et al., 2001), it was thought that Fe cylinder treatment could lead to PI having to compete to be able to bind to DNA. Indeed, this was proven to be the case by other members of the laboratory (Hotze et al., 2008). As this was not seen in the lower dose, it is possible that the amount of DNA binding is attributable to the amount of cell death seen. It is probable that Fe cylinder induces cell cycle arrest, rendering the cells in senescence and, upon DNA intercalation, induces some cell death.

Using the above data it was hypothesised that combining BEZ+MPA with Fe cylinder may be more effective at reducing cell proliferation of the CD40L stimulated CLL cells. If the cells were to become cytostatic it was hypothesised that BEZ+MPA could induce apoptosis of the CLL cells. A dose titration of Fe cylinder with BEZ+MPA revealed that no doses of this combination exerted pro-apoptotic effects (figure 49). Therefore, whilst Fe cylinder may be worth further investigation in AML, it is unlikely to be of benefit in CLL.
Figure 49. Fe Cylinder does not potentiate BEZ+MPA to induce apoptosis in the presence of CD40L. CLL cells were cultured on CD40L expressing stroma in a ratio of 10:1 in 96 well plates. Cells were treated in triplicate with a BEZ+MPA in the absence (blue) and presence (purple) of a 2-fold titration Fe Cylinder (from 50µM to 6.25µM). After 72 hours, triplicate wells were harvested, pooled and analysed for the binding of AV and uptake of PI using FC. Data are the mean ± s.e.m. from n=6 patients.
5.3. Use of plant derived compounds

5.3.1. Jasmonates

Jasmonates are fatty acid–derived cyclopentanones and have been investigated for their potential use as cancer therapy (Heyfets and Flescher, 2007, Flescher, 2007, Flescher, 2005). They have also been reported to have mitochondrial effects, most notably in CLL cells (Rotem et al., 2005). JA and MeJ have structural similarity to PGD$_2$ and this led this group to study these compounds in relation to their ability to inhibit AKR1C3 in AML cells. In addition, the cellular effects of JA and MeJ on cell viability, cell differentiation and the production of both ROS and MSO were investigated.

Using KG1a, both JA and MeJ reduced cellularity in a dose dependant manner (figure 50). In JA treatment only 4mM proved to be lethal to the whole culture with around 50% cellular activity at doses of 2 and 1mM. Conversely, MeJ was far more potent, reducing cellularity to almost 0 at a dose of 0.5mM. Comparing JA and MeJ at 0.5mM on $\Delta\psi_m$ showed that JA had no effect on the $\Delta\psi_m$ (figure 51a), whereas MeJ significantly reduced $\Delta\psi_m$. Analysis of MSO production demonstrated that JA did not induce MSO generation at doses lower than 4mM (figure 51b) and that very little MSO was detected at 4mM. Conversely, MeJ was far more effective at inducing MSO, with 0.5 to 4mM showing dose dependent increases in MSO produced. Following the CLL data in chapter 4 (section 4.3.10.), this supports the proposed importance of the induction MSO in order to induce cell death.
Figure 50. Jasmonates reduce cellularity of myeloid KG1a cells. KG1a cells were seeded at a density of 2.5x10^5/ml and cultured for 5 days in the presence of a 2-fold titration of JA (blue) and MeJ (purple) before analysis of cellularity by cell titre blue. Data are the mean ± s.e.m. from n=3 experiments.
Figure 51. Jasmonates adversely affect mitochondria and induce MSO in KG1a cells. KG1a cells were seeded at a density of 2.5x10^5/ml and treated with solvent control, 0.5mM JA or 0.5mM MeJ and a) cultured for 5 days prior to analysis of Δψm; (top) representative FC plots are shown; (bottom) mean percentage of apoptotic cells ± s.e.m. from n=3 experiments; b) cultured for 24 hours in the presence of a 2-fold titration of JA (blue) and MeJ (purple) before analysis of MSO generation by MitoSOX Red and FC. Data are the mean ± s.e.m. from n=3 experiments.
Although Jasmonates have been researched for their use in CLL, this was on quiescent cells only (Rotem et al., 2005). Following the data of MeJ inducing MSO in AML cells, it was hypothesised that MeJ may also be effective at inducing MSO in the presence of CD40L and that, when combined with BEZ+MPA, could lead to apoptosis of the proliferating CLL cells. A dose titration of MeJ without BEZ+MPA (figure 52) revealed that MeJ alone did not induce any apoptosis of CD40L protected CLL cells. When BEZ+MPA was combined with MeJ (figure 52) the level of apoptosis was increased but even at 1mM only reached around 32%. In the absence of BEZ+MPA, MeJ was additionally unable to induce MSO (figure 53). Upon the addition of BEZ+MPA only 1mM produced any significant level of MSO. This data correlates with cell death only being significantly detected in BEZ+MPA+1mM MeJ. Following Rotem’s studies (2005), it was surprising that low doses of MeJ could not potentiate BEZ+MPA. Whilst 1mM MeJ could potentially be combined with BEZ+MPA to treat CLL, this dose is high and the levels of apoptosis seen in vitro are not substantial enough for this to be likely to be successful clinically. Nonetheless, the data did contribute to the data presented thus far; that CD40L-mediated protection can be overcome and apoptosis instigated together with the generation on MSO. It also highlighted the potential of naturally derived compounds to exert these activities.
Figure 52. HD MeJ potentiates BEZ+MPA to induce apoptosis in the presence of CD40L. CLL cells were cultured on CD40L expressing stroma in a ratio of 10:1 in 96 well plates. Cells were treated in triplicate with a 2-fold titration (1mM to 0.125mM) of MeJ in the absence (blue) and presence (purple) of BEZ+MPA. After 72 hours, triplicate wells were harvested, pooled and analysed for the binding of AV and uptake of PI using FC. Data are the mean ± s.e.m. from n=4 patients.
Figure 53. HD MeJ potentiates BEZ+MPA to produce MSO in the presence of CD40L. CLL cells were cultured on CD40L expressing stroma in a ratio of 10:1 in 96 well plates. Cells were treated in triplicate with a 2-fold titration (1mM to 0.125mM) of MeJ in the absence (blue) and presence (purple) of BEZ+MPA. After 48 hours triplicate wells were harvested, pooled and analysed for MSO positivity using MitoSOX Red and FC. Data are the mean ± s.e.m. from n=4 patients.
5.4. **Lycorine.**

Lycorine is one of many alkaloids found in the plant family *Amaryllidaceae*. Several *Amaryllidaceae* alkaloids have been found to have anti-cancer properties and lycorine in particular has been investigated for its use in AML (McNulty et al., 2009) and ALL (Liu et al., 2007). Its potential use as a therapeutic has recently led to studies into synthetic production of the compound (Jones et al., 2009). The anti-apoptotic protein MCL-1 is known to be upregulated in CLL cells exposed to CD40L. Studies to date have shown that lycorine causes the down regulation of this protein (Liu et al., 2009), as well as inducing cell cycle arrest and mitochondrial damage (Liu et al., 2004). These three documented effects, led to the hypothesis that lycorine may be able to overcome the protection of CD40L and induce apoptosis either alone or in combination with BEZ+MPA.

