Targeting CD40 stimulation-mediated resistance to ABT-199 (venetoclax) with a combinational therapeutic approach in chronic lymphocytic leukaemia

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By

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Abstract

Chronic lymphocytic leukaemia (CLL), the most common form of adult leukaemia in the West including the UK, is characterised by accumulation of the malignant mature B lymphocytes in the blood, bone marrow and secondary lymphoid tissues. Overexpression of BCL-2 is a feature of CLL cells, making BCL-2 a valid target for therapeutic intervention. Indeed, ABT-199 (also known as venetoclax), a BH3 mimetic specifically interrupting the interaction of BCL-2 with apoptotic members of BCL-2 family of proteins, has been developed for clinical use and displayed remarkable anti-tumour activity in CLL. However, resistance to ABT-199 in CLL patients receiving treatment has recently been reported. *In-vitro* studies have also shown that ABT-199 is not effective in inducing cell death in CLL cells that have encountered the pro-survival stimuli present in the CLL microenvironment. One such stimulus involves the engagement of CD40 on CLL cells by CD154 from the non-malignant T cells in the lymph node microenvironment, resulting in strong survival signalling in CLL cells. Previous studies in the laboratory have shown that CD40 stimulation potently protect CLL cells from spontaneous and drug-induced cell death and that the protection is associated with up-regulated expression of anti-apoptotic proteins of BCL-2 family such as MCL-1 and BCL-XL. The aim of my study is thus to investigate whether CD40 stimulation will lead to the resistance of primary CLL cells to ABT-199-induced apoptosis and, if so, whether inhibition of MCL-1 or BCL-XL can reinstate the sensitivity of CD40-stimulated CLL cells to ABT-199. Here, I have shown that CD40 stimulation indeed significantly reduced the sensitivity of CLL cells to ABT-199 and that the reduction in sensitivity is associated with upregulated expression of BCL-XL and MCL-1 in CD40-stimulated CLL cells. Inhibition of MCL-1 by voruciclib (a small molecule flavone derivative potently inhibiting the expression of MCL-1) neither induced a significant amount of cell death nor sensitised CD40-stimulated CLL cells to ABT-199. In contrast, inhibition of BCL-XL by A-1331852 (a novel BH3 mimetic specifically targeting BCL-XL) significantly increased sensitivity of CD40-stimulated CLL cells to ABT-199. From a mechanistic point of view, I showed that A-1331852 acted through disrupting the interaction of BCL-XL with BIM in CD40-stimulated CLL cells, thus enabling released BIM to activate cell death. I have further demonstrated that BIM was required for ABT-199-induced cell death since reduction in BIM expression using siRNA knockdown technique led to significant decrease in cell death induced by ABT-199. Therefore, my study provided experimental evidence that pharmacological inhibition of BCL-XL can potentially overcome CD40 stimulation-mediated resistance to ABT-199 in CLL.

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Declaration

I declare that all of the work presented in this thesis is my own work.

Chapter 1. Introduction

1.1 Chronic lymphocytic leukaemia

Chronic lymphocytic leukaemia (CLL) is a malignant tumour characterized by the accumulation of mature B lymphocytes in the blood, bone marrow, and secondary lymphoid tissues, resulting in lymphocytosis, bulky lymph nodes and enlarged spleen (Kipps et al., 2017).

CLL is a highly heterogeneous disease, with individual patients following different disease courses. Some patients can live almost a normal life without need for treatment, but others develop aggressive disease rapidly, undergo repeated treatment episodes, and eventually develop relapsed disease, resulting in mortalities (Kipps et al., 2017).

CLL patients can now be divided into two main subgroups, due to different mutation states of the immunoglobulin heavy-chain variable region gene (IGHV) from the CLL cells. Patients with CLL cells expressing IGHV mutations have worse prognosis.(Hamblin et al., 1999, Damle et al., 1999, Tobin et al., 2002, Ghia et al., 2008).

1.1.1 Epidemiology

CLL is a common adult leukaemia, especially in Europe and the Americas. According to 2009-2015 dates from the National Cancer Institute in the United States of America (USA) (http://seer.cancer.gov/statfacts/html/clyl.html), the number of new cases of CLL was 6.8 per 100,000 for males and 3.5 per 100,000 for females per year. The number of deaths was 1.8 per 100,000 for males and 0.8 per 100,000 for females per year. According to the data of the National Cancer Institute, the diagnosis rate of CLL is about 0.6%. In 2016, approximately 178,206 people in the United States had CLL, and the new CLL case has maintained a stable ratio over the past decade. Due to the maturity of treatment options, CLL mortality rates have declined by an average of 2.9% per year between 2007 and 2016. (http://seer.cancer.gov/statfacts/html/clyl.html).

In the United States, in 2019, the number of new cases of CLL is 20,720. It accounts for 1.2% of all complementary cancer cases. CLL deaths were 3,930, which accounts for 0.6% of all cancer deaths. From 2009 to 2015, the 5-year survival rate was 85.1% (http://seer.cancer.gov/statfacts/html/clyl.html).

CLL is also a very common type of leukemia in the UK. According to data from Cancer Research UK 2014-2016, CLL increased patients accounted for about 1% of all new

cancer cases, and about 3,800 newly diagnosed cases. Among them, about 2,300 were males and about 1,400 were females. The highest age of onset of CLL is 85 to 89 (2014-2016).(https://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/leukaemia-cll/incidence#heading-Zero).

1.1.2 Clinical staging systems

Two main types of CLL clinical staging systems: Rai staging and Binet staging.

1.1.2.1 Rai staging system

Dr Rai and his team developed a clinical staging method based on the characteristics of CLL disease in 1975. This method mainly diagnoses the disease based on the clinical manifestations of patients and predicts the prognosis of patients (Rai et al., 1975). It is described as follows:

Stage 0--characterised by absolute lymphocytosis (i.e. >15,000/mm^3) without lymphadenopathy, hepatosplenomegaly, anaemia, or thrombocytopenia. Median survival from diagnosis is usually >150 months.

Stage I--characterised by absolute lymphocytosis with lymphadenopathy but without hepatosplenomegaly, anaemia, or thrombocytopenia. Median survival from diagnosis is about 101 months.