5.4.1. **Lycorine has little pro-apoptotic effect when used alone but the combination of BEZ+MPA+lycorine is potent.**

Surprisingly, lycorine alone was found to have very little pro-apoptotic effect on CD40L-stimulated CLL cells (figure 54). However, doses of 5µM induced higher apoptosis than was seen previously with either 1mM MeJ or 50µM Fe cylinder alone. This result was encouraging and thus, the effects of combining this agent with BEZ+MPA were analysed. When combined with BEZ+MPA the results showed a dose-dependent additive effect of BEZ+MPA to lycorine (figure 54). Even at the lowest dose tested, of 0.6µM, the BEZ+MPA pro-apoptotic effect was potentiated to give apoptosis of around 36%, compared to 17% in the controls. This apparent synergistic action of lycorine on BEZ+MPA, effectively plateaued beyond 2.5µM (figure 54).
Figure 54. Lycorine potentiates BEZ+MPA to induce apoptosis in the presence of CD40L in a dose dependent manner. CLL cells were cultured on CD40L expressing stroma in a ratio of 10:1 in 96 well plates. Cells were treated in triplicate with a 2-fold titration (10µM to 0.06µM) of lycorine in the absence (blue) and presence (purple) of BEZ+MPA. After 72 hours triplicate wells were harvested, pooled and analysed for the binding of AV and uptake of PI using FC. Data are the mean ± s.e.m. from n=6 patients.
Figure 55. Lycorine potentiates BEZ+MPA to produce MSO in the presence of CD40L, in a dose dependent manner. CLL cells were cultured on CD40L expressing stroma in a ratio of 10:1 in 96 well plates. Cells were treated in triplicate with a 2-fold titration (10µM to 0.06µM) of lycorine in the absence (blue) and presence (purple) of BEZ+MPA. After 48 hours triplicate wells were harvested, pooled and analysed for MSO positivity using MitoSOX Red and FC. Data are the mean ± s.e.m. from n=6 patients.
As a result of the lack of cell death induced by lycorine alone, it was predicted that this agent on its own would not induce MSO production. This indeed was the case (figure 55), with only 10µM able to cause detectable MSO. Even at this dose the amount produced was the same as that produced by BEZ+MPA alone. The combination of BEZ+MPA+lycorine induced far more MSO. This generation correlated with the level of total AV seen, with 0.6 to 1.25µM inducing a significant level of MSO, and doses higher than 2.5µM levelling out. Additionally, when cells were observed for viability, as measured by FSC and SSC, the number of viable events was reduced in BEZ+MPA+lycorine dose dependently (figure 56a). Further, correlation analysis of total MSO produced against viable events (figure 56a; right panel) and total AV (figure 56b; right panel) demonstrated that an increase in MSO correlated with loss of viability and apoptosis. Together, these data strongly suggest that the apoptosis caused by the combination of BEZ+MPA+lycorine is due to the reinstated accumulation of MSO by the treated cells.

5.4.2. Low dose lycorine+BEZ+MPA is targeted against malignant B cells.

Following the observation that lycorine could be combined with BEZ+MPA to induce apoptosis of the PC CLL cells, the effect on normal cells was investigated. Using MNCs isolated from normal donors, two doses of lycorine with and without BEZ+MPA were analysed on both CD40- stimulated cells and non-CD40- stimulated. The lower dose of 1.25µM lycorine did not induce apoptosis of either the CD40- protected cells (figure 57a) or MNCs without CD40- (figure 57b). However, lycorine used at the higher dose of 10µM, however, did cause some apoptosis of the normal cells. This apoptotic response was further potentiated by the addition of BEZ+MPA.
Figure 56. Cell viability and total AV correlates with MSO production. CLL cells were cultured in a 10:1 ratio on stromal cells expressing CD40L and were treated with solvent control or BEZ+MPA with a 2-fold dose titration of lycorine. a) At 72 hours cells were analysed for cell viability by FSC and SSC by FC and the viable events gated as shown (left panel), as well as for the binding of AV and exclusion of PI. b) At 48 hours cells were analysed for the generation of MSO. Graph a) shows total % MSO plotted against % viable cells and graph b) shows total % MSO plotted against total AV for each lycorine concentration. Data are the mean ± s.e.m. from n=6 patients.
Figure 57. LD lycorine+BEZ+MPA exerts minimal effects against normal cells. MNCs from normal donors were cultured on a) CD40L expressing stroma or b) non-CD40L expressing stroma in a ratio of 10:1 in 96 well plates. Cells were treated in triplicate with 1.25µM or 10µM lycorine in the absence (blue) and presence (purple) of BEZ+MPA. After 72 hours, triplicate wells were harvested, pooled and analysed for the binding of AV and uptake of PI using FC. Data are the mean ± s.e.m. from n=6 patients.
Together with the dose titration, this indicates that, for CLL cells to be sufficiently targeted with low effects on normal haemopoietic cells, that the best combination to use as a treatment would be BEZ+MPA+1.25µM lycorine.