Stage II--characterised by absolute lymphocytosis with either hepatomegaly or splenomegaly with or without lymphadenopathy. Median survival from diagnosis is >71 months.

Stage III--characterised by absolute lymphocytosis and anaemia (haemoglobin <11 g/dL) with or without lymphadenopathy, hepatomegaly, or splenomegaly. Median survival from diagnosis is about 19 months.

Stage IV--characterized by absolute lymphocytosis and thrombocytopenia (<100,000/mm3) with or without lymphadenopathy, hepatomegaly, splenomegaly, or anaemia. Median survival from diagnosis is about 19 months (Rai et al., 1975).

1.1.2.2 Binet classification

Dr Binet and his team proposed a new CLL prognostic classification method. This classification considers both thrombocytopenia and anaemia to be the most relevant factors for the prognosis of CLL disease. Moreover, this new classification method is simpler and provides more accurate prognostic value. It only requires simple clinical examination and routine blood hemogram examination (Binet et al., 1981). It is described as follows:

Clinical stage A--characterised by no anaemia or thrombocytopenia and fewer than three areas of lymphoid involvement (Rai stages 0, I, and II).

Clinical stage B--characterised by no anaemia or thrombocytopenia with three or more areas of lymphoid involvement (Rai stages I and II).

Clinical stage C--characterized by anaemia and/or thrombocytopenia regardless of the number of areas of lymphoid enlargement (Rai stages III and IV) (Binet et al., 1981).

1.1.3 Prognostic indicators in CLL

1.1.3.1 Chromosomal abnormalities

The four most common chromosomal abnormalities in CLL patients are: deletions in chromosome 13q14 (del 13q), 11q22-23 (del 11q), or 17p13 (del 17p), or trisomy 12 (Döhner et al., 2000). Del 13q can be seen in over than 50% of patients, making it the most common chromosomal alteration in CLL (Klein et al., 2011).

1.1.3.2 Mutational status of immunoglobulin heavy chain variable region gene (IGHV)

The relationship between IGHV mutations and prognosis was first reported in the 1990s by two independent groups (Hamblin et al., 1999, Damle et al., 1999). The definition of mutation in IGHV is to compare the IGHV gene of CLL cells with the recent gene sequence, and divide it into non-mutated and mutated according to the comparison of the somatic mutations, which less than 2% of the difference is unmutated (UM-CLL) and over than 2% is mutated (M-CLL). (Kipps et al., 2017). Regardless of the stage, the survival rate of patients with UM-CLL is significantly worse than those with M-CLL (Hamblin et al., 1999).

Research by Hamblin et al showed that UM-CLL is associated with genes in V1-69 and D3-3. It is also related to isolated trisomy 12 (Hamblin et al., 1999). Another reported phenomenon was that patients with UM-CLL had a higher percentage of $CD38^+$ B-CLL cells (>30%) than patients with M-CLL (Damle et al., 1999). Patients with UM-CLL and also having >30% $CD38^+$ B-cells were found not responding well to the treatment with chemotherapy drugs, and their survival was shorter (Damle et al., 1999).

In 2002, it was reported that patients with M-CLL and with gene usage in V3-21 had significantly shorter survival as compared to those with M-CLL without using V3-21 gene segment (Tobin et al., 2002). Indeed, this group of patients had an overall survival similar to patients with UM-CLL (Tobin et al., 2002). A later study confirmed this finding by a large, independent study consisting of 2,457 patients with CLL in which patients with M-CLL who used V3-21 had significantly shorter time from diagnosis to first treatment than patients with M-CLL who used other segments, although the number of the CLL patients using V3-21 was relatively small with only 63 detected out of 2457

cases studied (2.6%) from this study (Ghia et al., 2008). Patients with M-CLL using V3-21 gene segment were also found to be at an increased risk of relapse following treatment with front-line chemoimmunotherapy (Lin et al., 2009). Therefore, use of V3-21 in M-CLL predicts high-risk disease and patients with M-CLL using V3-21 gene are managed similarly to those patients with UM-CLL (Zenz et al., 2010b).

1.1.3.3 Recurrent mutations.

The new generation of sequencing technology provides us with new information in understanding the genetic changes of CLL. Existing evidence showed that CLL cells have high genetic variability (Fabbri et al., 2011, Pleasance et al., 2010, Puente et al., 2011, Wang et al., 2011). The factors affecting the prognosis have been previously determined including the doubling time of lymphocytes, the status of IGHV mutations, chromosome aberrations, and the expression level of CD38. The latest sequencing technology has confirmed new multiple gene mutations in CLL cells. These newly discovered mutations are valuable for judged prognosis (Wang et al., 2011, Puente et al., 2011).

According to Wang's group research results, genes that frequently mutate include *TP53, ATM, MYD88, SF3B1, NOTCH1, DDX3X, ZMYM3,* and *FBXW7*. In another study by Puente et al., consisting of more than 360 CLL samples, recurrent mutations in *NOTCH1*, *XPO1, MYD88* and *KLHL6* were found (Wang, 2001, Puente et al., 2011).

In the following part, I will discuss recently reported recurrent mutations in several genes and their associated biological significance. They include *TP53, NOTCH1, BIRC3,* and *SF3B1*.

1.1.3.3.1 *TP53*

The *TP53* gene is a well-known tumour suppressor gene which is located on chromosome 17p13 (Isobe et al., 1986). *TP53* encodes the p53 protein as a transcription factor. It can up-regulate genes that prevent cell cycle progression, enabling cells to repair damaged DNA. In the case of severe DNA damage, p53 can transcribe pro-apoptotic genes, which makes these DNA damaged and irrecoverable cell apoptosis (Wickremasinghe et al., 2011). Research results show that the direct interaction between p53 and mitochondrial anti-apoptotic proteins (including BCL-2) is the pathway of apoptosis (Steele et al., 2008).