5.4.3. The Mitochondrial specific antioxidant, MitoQ, is unable to rescue the cells from MSO production.

The anti-oxidant MitoQ, a ubiquinone derivative, was developed in 2001 to specifically locate to the mitochondria (Kelso et al., 2001). Subsequent studies have shown that MitoQ can successfully reduce myocardial ischemia (Neuzil et al., 2007), which has been linked with mitochondrial damage (Rodriguez-Cuenca et al., 2009). To date, no studies have been published using MitoQ to counteract mitochondrial damage exerted by exogenously added therapies. Nevertheless, it was hypothesised that MitoQ may be able to inhibit MSO production by cells treated with BEZ+MPA+2.5µMlycorine. A dose titration of MitoQ against CLL cells showed a mild dose dependent increase in MSO when MitoQ was used alone (figure 58). At the doses of 0.2 µM, very little MSO was induced alone but this dose was unable to counteract the MSO produced by BEZ+MPA+lycorine (figure 58).
Figure 58. MitoQ is unable to reduce the levels of MSO produced by BEZ+MPA+lycorine. CLL cells were cultured on CD40L-expressing stroma in a ratio of 10:1 in 96 well plates. Cells were treated in triplicate with a 2-fold titration (1μM to 0.2μM) of mitoQ in the absence (blue) and presence (purple) of BEZ+MPA+2.5μM lycorine. After 48 hours triplicate wells were harvested, pooled and analysed for MSO positivity using MitoSOX Red and FC. Data are the mean ± s.e.m from n=4 patients.
5.4.4. **When the CD40L is removed BEZ+MPA is as good at inducing apoptosis as the published study of dasatinib + fludaribine.**

As described in chapter 1, sections 1.10.1 and 1.21, in 2008 Hallaert et al published the potential use of the DSN to sensitise CLL cells to F-ara-A. Owing to the way the experiments had been carried out, attempts were made to recapitulate them, together with BEZ+MPA. Following Hallaert’s protocol of removing the CD40L signal and culturing the cells with treatments for a further 24 hours prior to analysis, DSN did not induce apoptosis alone, but the combination of DSN and F-ara-A did (figure 59). This confirmed the observation of Hallaert et al. Nevertheless, BEZ+MPA was equally as good at inducing apoptosis in this setting. Additionally, DSN did not have any additive effect on BEZ+MPA (figure 59).

5.4.5. **When the protective CD40L is maintained, unlike lycorine, DSN+F-ara-A no longer induce apoptosis.**

Whilst the above experiment serves to prove that DSN+F-ara-A could induce apoptosis of circulating B-CLL cells on release from the PC, it does not serve to prove that this combination would be effective at targeting cells within the PC. Indeed, as the combination was only effective as BEZ+MPA, it was hypothesised that in the same way as BEZ+MPA is unable to, the Hallaert combination would not be effective at inducing apoptosis within the PC. To investigate this, the treatments were repeated without the removal of CD40L thereby mimicking the PC, as already shown in this chapter.
Figure 59. DSN, BEZ+MPA and DSN+BEZ+MPA sensitise previously, CD40L stimulated CLL cells to F-ara-A 24 hours after the signal is removed. CLL cells were cultured on CD40L expressing stroma in a ratio of 10:1 in 96 well plates. Cells were treated in triplicate with solvent control, 30µM DSN, 5mM F-ara-A, DSN+F-ara-A, BEZ+MPA or BEZ+MPA+DSN. After 48 hours the cells were harvested from the stroma, washed and re-cultured with the appropriate treatments for a further 24 hours, as described in Hallaert et al. Triplicate wells were harvested, pooled and analysed for the binding of AV and uptake of PI using FC. Data are the mean ± s.e.m. from n=3 patients.
Figure 60. If the CD40 is not removed DSN does not sensitise CLL cells to F-ara-A. CLL cells were cultured on CD40 expressing stroma in a ratio of 10:1 in 96 well plates. Cells were treated in triplicate with solvent control, 1.25µM lycorine, 30µM DSN, 5µM F-ara-A, BEZ+MPA, BEZ+MPA+1.25µM lycorine, DSN+F-ara-A, or BEZ+MPA+DSN. After 72 hours, the cells were harvested, pooled and analysed for the binding of AV and uptake of PI using FC. Data are the mean ± s.e.m. from n=3 patients.
After 72 hours treatment, BEZ+MPA predictably did not induce any apoptosis of the CD40L stimulated CLL cells (figure 60). Additionally, and again as expected, DSN did not sensitise the CD40L protected cells to BEZ+MPA. As already shown, the combination of BEZ+MPA+1.25µM lycorine did successfully induce apoptosis of the cells. The lack of apoptosis caused by DSN+F-ara-A (figure 60) was particularly interesting. Consequently, the combination studied by Hallaert et al can only induce apoptosis of non-CD40L protected cells, in the same was as BEZ+MPA alone. However, BEZ+MPA+1.25µM lycorine consistently causes apoptosis of these cells.

5.4.6. **DSN+F-ara-A are not specifically targeted to CLL cells.**

Whilst DSN+F-ara-A did not cause apoptosis of CLL cells *in vitro* when the CD40L is maintained, it could be therapeutically useful to treat CLL cells that are in the periphery. The level of apoptosis induced by DSN+F-ara-A on the removal of CD40L was similar to that of BEZ+MPA alone. In a chapter 3, figures 12, 13 and 20, it was shown that BEZ+MPA had minimal effects on normal B cells. Therefore, the effects of DSN+F-ara-A against normal cells were investigated.

Both in the presence (figure 61a) and the absence (figure 61b) of CD40L DSN induced apoptosis of normal MNCs. It is well documented that F-ara-A is non-targeted and will induce death in many cell types, not only the malignant cells. Therefore, the level of apoptosis induced by F-ara-A in both the CD40L protected cells and the cells cultured without CD40L was to be expected (figure 61). This non-discriminatory induction of apoptosis seen in DSN or F-ara-A alone was increased when both agents were used together (figure 61). This was the same for the presence (figure 61a) and absence (figure
61b) of CD40, with the total AV positivity of the combination reaching around 60% in both culture conditions.

This data suggests that, while DSN+F-ara-A induce apoptosis of CLL cells, it is untargeted. The level of cell death of CLL cells using this published combination is no better than that of BEZ+MPA. As BEZ+MPA exert little effect on non-malignant cells the combination of BEZ+MPA would seem to be more beneficial than that of DSN+F-ara-A.

Further, the combination of BEZ+MPA+1.25µM lycorine *in vitro* is able to target the PC whilst leaving the normal cells intact.
Figure 61. DSN alone induces apoptosis of normal cells, which is potentiated by F-ara-A. MNCs from normal donors were cultured on a) CD40L expressing stroma or b) non-CD40L expressing stroma in a ratio of 10:1 in 96 well plates. Cells were treated in triplicate with solvent control or 30µM DSN in the absence (blue) and presence (purple) of 5µM F-ara-A. After 72 hours, triplicate wells were harvested, pooled and analysed for the binding of AV and uptake of PI using FC. Data are the mean ± s.e.m. from n=3 patients.
5.5. Discussion.

Targeting the PCs in CLL in vivo remains a challenge. In chapter 3, it was shown that BEZ+MPA induced apoptosis in the absence of CD40L, but not in its presence.