Loss of *TP53* gene is associated with poor clinical prognosis and disease resistance (Dohner et al., 1995, Döhner et al., 2000), At the time of diagnosis, about 5-15% of CLL patient cells were deficient in *TP53*. Another 80% of CLL patients with mono-allelic 17p deletion also had *TP53 deletions* (Zenz et al., 2008, Rossi et al., 2009, Dicker et al., 2009,

Malcikova et al., 2009). Recently it has been shown that *TP53* mutation alone is also associated with poor clinical outcome. For example, *TP53* mutations without 17p deletion were detected in 3-8.5% of CLL patients who had a short survival regardless of clinical stage, IGHV mutational status, and presence of 11q and 17p deletion (Zenz et al., 2008, Zenz et al., 2010a, Gonzalez et al., 2011). And after receiving chemotherapy, mutations in *TP53* or deletion of the 17p gene were observed in cells of patients with refractory disease, which also shows that there is a certain relationship between the two gene with refractory disease (Malcikova et al., 2009). Although just short number of patients about 5-10% develop *TP53* abnormalities at the time of diagnosis, the abnormality can be detected in 40-50% of patients with refractory diseases (Rossi et al., 2009, Zenz et al., 2010a, Gonzalez et al., 2011, Zainuddin et al., 2011).

Because of the importance of p53 in tumour suppression and the prognostic value of *TP53* mutations, screening for *TP53* mutations can be performed using direct Sanger sequencing technique mainly in exons 4-9 of *TP53* gene, which encode the DNA binding region of p53 (Pospisilova et al., 2012). According to the above, the mutation of p53 gene is closely related to the poor prognosis of CLL (Trbusek et al., 2011).

1.1.3.3.2 *NOTCH1*

NOTCH1 is one of four known genes encoding the NOTCH protein family, which is a group of receptors involved in the NOTCH signalling pathway. Studies have found that the NOTCH signalling pathway plays an important role in cell differentiation, proliferation, and survival, as well as in the development and function of lymphocytes (Villamor et al., 2013, Radtke and Raj, 2003, Pear and Radtke, 2003).

Studies have shown that *NOTCH1* and *NOTCH2* are active in CLL cells, and they also have important effects on CLL cell survival and resistance to apoptosis. This activity is not shown in normal B cells, suggesting that the NOTCH1 protein may become a new therapeutic target (Rosati et al., 2009).

At the time of diagnosis, mutations in the *NOTCH1* gene were detected in cells in about 10% of CLL patients, and these patients were usually in the late stages of the disease and had shorter survival. (Puente et al., 2011, Fabbri et al., 2011, Rossi et al., 2012b, Oscier et al., 2013). Furthermore, we often detect *NOTCH1* mutations in the cells of patients with unmutated IGHV and trisomy 12, which suggest a poor prognosis. This suggests that *NOTCH1* can be used as an independent predictor of survival in patients with CLL (Oscier et al., 2013, Puente et al., 2011, Rossi et al., 2012b).

1.1.3.3.3 *SF3B1*

SF3B1 is one of several genes involved in RNA splicing. It has been repeatedly mutated in several malignancies. The latest research found that *SF3B1* mutations in about 9.7% of CLL patients. Occurs in patients who have unmutated IGHV gene (Quesada et al., 2012, Malcovati et al., 2011, Network, 2012). Although how mutations in *SF3B1* occur is still unclear, current research has identified several hotspots of point mutations mainly involving R625H and K700E (Schwaederle et al., 2013).

SF3B1 protein is a key component of the RNA splicing machinery and its role is to ensure successful transcription (Wan and Wu, 2013). In a clinical study, *SF3B1* mutations were found in 17% of CLL patients, and this mutation was associated with high expression of CD38 and poor prognosis (Oscier et al., 2013). A study by Schwaederle‘ teams in 2013 found that CLL patients with *SF3B1* mutations grew faster than other types and caused their clones to proliferate. These studies have shown that the *SF3B1* mutation is an independent indicator of poor prognosis (Schwaederle et al., 2013).

It has long been suspected that *SF3B1* mutations, especially abnormal cleavage of specific transcripts, may be critically involved in the pathogenesis of CLL. In 2016, Larrayoz and colleagues explored therapeutic potential of targeting the spliceosome. Their study found that inhibition of *SF3B1* by spliceostation A (SSA) induced higher levels of apoptosis in CLL cells to compared with normal B cells, but this increase was not related to the status of *SF3B1* mutations (Larrayoz et al., 2016). Nonetheless, an interesting observation was noted that SSA-induced apoptosis was associated with changes in MCL-1 splicing and down-regulation of MCL-1 protein, suggesting that cell death is regulated by changes in MCL-1 splicing (Larrayoz et al., 2016). Other studies also revealed recurring mutations affecting the other components of the splice machinery in CLL, however the mutations in splicing factor *SF3B1* are the most frequent, occurring in 10% of CLL cases and mainly in the UM-CLL subgroup (Puente et al., 2015, Puente et al., 2011, Landau et al., 2015, Cazzola et al., 2013).

1.1.3.3.4 *BIRC3*

The gene *BIRC3* is an inhibitor of apoptotic protein 2 (c-IAP2). It is located on chromosome 11q22 and contains three baculoviral IAP repeats (Rossi et al., 2012a). In a 2012 study by Rossi's team, they found that the loss of BIRC3 gene function in CLL cells affects the expression of c-IAP2 protein, which subsequently leads to the activation of NF-κB signalling, which also leads to prolonged survival of leukaemia cells (Rossi et al., 2012a). Interestingly, *BIRC3* mutations are rare in the diagnosis of CLL, but they are present in 24% of fludarabine-refractory CLL cases, which showed that *BIRC3* mutations are likely to be associated with fludarabine resistance, which is likely associated with increased NF-κB activity (Rossi et al., 2012b).

Another study found that *BIRC3* mutations are associated with UM-CLL, del (11q) and trisomy 12, and these influencing factors all suggest a poor prognosis for CLL (Baliakas et al., 2015). All these indicate that it may be related to the poor prognosis of CLL patients, and it can also be an important influential factor for the diagnosis of CLL prognosis.