Investigations into the use of novel compounds has revealed that whilst agents such as the DNA intercalator Fe cylinder can reduce proliferation of the AML cell line HL-60, it is unlikely to be of therapeutic use in CLL.

Plant compounds and their use in cancer, are a current area of research. The use of jasmonates have been investigated in leukaemia including CLL by other research groups (Rotem et al., 2005) and have been shown to cause mitochondrial damage. Here we have shown in the AML cell line KG1a that, MeJ causes ΔΨm depolarisation, and induces apoptosis with MSO dose dependently. However, on investigation in CLL, MeJ alone exerted very little effects and in combination with BEZ+MPA only the highest dose tested (of 1mM) was able to induce apoptosis and MSO.

The experiments shown here, using lycorine and BEZ+MPA, highlight that as a proof of principle, apoptosis can be induced when CD40L is continually present. This is significantly different to other studies on DSN+F-ara-A, where apoptosis is dependent on the removal of the protective signal. Additionally, normal cells remain relatively unaffected by the combination of BEZ+MPA+1.25µM lycorine, whereas DSN and F-ara-A cause significant apoptosis of normal cells.

The data presented here, strongly suggests that the apoptosis caused by the combination BEZ+MPA+1.25µM lycorine is most likely to be due to the production of MSO by the treated cells.
6. General Discussion
6.1. The case for rational drug redeployment in CLL.

To date CLL remains a disease that requires new therapies that aim to improve response rates, progression free survival and OS. Whilst research into new drugs should provide improvements, the time from concept to clinic is likely to be lengthy. Therefore, it is pertinent to consider the redeployment of existing drugs, either alone or in combination with other therapies for the treatment of this disease. Such strategies in haematological malignancies have demonstrated the substantial impact that existing agents can exert. Examples include the use of retinoic acid (RA) (Fenaux et al., 2007) and arsenic trioxide (Chen et al., 2007) in APL and thalidomide in multiple myeloma (MM) (Prince et al., 2007). These paradigms illustrate that drugs, not originally considered as cancer therapeutics, can provide genuine benefit. Screening ‘old’ therapies in CLL in vitro has already proven to be beneficial in relation to bendamustine. The in vitro studies of this agent alone against CLL cells (without CD40L) identified that at 72 hours this agent was able to induce a median apoptosis of around 40% (Schwanen et al., 2002). Such studies have led to the use of this agent in clinical trials, which have shown around a 30% CR (Kath et al., 2001, Knauf et al., 2009).

Other studies from our group have highlighted the potential of BEZ and MPA in AML and BL (Fenton et al., 2003, Khanim et al., 2009, Murray et al.). Here the potential for redeployment of the same drugs in CLL was investigated.

6.1.1. Is the redeployment of MPA and BEZ feasible?

MPA, best known as the contraceptive Provera, has been successfully used to treat breast (Davila et al., 1988) and endometrial (von Minckwitz et al., 2002) cancers. The doses used in these settings (up to a 1000mg/day) achieve plasma levels up to 15µM
(Ohtsu et al., 1998). The dose titration of MPA against CLL shown in figures 6 and 15 indicates that the doses of 5µM MPA that were subsequently selected for further study, were sub-optimal in the generation of apoptosis or the arrest of cell division. However, this dose of 5µM is commensurate with that achievable in patients. Notably, with BEZ the standard dose used to treat hyperlipidemia is 400 mg of Bezalip Mono, taken once a day. This dose is associated with a plasma level of 25µM. Although the in vitro dose of 0.5mM used here to exert the pro-apoptotic (figure 6) and anti-proliferative actions (figure 15) of BEZ exceed this by twenty fold, investigations of overdose in humans and exposure in rhesus monkeys indicates that thirty times the standard daily dose is tolerated without ill effect, for up to a year (Yoshikuni et al., 1988). It seems likely, therefore, that the drug dose used herein can be achieved and tolerated in patients.

Following the parallel laboratory study of the actions of BEZ and MPA in AML (Khanim et al., 2009), a pilot phase I/II study has been undertaken in 20 elderly or relapsed AML patients (Murray et al.). This study aimed at investigating the safety of combining BEZ and MPA in vivo and was restricted to using only those maximal doses that had been previously administered to patients. Despite the known shortfall in the drug doses achieved, compared to those used both in the in vitro studies of AML and this study of CLL, strong indications of potential clinical benefit were observed. This included one patient who, although classified as a PR, had reduced blast count and was both platelet and red cell-transfusion independent for over 201 weeks and is currently fit and well (Murray et al; 2009 in press). These benefits observed following BEZ+MPA therapy were associated with very little toxicity. Despite the positive outcomes observed, the investigation was stopped due to the fact that little or no toxicity was observed and owing
to the concomitant laboratory studies that, as discussed here, achieved better responses at
higher does. Murray et al (2010) therefore proposed the use of six 400mg tablets twice
daily. This dose has been demonstrated to give peak bezafibrate plasma concentration of
264µM, without significant toxicity (Abshagen, et al 1980, Dollery 1999). Therefore, if
trialled in CLL, the doses of Provera and Bezalip Mono should be 1000mg and 3600mg per
day respectively.

6.2. The potential of the combination of BEZ+MPA and its use in combination with
chlorambucil.

The in vitro studies shown here demonstrated that while the overall response rate of CLL
cells to BEZ and MPA was high, the combination of BEZ+MPA proved to be most
effective at inducing apoptosis and reducing proliferation in the absence and presence of
CD40, respectively. Nonetheless, there was some underlying heterogeneity in responses,
especially in the absence of CD40. Extensive statistics revealed that this heterogeneity
did not correlate with acknowledged prognostic indicators namely of Binet stage, LDT,
CD38 expression, ZAP-70 positivity, chromosomal abnormalities or IgVH mutations.
Considering chromosomal alterations, in particular the TP53 deletion, the finding that
BEZ+MPA response does not correlate with any investigated deletion could be
encouraging. This indicates that BEZ+MPA, unlike most common therapies (Pepper,
2008) (except flavopridol and antibody therapies), is unlikely to be dependent on the P53-
apoptosis pathway. Nevertheless, as yet, the cellular and/or molecular basis of the
heterogeneous response to BEZ+MPA remains unknown. As with all current CLL
therapy, it is likely that if BEZ+MPA became an established form of treatment some
patients would benefit from this therapy more than others. Forthcoming publications on
risk and prognosis in CLL could elucidate this. For example, new risk variants in CLL, following SNPs analysis, such as 2q37.3, 8q24.21, 15q21.3 and 16q24.1 (Crowther-Swanepoel et al.) and/or the newly modified prognostic indicator glomular filtration rate (GFR) adjusted B2M (GFR-B2M) (Delgado et al., 2009b) were not assessed here. Such comparisons, theoretically, may identify patients likely to respond to therapy. However, such methods, especially SNPs analyses are expensive, therefore, it would be less costly, and interesting to consider whether in vitro responses to BEZ+MPA were to reflect in vivo responses. In this regard, if a clinical trial on refractory and relapsed patients were to be undertaken, patients’ pre-treatment samples should first be subjected to BEZ+MPA in vitro and analysed by the assays used here such as MitoSOX. This will determine whether these in vitro analyses will, in future, be able to identify patients likely to benefit.