1.1.3.3.5 Prognostic value of CLL-associated mutations

In recent years, several recurrently mutated genes detected at diagnosis, such as *NOTCH1*, *SF3B1* and *BIRC3*, were shown to independently confer a poor prognosis in patients with CLL (Puente et al., 2015, Fabbri et al., 2011, Quesada et al., 2012, Landau et al., 2015, Rossi et al., 2012a, Rossi et al., 2012b, Rossi et al., 2011). These new discoveries in gene mutations, which have stimulated enthusiasm within the CLL community for the development of new models for high-risk patient classification to make the prognosis of CLL patients more accurate (Döhner et al., 2000)(Döhner et al., 2000)(Döhner et al., 2000)(Döhner et al., 2000)(Döhner et al., 2000).

Rossi and his team proposed a new prognostic system through an analytical study of more than 1,000 newly diagnosed and untreated CLL patients. They used traditional fluorescence in situ hybridization (FISH) to the prognosis-related mutations and cytogenetics were analysis (Rossi et al., 2013). According to the analysis results, the disease prognosis is classified into four subgroups: a. high-risk CLL: those carrying *TP53* and/or *BIRC3* abnormalities; b. intermediate-risk CLL: those with *NOTCH1* and/or *SF3B1* mutations and/or del 11q; c. low-risk CLL: those having trisomy 12 or a normal karyotype; and d. very low-risk CLL: those with del 13q14 only (Rossi et al., 2013).

Importantly, approximately 20% of patients were previously classified as patients in the low-risk category were reclassified as high-risk categories due to new influencing factors such as *NOTCH1*, *SF3B1* and *TP53* mutations, and *BIRC3* disruption, thereby improving independent predictions of disease progression and response to therapy (Rossi et al., 2013).

1.1.4 Pathogenesis of CLL

1.1.4.1 Cell origins of CLL

There is still no definitive answer for the origin of CLL cells. According to recent research and clinical tests, there are different origin cells for M-CLL and UM-CLL, and the prognosis of the two group are also significantly different (Zhang and Kipps, 2014, Gaidano et al., 2012, Chiorazzi and Ferrarini, 2011).

For the duration normal B cell development, immature B cells undergo positive and negative selection in the bone marrow (BM) and subsequently move into the secondary lymphoid organs/tissues including spleen, lymph nodes and pleural cavity where they go through further selection before becoming mature B cells (LeBien and Tedder, 2008, Rickert, 2013). It is within the secondary lymphoid organ such as lymph nodes where a B cell encounters an antigen through its B cell receptor (BCR) and communicate with $CD4^+$ T helper cells, it also known as T follicle helper cells (Tfh) in the germinal centre (GC) of the lymph nodes. In other costimulatory factors, B cells proliferate in the GC. In order to increase the affinity of BCR for antigens, B cells also undergo point mutations in the V gene segment of IGHV by somatic hypermutation (SHM) and class-switch recombination (CSR) that are both mediated by activation-induced cytidine deaminase (AID) (Rickert, 2013). Finally, depending on the type and strength of signals received via the BCR, co-receptors, or from neighbouring T cells, B cells differentiate into plasma cells, GC B cells, and/or memory B cells (Cyster and Allen, 2019).

In CLL, the gene expression profile (GEP) shows that among the 12,000 genes that have been identified, the difference between M-CLL and UM-CLL is very small, only 23 genes have different expressions, but the prognosis between M-CLL and UM-CLL is so different (Klein et al., 2001, Rosenwald et al., 2001).

An existing gene expression profile (GEP) study showed that UM-CLL cells are similar to pre-GC CD5 + CD27-B cells, while M-CLL genes are more similar to post-GC CD5 + CD27 + B cells (Seifert et al., 2012). The results also indicate that the IGHV rearrangements common in CLL are consistent with mature $CD5^+$ B cells from healthy cells, which indicates that the source of CLL cells is $CD5^+$ B cells (Seifert et al., 2012).

1.1.4.2 The CLL microenvironment

The lymph node microenvironment has been shown to be the primary site of CLL-cell proliferation (Herishanu et al., 2011). The supportive niche of the tumour microenvironment (TME) has been recognised as a critical factor in the pathogenesis of the disease (Caligaris-Cappio et al., 2014, Burger, 2011). CLL cells can survive for a long time *in vivo*, but when cultured *in vitro*, they experience rapid spontaneous apoptosis (Coscia et al., 2011, Collins et al., 1989). These studies have shown that the machinery of apoptosis is intact in CLL cells and that survival of CLL cells is dependent on the microenvironmental factors. Studies have shown that culturing CLL cells with accessory cells or cytokines can increase their survival time and possibly prevent spontaneous apoptosis. This shows that new CLL treatment drugs should not only target CLL cells circulating in peripheral blood, but also be able to target non-circulating CLL cells in the tissue microenvironment (such as bone marrow and lymph nodes) (Caligaris-Cappio et al., 2014, Burger, 2011).

The CLL microenvironment plays an important role in CLL disease. CLL cells proliferate

in the microenvironment, and the protective effect of the microenvironment on CLL cells can make CLL cells survive better and become resistant to chemotherapy drugs (Meads et al., 2009).

In the CLL microenvironment (Figure 1), CLL cells interact with many other cells, including T cells, bone marrow stromal cells (BMSC), nurse-like cells (NLC), and endothelial cells (EC). Many experimental results show that the activation of BCR will lead to the survival, proliferation and drug resistance of CLL cells, which needs further experimental proof. Similarly, the activation of NF-κB occurs in the proliferation centres of CLL lymph nodes, which is also considered to be a cause of CLL proliferation and inhibition of apoptosis (Herreros et al., 2010, Herishanu et al., 2011).

In the following sections, the molecular interactions of CLL cells with the main components of the TME, including antigens via BCR, T cells and NLCs will be discussed in more detail.