Despite the ability of BEZ+MPA to successfully induce apoptosis, in most patient samples tested, in the absence of CD40L, and reduce proliferation in its presence, it is likely that to proceed with a phase I/II trial, ethically, additional chemotherapeutics may have to be co-administered. Whilst F-ara-A is the most commonly used agent, this was not assessed here. There is also some clinical debate over the advantage of F-ara-A over chlorambucil, especially in elderly patients (Jaksic and Brugiatelli, 1988, Jaksic et al., 1997). Additionally bendamustine was compared clinically against chlorambucil rather than F-ara-A (Knauf et al., 2009). Therefore the studies here only utilised chlorambucil, in comparison and as adjunctive treatment with BEZ, MPA and BEZ+MPA. Whilst chlorambucil was effective alone, its addition to BEZ+MPA generated the most apoptosis and the highest reduction in proliferation. This additive or perhaps synergistic effect is likely to be due to different mechanisms of the agents. In this regard, BEZ+MPA successfully induced MSO in the
absence of CD40L whereas chlorambucil did not. It has previously been suggested that inducing oxidative stress within cells, sensitises them to chlorambucil (Boldogh et al., 2003). BEZ, MPA and BEZ+MPA all increased ROS in both the presence and absence of CD40L. Therefore, it is possible that the combination of BEZ+MPA+chlorambucil is effective due to the elevation of ROS by BEZ+MPA and, thereby, the sensitisation of CLL cells to chlorambucil. To make the combination of BEZ+MPA more attractive to clinicians further studies should investigates its combinatorial effects with the more commonly used F-ara-A.

6.2.1. The proliferative and protective properties of CD40L render the effects of BEZ+MPA different to those observed in the absence of CD40L.

Whilst BEZ+MPA induced apoptosis in culture conditions used to represent CLL cells in the periphery, apoptosis was not observed where CD40L was used to mimic the PC niche. It is possible that apoptosis was not observed, owing to its delay by the protective action of CD40L rather than its prevention. A time course revealed that whilst apoptosis in the absence of CD40L peaked between 24 and 48 hours, even at 72 hours no apoptosis could be seen in the presence of CD40L. It was not possible to extend this time course due to the culture system being over stimulated and apoptosis occurring in the controls. This culture exhaustion was not surprising as studies have alluded to the peaking of gene expression at 72 hours (Gricks et al., 2004) and thymidine incorporation has been demonstrated to peak at 72 hours and rapidly decline after one week in culture (Crawford and Catovsky, 1993).

This study was able to recapitulate the observations of others that co-culture of CLL cells with stromally delivered CD40L and IL-4 (Willimott et al., 2007a, Willimott et al., 2007b, Willimott et al., 2007c, Hallaert et al., 2008, Vogler et al., 2009) induced cell proliferation.
The level of proliferation was extremely variable. However, one of the first studies on the *in vitro* use of anti-CD40 antibodies and IL-4 additionally showed patient to patient variability. Such increased proliferation could be postulated to be due to variation in CD38 level or CD38 induction owing to the increasing evidence of a CD38 role in the microenvironment (Calissano et al., 2009, Patten et al., 2008, Pepper et al., 2007). However, studies by Willimot *et al* have shown that this is unlikely to be the case (Willimott et al., 2007a). The proliferation of CLL cells when engaged by CD40L and aided by IL4 was reduced by both BEZ and MPA but most significantly the combination of the agents (figure 19). Once again, this was not associated with any of the known prognostic indicators and, notably, there was less heterogeneity in this response between individuals compared with their apoptotic response. Previous studies have calculated the clonal expansion within PCs to be around $10^9$ to $10^{12}$ cells per day. Therefore, despite the lack of apoptosis induced by BEZ+MPA in the presence of CD40L they could have significant impact on reducing the CLL clone, in turn affecting the number of CLL cells entering the circulation.

In addition to the increase in proliferation afforded by CD40L and IL-4, there have been reports that the CD40L provides CLL cells with chemoresistance (Kater et al., 2004, Kitada et al., 1999) and in 2009, Vogler *et al* demonstrated that CLL cells exposed to CD40L have 1000-fold resistance to the novel therapeutic ABT-737 (Vogler et al., 2009). Microarray studies have shown that CLL cells exposed to CD40L upregulate many genes encoding for anti-apoptotic proteins (Gricks et al., 2004) which is likely to contribute to the lack of apoptosis observed in this environment. A recent suggestion is that circulating CLL cells are able to migrate to and re-engage with PCs within the LN. The LN provides these CLL
cells with survival signals from CD40L, causing the development of pro-survival niches that are widely believed to be the source of relapse and therapy resistance (Hallaert et al., 2008, Hartmann et al., 2009). The lack of apoptosis induced by BEZ+MPA in the presence of CD40L fits with this theory.

6.3. The possible mechanism of BEZ activity against CLL cells.

The actions of BEZ when used alone or in combination with MPA were associated with marked elevations of PGD₂ compared to the untreated controls. The actions of MPA were not associated with the elevation of PGD₂, nor did MPA enhance its elevation by BEZ. The argument that BEZ exhibits anti-leukaemic actions against CLL cells via PGD₂ is supported by the observations that exogenously added PGD₂ or its dehydration product 15dΔ¹²,¹⁴PGJ₂ induced CLL cell apoptosis (in the absence of CD40L) in the micromolar range. Similar pro-apoptotic and anti-neoplastic actions of 15dΔ¹²,¹⁴PGJ₂ have been demonstrated against multiple myeloma cells, non-Hodgkin lymphomas, including BL (Eucker et al., 2004, Eucker et al., 2006, Higashiyama et al., 1996, Padilla et al., 2000b, Ray et al., 2006, Ray et al., 2004) and a number of non-haemato-lymphoid cancers, including neuronal (Kondo et al., 2002), oral sarcoma (Nikitakis et al., 2002), hepatoma (Date et al., 2003), breast cancer (Liu et al., 2003), anaplastic thyroid carcinoma (Hayashi et al., 2004), glioblastoma (Morosetti et al., 2004) and colorectal cancer (Lin et al., 2007). However, it should be noted that these effects were seen at lower doses than those used here. Previously, BEZ has been reported to induce oxidative stress (Qu et al., 2001). This has been further demonstrated here, with BEZ and BEZ+MPA inducing ROS within CLL cells. Oxidative stress has been strongly linked to LP (Montuschi et al., 2007, Montuschi et al., 2004), which in turn has been linked with the formation of IoP-PGs via the COX
independent LLP (Morrow et al., 1992a, Morrow et al., 1992b). Therefore, the elevated levels of \( \text{PGD}_2 \) following BEZ and likewise, the BEZ component of BEZ+MPA treatment are most likely due to this pathway.