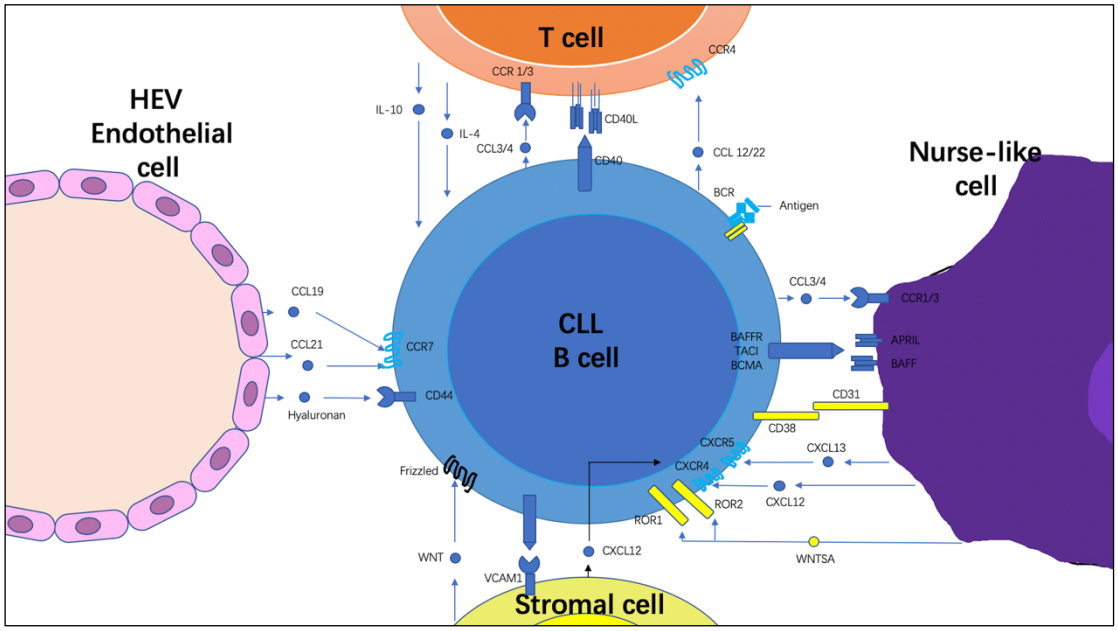


Figure 1. CLL microenvironment. Inside the CLL microenvironment, CLL cells cross-talk with many types of cells, including T cells, bone marrow stromal cells (BMSC), monocyte-derived nurse-like cells (NLC), and high endothelial venules (HEVs) endothelial cells, The activation of BCR is related to the interaction of certain antigens, which is also an important reason why CLL cells can survive, proliferate, and develop drug resistance. The figure is adapted from a recent review paper by Thomas Kipps and colleagues (Kipps et al., 2017).

1.1.4.2.1 B cell receptor (BCR) signalling

The composition of BCR includes transmembrane immunoglobulin molecules IgA, IgD, IgE and IgM, and the signally molecules CD79A and CD79B. These signalling molecules

often form heterodimers with CLL cells and co-express the immunoglobulin molecules IgD and IgM (Kipps et al., 2017). BCR is common in most mature malignant B cells, including CLL cells. In CLL, CLL B cell surface immunoglobulins can bind to their own antigens, which creates a BCR signalling pattern (Herishanu et al., 2011, Mockridge et al., 2007, Dühren-von Minden et al., 2012).

CLL is a heterogeneous disease, and the clinical manifestations of different patients are often different. Therefore, the degree of activation of BCR signalling is also different. Factors related to BCR signalling include mutations and un-mutated of IGHV. Studies have shown that BCR signalling activation will be stronger in CLL cells expressing un-mutated IGHV (Packham et al., 2014).

BCR signalling may be an important pathway in the malignant expansion of CLL. Compared with normal B cells, the expression of IgM and CD79b in CLL is relatively low (Sigalov, 2008). In response to IgM stimulation, the calcium flux in CLL cells is usually lower than that in normal B cells (Woyach et al., 2012). Moreover, the ectopic expression of LYN, SYK, and ZAP70 in CLL has been increased, and these factors have suggested poor prognostic indicators (Chen et al., 2002, Contri et al., 2005, Buchner et al., 2009). In a comparative test of M-CLL and UM-CLL, the SYK activation time of UM-CLL cells was also prolonged (Herman et al., 2010).

1.1.4.2.2 T-cell and CLL-cell interaction

Survival of CLL cells depends on signals received from adjacent non-neoplastic cells within the TME. CLL cells follow the chemokine gradient into the lymph nodes and form a proliferation centre (Herishanu et al., 2011). In the proliferation centre, CLL cells are in contact with different types of cells, mainly T cells, nurse-like cells, and stromal cells. During this process, binding of BCR to autoantigen may occur, if there are enough T cells, the activation and proliferation of CLL cells will increase (Packham et al., 2014). T cells secrete cytokines, such as IL-4, which upregulate surface IgM and may further promote autoantigen stimulation of CLL cells (Aguilar-Hernandez et al., 2016). Requirement of T cells in CLL development and progression was eloquently demonstrated by the seminal study using an adoptive transfer model (Bagnara et al., 2011). In that study, primary CLL cells transferred to nonobese diabetes/severe combined immunodeficient mice were able to proliferate only in the presence of autologous $CD4^+$ T cells whereas purified CLL cells transferred to the mice alone fail to expand (Bagnara et al., 2011).

The *in-vivo* evidence of interaction of CD40 on CLL cells with CD154 (also known as CD40 ligand) on activated $CD4^+$ T cells has been corroborated through histological studies. Initially, CLL cells have been shown to intermix with $CD4^+$ T helper (Th) cells in the lymph nodes and bone marrow (Pizzolo et al., 1983, Schmid and Isaacson, 1994). The association between activated $CD4^+$ T cells and CLL cells leads to increased expression of CD38 in CLL cells, which triggers the proliferation of CLL cells (Patten et

al., 2008). Studies have shown that CLL B cells can attract $CD4^+$, $CD154^+$ T cells, of which CCL22 plays an important role (Granziero et al., 2001, Ghia et al., 2002). The research from Herreros and colleagues found that the proliferation centres of CLL patients had more T cells ($CD3^+$ cells), many of which expressed CD40L (Herreros et al., 2010). These findings indicate that interaction between T cells and CLL cells is taking place in lymph nodes and bone marrow and that engagement of CD40 in CLL cells by CD154 in T cells mediates the interaction between T cells and B cells. Many of the biological effects of CD40-CD154 interaction can now be reproduced in *in-vitro* studies.

Thus, Tretter and colleagues cultured CLL cells with activated T cells and found that this can improve the survival and induce proliferation of leukaemia cells *in vitro* (Tretter et al., 1998). Subsequent *in-vitro* studies confirm CD40-CD154 interaction as a key molecular mechanism responsible for activation of survival signalling in CLL cells, resulting in their protection from spontaneous and drug-induced apoptosis *in vitro* (Grdisa, 2003, Kater et al., 2004, Zhuang et al., 2014, Purroy et al., 2015).

1.1.4.2.3 CD40 signalling

CD40, a 48-kDa type I transmembrane protein, belongs to the tumour necrosis factor receptor (TNFR) family and is expressed on B cells (Quezada et al., 2004). The ligand of CD40 is CD154 (or CD40L) which is a type II transmembrane protein, with a varied molecular weight of 34-39 kDa due to post translational modification (Quezada et al., 2004). CD154 belongs to the tumor necrosis factor (TNF) family, and its expression mode is mainly the activation of T cells as well as some other immune cells (Lederman et al., 1992). Ligation of CD40 by CD154 leads to trimeric clustering of the receptor. Since the cytoplasmic domain of CD40 lacks kinase activity, it relies on recruiting the cytoplasmic adaptor molecules such as TNF receptor associated factors (TRAFs) to propagate the signalling cascade (Bishop et al., 2007). Among the signalling pathways initiated following CD40 ligation, activation of the canonical and non-canonical NF-κB pathways is the best characterised signalling pathway following of the recruitment of the TRAF proteins to the CD40 cytoplasmic domains (Elgueta et al., 2009, Rickert et al., 2011).

NF-κB is a transcription factor, initially thought to be specifically expressed by B cells, but later found to be ubiquitously expressed in most cell types (May and Ghosh, 1998). It regulates many target genes critically involved in inflammation and innate immunity. Recently it has been shown that NF-κB also plays an important role in cancer initiation and progression as activation of NF-κB pathway leads to inhibition of apoptosis, increased cell proliferation, and enhanced migratory and invasive phenotype of cancer cells (Hoesel and Schmid, 2013, DiDonato et al., 2012, Taniguchi and Karin, 2018).

Thus, it is now established that, CD40 signalling plays an important role in B cell survival and proliferation as a result of CD40-CD154 interaction between T cells and B cells (Elgueta et al., 2009, van Kooten and Banchereau, 2000, Rickert et al., 2011).

1.1.4.2.4 Biological effects of CD40 stimulation in CLL cells

CD40 stimulation in CLL cells will activate NF-κB. Activated NF-κB will increase the expression of anti-apoptotic proteins, such as BCl-2, BCL-XL, c-IAP2 and A1 / BFL1. (Schattner, 2000, Furman et al., 2000), Increased expression of these apoptotic proteins leads to inhibition of apoptosis. Research from the Tromp team showed that after co-culture with CD154-expressing fibroblasts, CLL cells can proliferate and proliferation was also observed in 30% of UM-CLL cells, indicating that the stimulation of CD40 may promote the CLL cell proliferation (Tromp et al., 2010).

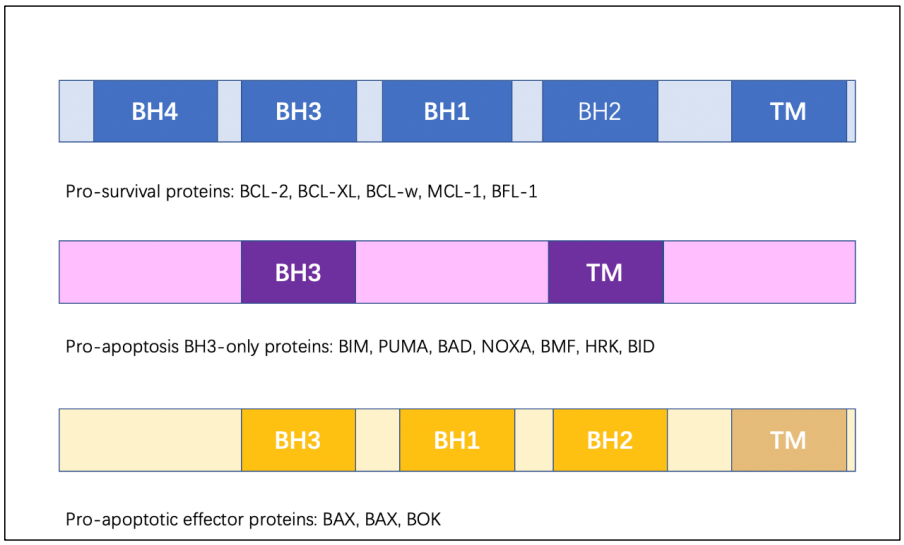
To study the effect of CD40 stimulation on gene expression of CLL cells, CLL cells were cultured with CD154-expressing fibroblasts and their gene expression was found to be similar to the CLL cells which were stimulated by activated T cells (Pascutti et al., 2013). Another interesting finding is that the expression of apoptosis related genes (pro-apoptotic and anti-apoptotic) is also observed, such as BCL-XL, BID, CD95, c-IAP1, A1 / BFL1, p21, and SERPIB9 (Pascutti et al., 2013).

CD40-stimulated CLL cells also developed resistance to therapeutic drugs. Previous research from our laboratory found that after co-culture with CD40L-expressing fibroblasts, CLL cells developed resistance to the chemotherapy drug fludarabine (Zhuang et al., 2014). Another study found that CLL cells developed resistance to apoptosis induced by the BCL-2 inhibitor ABT-737 after CD40 stimulation (Vogler et al., 2009b).