Collectively this study and those of others indicate that drugs leading to the accumulation of \( 15\Delta^{12,14}\text{PGJ}_2 \) by tumour cells may have widespread clinical benefit. In pursuit of this, a recent study identified that a novel cycloanthranilylproline derivative, Fuligocandin B (FCB) increased \( 15\Delta^{12,14}\text{PGJ}_2 \) levels in adult T cell leukaemia cells and thereby increased their apoptotic response to TNF–related apoptosis-inducing ligand (TRAIL) (Hasegawa et al., 2007). However, the data shown here and this group’s studies in AML, indicate that similar elevation of \( 15\Delta^{12,14}\text{PGJ}_2 \) can be achieved using a drug that is already available.

On a cautionary note, it has been demonstrated that elevated LpL is a poor risk factor in CLL (Heintel et al., 2005), that the LpL-inhibitor orlistat induces apoptosis of CLL cells \textit{in vitro} (Pallasch et al., 2008) and that BEZ elevated LpL in hypertriglyceridemic patients during BEZ administration (Totsuka et al., 2000). The effects of BEZ against LpL should be investigated further.

6.4. The possible mechanism of MPA activity against CLL cells.

The anti-leukaemic actions of MPA against AML cells has been shown to include the inhibition of the \( \text{PGD}_2 \) ketoreductase activity of AKR1C3 (Khanim et al., 2009). However, whilst AKR1C3 was readily detected in CLL cells, \( \text{PGD}_2 \) ketoreductase activity could not be detected; an observation consistent with the fact that MPA alone or in combination with BEZ did not generate an increase in \( \text{PGD}_2 \) levels. Investigations using other known AKR1C3 inhibitors also revealed that none tested were able to recapitulate the effects of MPA alone or when combined with BEZ. These observations indicate that AKR1C3 is
unlikely to be the target of MPA in mediating its activity against CLL cells and indicates the likelihood of an alternative and PGD2 ketoreductase independent target. The presence of a non AKR1C3 target for MPA in B-cell malignancies was further supported by observations that the AKR1C3 negative line Nalm6 was sensitive to both MPA and BEZ+MPA treatment.

Other known targets of MPA include GR and PRs (Bentel et al., 1999, Kawaguchi et al., 2006, Poulin et al., 1989). Experiments using ligands and antagonists of these receptors demonstrated that ML-PRD mimics the effects of MPA alone and in combination with BEZ. The apparent synergy of ML-PRD with BEZ is complementary to a recent clinical trial in CLL in which high dose ML-PRD was shown to increase patient response to rituximab, with low associated toxicity (Castro et al., 2009). However, investigations demonstrated that the actions of MPA and ML-PRD, although similar, are unlikely to be mediated by PR or GR, as investigations using PR antagonists and GR agonists and antagonists were unable to recapitulate or reduce their effects. Despite this it should be noted that the GR agonist DEX and the PR agonist PRD have been used as CLL therapy. HD SPN, a MR antagonist, also exerted similar levels of apoptosis to MPA but again the experiments shown here were unable to demonstrate that this activity is mediated via the MR. Whilst the effects of ML-PRD and SPN are small it should be noted that the level of apoptosis generated by either agent is in line with the average response seen to MPA. Furthermore, combining either ML-PRD or SPN with MPA or BEZ+MPA, failed to abrogate or add to the effect of MPA on CLL cells. Therefore, it is plausible that all three of these agents share an undocumented common target. However, owing to the
differences in doses required to exert their effects (5µM MPA, 20µM ML-PRD and 1µM SPN) it is probable that SPN and MPA have higher affinities for this target than ML-PRD. Others have identified that MPA enhances anthracyclin uptake by CLL cells and increases apoptosis of cultured CLL cells exposed to the anthracyclin idarubicin (Florio et al., 2003, Pagnini et al., 2000). These observations are possibly explained by the chemosensitising actions of MPA acting as an inhibitor of the ATP-binding cassette (ABC) drug resistance transporter p-glycoprotein (PgP; MDR1) (Zibera et al., 1995).

Further studies could investigate whether MPA is aiding the uptake of BEZ. However, this is unlikely to explain the MPA alone response.

The data demonstrating that MPA effects were not mediated via enhanced PGD2 synthesis were unexpected. Nevertheless, MPA did contribute to ROS and MSO generation in CLL cells and exerted both pro-apoptotic and anti-proliferative effects independently of BEZ. However the mechanism of the generation of ROS without an accumulation of isoprostane-PGD2 is uncertain.

**6.4.1. Dissecting the variability in response**

As previously discussed, the heterogeneity in response did not correlate with any known factors. The patient responses, in the absence of CD40L, could be categorised into non-responder, single agent responder, apparent synergistic responder or additive responder (figure 9). It would be of clinical and scientific relevance to elucidate the variance in drug response. Such studies could be investigated in one of the many CLL mouse models (Michie et al., 2007), such as NZB mice. However, in light of the hepato-toxicity of BEZ in mice this would be problematical. An alternative would be the use of metabolomic analyses which are gaining interest in CLL (Macintyre et al.). Similar profiling has been
employed in AML following BEZ+MPA treatments AML (Tiziani et al., 2009) and such profiling may give indications to the mechanism of action of MPA.