1.1.4.3. Regulation of apoptosis by BCL-2 family of proteins in CLL

The BCL-2 family of proteins can be divided into 3 functionally distinct groups; anti-apoptotic proteins and pro-apoptotic activators and effectors (Figure 2). Anti-apoptotic proteins include BCL-2, MCL-1, BCL-XL, A1/BFL-1 and BCL-w that share all 4 BCL-2 homologue (BH) domains. Pro-apoptotic effector proteins include BAX and BAK that contain multiple BH domains. Finally, pro-apoptotic activators are also known as BH3-only proteins (such as BIM, BID, PUMA, NOXA and BAD) that are activated in response to cellular stress and subsequently activate the pro-apoptotic effector proteins (such as BAX and BAK) (Adams and Cory, 2007, Youle and Strasser, 2008, Chipuk et al., 2010, Czabotar et al., 2014). Anti-apoptotic proteins sequester BH3-only proteins via binding to their BH3 motif. The activation/upregulation of BH3-only proteins during apoptosis induction enables them to overcome the suppression by the anti-apoptotic proteins. Subsequently, BH3-only proteins bind to BAX and BAK proteins and induce their conformation changes, leading to their oligomerisation to form pores in the mitochondrial outer membrane. Permeabilisation of the mitochondrial outer membrane leads to the release of cytochrome c, initiating a cascade of activation of effector caspases, leading to full-blown apoptosis (Shamas-Din et al., 2011, Delbridge et al., 2016b, Kale et al., 2018).

(A)



(B)

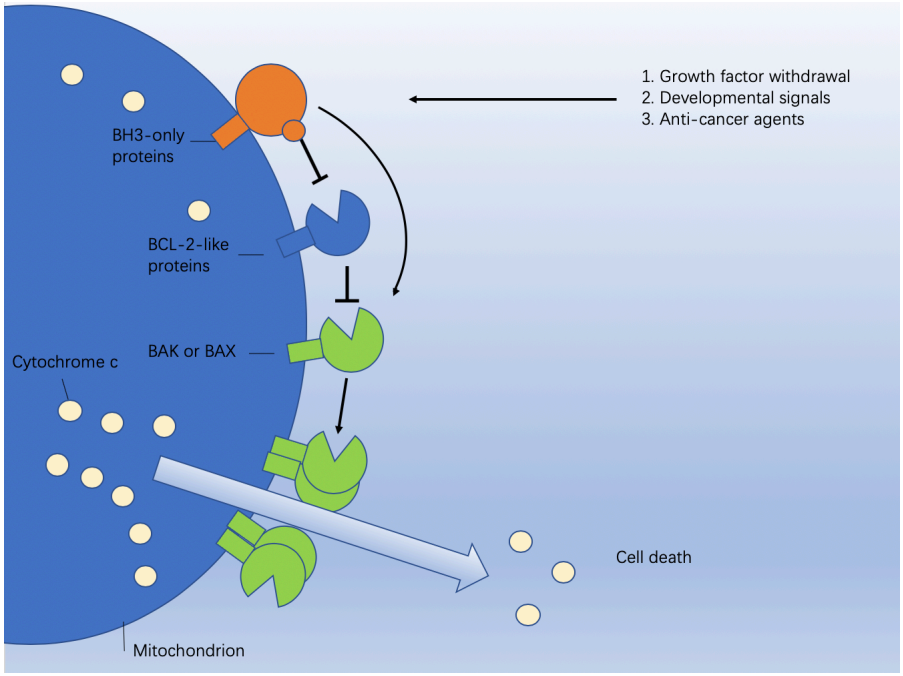


Figure 2. The BCL-2 family of proteins in regulating apoptosis (A) shows the presence of one or more of the BCL-2 homology (BH) domains, which subdivides BCL-2 family members into three major subsets of proteins. In the case of the BH3-only protein, the BH3 domain acts as a ligand domain to facilitate interaction with other subgroups. Many pro-apoptotic and anti-apoptotic BCL-2 family members also have a transmembrane (TM) domain to promote binding to the mitochondrial outer membrane; exceptions are A1/BFL1, BAD, BID, BIM, PUMA, NOXA and BMF. The diagram is modified from a review paper by (Valentin et al., 2018). (B) shows how the BCL-2 family of proteins regulate apoptosis signal. The diagram is modified from a review paper by (Delbridge et al., 2016a).

CLL cells have increased expression of BCL-2 protein in leukaemia compared to normal B cells (Pepper et al., 1997, Kitada et al., 1998). Studies show that increased BCL-2 expression indicates poor prognosis (Robertson et al., 1996, Faderl et al., 2002). MCL-1 is an anti-apoptotic BCL-2 family protein, which is also overexpressed in CLL cells (Pedersen et al., 2002, Bannerji et al., 2003). Over-expression of BCL-2 and MCL-1 has been associated with downregulation of the microRNA (miR)-15a and miR-16-1 in CLL (Pekarsky and Croce, 2015). The miR-15a/16-1 cluster is located at chromosome 13q14, a region which is commonly deleted in CLL cells (Calin et al., 2002). Later, it has been shown that miR-15a and miR-16-1 are a natural suppressor of the expression of BCL-2 protein as over-expression of miR-15a and miR-16-1 leads to apoptosis by targeting BCL-2 expression (Cimmino et al., 2005). The miR-15a and miR-16-1 also inhibit the expression of MCL-1 (Calin et al., 2008). Thus, it is now established that the miR-15a and miR-16-1 downregulation are closely related to 13q14 deletion, which leads to higher expression of BCL-2 and MCL-1 in CLL.

1.1.5 Current clinical treatment

In the past ten years, although there are many new treatment options and new drugs, except for the use of allogeneic hematopoietic stem cell transplantation, the disease cannot be cured when treated with drugs. Current general guidelines for treatment (Hallek et al., 2018) are summarised below.

Depending on the clinical stage of the patient, we use different treatment options. If the patient is in the Binet A-B stage or Rai 0-II stage, then no treatment is recommended. If the disease progresses to Binet C or Rai III-IV, treatment is recommended

The current standard treatment is immunochemotherapy, which includes a combination of fludarabine (F), cyclophosphamide (C), and rituximab (R). This treatment is suitable for young and fit patients without chromosome deletion within 17p.(Hallek, 2013, Jain and O'Brien, 2015). After assessing the patient's condition, if it is found that the patient cannot tolerate FC toxicity, a combination of prescription chlorambucil and rituximab (R) is required (Hallek, 2013, Jain and O'Brien, 2015).