6.5. The relative importance of induction of ROS and MSO for BEZ and BEZ+MPA induced CLL cell apoptosis

ROS has been shown to be increased in cells that have acquired mtDNA mutations following chemotherapy (Carew et al., 2003), thereby indicating an increased ROS to be advantageous to survival of the malignant clone. As increased ROS was also seen in CLL cells exposed to CD40L it is likely that ROS alone is not the mechanism of cell death. Therefore, as well as the generation of ROS, BEZ and BEZ+MPA were investigated for the production of MSO. BEZ has been reported to cause mitochondrial damage and mitochondria specific ROS in rodent hepatocytes (Qu et al., 2001). Therefore it was not surprising that BEZ alone and, most potently in combination with MPA, induced MSO. However, this was only the case in the absence of CD40L. Whilst ROS was generated in both culture conditions, MSO was only detected in the non-CD40L cultured cells. These data indicated that CD40-ligation protects CLL cells against BEZ+MPA induced MSO production and reciprocally that the induction of MSO is essential to achieve BEZ+MPA apoptosis. That BEZ+MPA induced apoptosis is particularly associated with an MSO mediated mechanism is supported by the observation that chlorambucil successfully induced apoptosis in the absence of CD40L without generating significant increases in MSO (chapter 3 figure 47). However, it should be noted that this observation is based on a small sample of CLL cells (n=4) amongst whom apoptosis responses were not as strong as the overall responses across the whole study. Consequently, it may remain a possibility that a strong chlorambucil response also requires high MSO production.
6.6. **Targeting the T cell-B cell interaction.**

Whilst BEZ+MPA may have therapeutic benefit in CLL by reducing the circulating CLL cells, the lack of cell death in the presence of CD40L limits the potential of the approach. For the best treatment outcomes it would be desirable to maintain the reduction in cell expansion by BEZ+MPA, whilst also reinstating the pro-apoptotic effects afforded by BEZ+MPA, observed when CD40L is present. Theoretically, in a clinical setting this could lead to reduced disease and ultimately CR with MRD negativity. It was hypothesised that the introduction of a third novel agent with a mechanism of action distinct but complementary to BEZ+MPA may achieve this.

Investigations into identifying this compound first led to the use of Fe cylinder, which has been suggested to be a possible alternative to cisplatin. Initial studies used this compound against the AML cell line HL-60 and showed that moderate apoptosis was induced, with cell cycle arrest (figure 48). Whilst this agent exerted no determinable effect against CLL cells, it should not be ruled out of the leukaemic arena completely. It could be exploited as combination therapy in AML and its intercalating DNA properties and potential induction of cellular senescence could make it a useful agent against malignant cells with a high proliferative index such as BL. Although on a cautionary note this would be expensive to develop and administer.

Previous studies have demonstrated that transcription of MSO antioxidant mnSOD is upregulated in cells exposed to TNF-α. As CD40 and its ligand are members of the TNF super-family, it is possible that CLL cells exposed to CD40L also upregulate mnSOD to levels sufficient to overcome the pro-apoptotic effects of BEZ+MPA induced MSO. Thus a
desired characteristic of an agent to be used alongside, or indeed in place of, BEZ + MPA, would be the induction of MSO despite the presence of CD40L.

Investigations into the plant hormones JA and MeJ against the AML cell line K562 revealed that, except at high doses, JA exerts little effects whilst MeJ causes Δψm depolarisation, and induces apoptosis with MSO dose dependently (figures 50 and 51). This was postulated to be due to the methyl group aiding cellular uptake. Indeed, investigations into this proved this to be the case (Davies et al., 2009). Owing to its PGD2-like structure and its ability to inhibit AKR1C3 (Davies et al., 2009), MeJ could be considered to be exploited as anti-AML therapy. It is most likely that this would be best used in combination with other agents, in order to reduce the concentrations required to generate the required effects. However, despite its reported interference on the mitochondrial membrane bound enzyme hexokinase (Goldin et al., 2008), on investigation in CLL, MeJ alone exerted very little effects and, in combination with BEZ+MPA, only the highest dose tested (of 1mM) was able to induce moderate apoptosis (figure 52) and MSO (figure 53). Additionally, whilst MeJ has been shown to be exerting activities against AKR1C3 in AML this was not the case here. Together with the current lack of FDA approval for the use of this agent, collectively these data preclude this agent as a viable option for further investigation for the targeting the PC in CLL.

Recapitulation of the work by Hallaert et al, using DSN to sensitise CLL cells to F-ara-A, demonstrated that this synergy only occurred upon the removal of CD40L (figures 59 and 60). This, in effect, correlates with Amrein et al (Amrein et al., 2008) who concluded that DSN sensitises CLL cells to chemotherapeutics in the absence of CD40L. Additionally it is worth considering that in the experiments shown here, the combination of F-ara-A+DSN
was only as good as BEZ+MPA at inducing apoptosis. Additionally, DSN did not sensitise CLL cells to BEZ+MPA in the continual presence of CD40L. Therefore, it is unlikely that combination therapy utilising DSN would target cells within the PC.

Lycorine has previously been shown to induce apoptosis of APL cells (Liu et al., 2004, Liu et al., 2007) and has been shown to exert its effects via mitochondria (Del Giudice et al., 2005) making it of interest to this study. It was observed that, whilst neither lycorine nor BEZ+MPA alone induced apoptosis in CD40L protected CLL cells, the combination of all three agents produced profound apoptosis (figure 54), which was associated with the reinstated generation of MSO (figure 55). The level of apoptosis observed was dose dependent and was tightly correlated with MSO (figure 56). These data strongly indicate that a mechanism that is able to generate cell death in the presence of CD40L is likely to sufficiently increase the level of MSO to that which the cell cannot overcome.

More elderly CLL patients are commonly excluded from clinical trials due to their susceptibility to the development of treatment-related cytopenia and infections. Thus in these patients in particular more targeted therapies that spare non-tumour cells are urgently required. Studies on normal donor cells indicated that BEZ+MPA had little effect on CD19+ve cells (figures 13 and 21). Additionally, MNCs isolated from normal donors remained relatively unaffected by the combination of BEZ+MPA+ lycorine (figure 57). However, the combination of DSN and F-ara-A caused significant apoptosis (figure 61). This further indicates that therapy utilising BEZ+MPA and lycorine has better potential for the treatment of CLL.
6.7. Conclusion.

New drug discovery is time consuming, expensive and has low success rates. Increasingly, there is an interest in utilising existing drugs to target different diseases (Keiser et al., 2009, Chong and Sullivan, 2007) and a call for a better ‘off-label’ FDA approved prescribing system (Tabarrok, 2009). Currently, the cure rate for CLL is low and many patients are not treated as a first line of therapy. Many patients are unable to tolerate intensive treatments because of advanced age or a poor performance status. The use of low-cost, low-toxicity, therapy could be advantageous both clinically and financially. The in vitro data acquired here demonstrates the powerful effects of BEZ+MPA by apoptosis in the absence of CD40L and reducing the proliferative capacity in the presence of CD40L. In vivo BEZ+MPA, with or without chemotherapy, are likely to exert these effects on the peripheral and PC compartments respectively with little side effect (as demonstrated in the AML phase I/II trial) and should be considered for a phase I/II clinical trial. Considering the possible transition of MBL to CLL, BEZ+MPA could also be considered in these patients, as a cost-effective attempt to cease the progression.

In order to fully target the PCs and gain a MRD negative state, further investigation into the addition of lycorine to BEZ+MPA should be considered. Currently, many plant compounds are being investigated with the intention of gaining FDA approval for their use therapeutically. Recently, the prescription drug, Reminyl was released for the treatment of Alzheimer’s disease. The active ingredient of this compound is galanthamine, another Amaryllidaceae alkaloid (McNulty et al., 2009, Houghton et al., 2006). Therefore, a precedent has been set for the use of this family of agents in the treatment of diseases. Thus, the concept of lycorine gaining FDA approval for therapeutic
use is a real possibility. Nonetheless, the time scale and cost of developing new drugs is limiting and, in addition, the \textit{in vivo} toxicity of lycorine is as yet unknown and will have to be determined prior to possible trials in combination with BEZ+MPA. However, the data shown here indicate that only a relatively low dose of lycorine of 1-2\(\mu\)M would be required to be effective.

Whilst it is certain that CD40\(_L\) and IL-4 play a role in the PC, there are likely to be other environmental factors that offer CLL cells protection. Until these are elucidated further, the targeting of the CD40\(_L\) protective niche is a positive starting point. As proof of principle, lycorine serves to show that the anti-apoptotic signal afforded by CD40\(_L\) can be overcome by the generation of MSO. Such targeting of the cell-cell interaction \textit{in vitro}, mimicking that of the B cell-T cell interaction \textit{in vivo} reinstates the pro-apoptotic actions of BEZ+MPA seen in the absence of CD40\(_L\). Utilising the concept, further proven here, that already existing drugs can be beneficial in new settings, it is likely that an agent that is already available can target this cell-cell interaction, in the same way as lycorine. With the importance of MSO generation highlighted here, drug screening together with BEZ+MPA should be undertaken with MSO as the readout in an attempt to find a suitable candidate.
Figure 62. A proposed schematic of the pro-apoptotic mechanism of BEZ against CLL cells cultured in the absence of CD40L. BEZ induces the production of ROS, as measured by H2DCFDA staining and FC. We propose that, like in our AML studies, ROS attacks membrane lipids, inducing lipid peroxidation and the production of PGD$_2$, most likely via the isoprostane pathway. PGD$_2$ was found to be elevated in extracts of CLL cells with their supernatants. If not exported the PGD$_2$, will spontaneously convert to 15d$\Delta^{12,14}$PGJ$_2$ which causes damage to mitochondrial membranes and thus the production and leaking of MSO as detected by MitoSox Red and FC. A high level of MSO could inhibit mitochondrial metabolism and ultimately cause cell death. Note that in CLL both BEZ and MPA induce the production of MSO, that is increased in the combination of BEZ+MPA.
References


References

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References


Appendix A1: Buffers and Recipes

2.1.2. MACS buffer
PBS with the addition of 2mM EDTA (filtered to sterilise and degas) and 1% (v/v) FBS. Keep at 4ºC.

2.2. Freezing mix
FBS supplemented with 5% (v/v) DMSO to be made fresh each time

2.4.2. FACS Fix
PBS with the addition of 1%(v/v) formaldehyde and 2% (v/v)FBS. Keep at 4ºC.

2.8.3. Cell Cycle Buffer
30µg PI, 0.1mM NaCl, 1% (w/v) sodium citrate, 0.1% Triton X100 in ddH₂O

2.11.4. 10x TBE
108g Tris base, 55g Boric acid, 9.3g EDTA made up to 1L in ddH₂O. Dilute to 1 x before use.

2.14.1. RIPA buffer
1% (v/v) NP40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS in distilled water. Keep at 4ºC. Add protease inhibitor prior to use.

2.14.2. 4x SDS gel loading buffer
62.5mM Tris HCl pH6.8, 25% (v/v) glycerol, 2% (v/v) SDS, 5% (v/v) 2-β-ME, Bromophenol Blue, in distilled water. Store at RT.

2.14.2. 10% Resolving gel

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% Bis/Acrylamide (Geneflow)</td>
<td>3.3ml</td>
</tr>
<tr>
<td>1.5M Tris HCl pH8.8</td>
<td>2.5ml</td>
</tr>
<tr>
<td>10% (v/v) SDS</td>
<td>0.1ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>4.1ml</td>
</tr>
<tr>
<td>10% Ammonium persulphate ((w/v)APS; Sigma)</td>
<td>60µl</td>
</tr>
<tr>
<td>TEMED (VWR international)</td>
<td>4.5µl</td>
</tr>
</tbody>
</table>
2.14.2. **Stacking gel**

30 % Bis/Acrylamide 440µl
0.5M Tris HCl pH6.8 830µl
10% (w/v)SDS 33µl
distilled water 2.03ml
10% (w/v)APS 16.7µl
TEMED 1.7µl

2.14.3. **1x SDS gel running buffer**

25mM Tris, 192mM glycine, 3.5mM SDS in distilled water

2.14.3. **Towbin’s transfer buffer**

25mM Tris, 192mM glycine, 20% (v/v) methanol in distilled water

2.14.4. **TBS-T**

137mM NaCl, 20mM Tris HCl pH 7.6, 0.2% (v/v) Tween20 in distilled water

2.14.4. **5% blocking solution**

5% (w/v) milk powder (Marvel) in TBS-T
Appendix A2: Manuscripts relating to the contents of this thesis.

Chapters 3 & 4.


http://www.nature.com/leu/journal/v23/n2/full/leu2008283a.html

Chapter 5.


http://www.sciencedirect.com/science?_ob=ArticleURL&_udi=B6VRP-4V5XG3C-5&_user=122868&_coverDate=12%2F22%2F2008&_alid=1372740809&_rdoc=2&_fmt=high&_orig=search&_cdi=6240&_sort=r&_docanchor=&view=c&_ct=2&_acct=C000010083&_version=1&_urlVersion=0&_userid=122868&md5=e8327b9c195d68e03a0d67a765f68856


http://cancerres.aacrjournals.org/content/69/11/4769.full?sid=5451722f-8139-4bee-a2c3-7b5183cc68b5