For other patients, further assessment is needed to determine what stage the patient is in and what corresponding treatment should be offered. Allogeneic hematopoietic stem cell transplantation is recommended if the 17p deletion or p53 mutation is detected. Alternatively, an antibody therapy that targets specific antigen expressed in CLL cells such as CD20 or CD52 can be used (Hallek, 2013, Jain and O'Brien, 2015). Such antibodies include rituximab or ofatumumab and alemtuzumab that target CD20 and CD52, respectively.

A very common situation is that most patients will relapse after the first treatment. At this time, the guidelines require repeating first-line treatment for relapsed patients.

Alternatively, depending on the fitness of patients, other treatment possibilities may be considered (Hallek, 2013).

1.1.5.1 Chemotherapeutic agents

1.1.5.1.1 Chlorambucil

Chlorambucil is an alkylating agent that has been used as first-line therapy for CLL for many years, until it is replaced by fludarabine-cyclophosphamide (FC). Its mechanism of action is to alkylate DNA, causing cross-linking between DNA strands, which prevents DNA replication and inducing apoptosis (Panasci et al., 2001). This drug is still in use today to treat people with intolerance to fludarabine-cyclophosphamide FC toxicity, and can be combined with rituximab or ofatumumab (Goede et al., 2015, Hillmen et al., 2014, Hillmen et al., 2015).

1.1.5.1.2 Fludarabine and cyclophosphamide

Fludarabine(F) is a purine nucleoside analogue that effectively induces apoptosis in CLL cells by activating the mitochondrial apoptotic pathway (Robertson and Plunkett, 1993, Genini et al., 2000). It is a fluorinated analogue of adenine that is absorbed by cells and converted to fludarabine triphosphate, It will enter the DNA, thereby inhibiting the synthesis of DNA and RNA (Adkins et al., 1997). And fludarabine triphosphate can also inhibit the activity of ribonucleotide reductase and DNA polymerase (Pettitt, 2003).

Cyclophosphamide (C) is an alkylating agent, which works by inducing cross-linking between DNA strands and causing DNA damage (Hall and Tilby, 1992). Several experiments have proven that when used in combination with fludarabine (F), it can improve the complete and overall response rate, which as a result becomes the first-line treatment of choice in many CLL clinics (Eichhorst et al., 2006, Flinn et al., 2007, Catovsky et al., 2007).

1.1.5.1.3 Bendamustine

Bendamustine is an alkylating agent that induces cross-links between DNA bases, thereby inhibiting DNA repair (Burger and Gandhi, 2009). When used in combination with rituximab (R), it has improved the treatment of patients with relapsed and refractory CLL (Fischer et al., 2011). Comparing with other alkylating agent such as chlorambucil, Bendamustine has shown a distinct pattern of cytotoxicity. In particular, it can induce apoptosis through both apoptotic and nonapoptotic mechanisms, thus maintaining activity in cells with no functional apoptotic pathway(Cheson and Rummel, 2009).

1.1.5.2 Immunochemotherapy

Immunochemotherapy is combined with immune antibody therapy on the basis of chemotherapy. The current standard first-line treatment FCR for CLL patients is composed of fludarabine (F), cyclophosphamide (C) and rituximab (R) (the monoclonal antibody against CD20 antigen expressed in B cells). The combination of these three can significantly improve the survival of CLL patients (Hallek et al., 2010).

In addition to rituximab, other monoclonal antibodies against CD20 include ofatumumab, which, when used in combination with chlorambucil, has shown to significantly improve response in treatment-naive CLL patients when compared to chlorambucil alone (Hillmen et al., 2015). Other antibodies have also been developed including alemtuzumab which targets CD52 antigen that is expressed in normal and malignant lymphoid cells (Robak, 2008). Alemtuzumab has been shown to improve treatment in patients with relapsed/refractory CLL who also display high-risk features (e.g. 17p deletion, elderly and with comorbidity) (Schweighofer and Wendtner, 2010).

1.1.5.3 Immunomodulatory drugs

Immunomodulatory drugs include thalidomide, lenalidomide and pomalidomide. Although exact mode of action of these drugs is still unclear, they have been shown to display clinical activity in CLL (Kater et al., 2014b).

Lenalidomide inhibits CLL cell proliferation by disrupting the interaction of CLL cells with other cells in the microenvironment, such as nurse-like cells, and mesenchymal stromal cells, and its mode of action is independent of p53 (Kater et al., 2014b). Recent clinical studies have shown that lenalidomide has a partial response in the treatment of fludarabine-refractory CLL patients, but the specific use method requires further clinical validation (Kater et al., 2014a).

1.1.5.4 Novel, molecularly-targeted therapeutic agents

1.1.5.4.1 BCR signalling inhibitors

1.1.5.4.1.1 Ibrutinib

Ibrutinib is a small molecule drug that permanently binds to the important protein Bruton tyrosine kinase (BTK) in B cells. BTK is critical in mediating BCR signalling. By inhibiting the effect of BTK, the effect of blocking BCR signalling is achieved (Woyach et al., 2012, Kipps et al., 2017). Ibrutinib can also reduce the chemotaxis of CLL cells to the chemokines CXCL12 and CXCL13, and can inhibit cell adhesion after cell receptor (BCR) stimulation. (Ponader et al., 2012; de Rooij et al., 2012)

Studies have shown that Ibrutinib can promote apoptosis, inhibit cell proliferation, and also block the response of CLL cells to pro-survival stimuli in the microenvironment (Pavlasova et al., 2016). It also effectively prevents the microenvironment Survival signals provided to the outside of CLL cells, including multiple signalling pathways such as soluble factors (CD40L, BAFF, IL-6, IL-4, and TNF-α), fibronectin binding, and stromal cell contact (Herman et al., 2011).

Ibrutinib was initially used to treat mantle cell lymphoma, following approval by the US FDA in 2013 (Food and Administration, 2013). In 2014, the FDA approved Ibrutinib as one of the drugs for CLL (Davids and Brown, 2014).

1.1.5.4.1.2 Idelalicib

Idelalisib, also known as GS-1101 and CAL-101, is a selective small molecule inhibitor targeting the PI3K p110δ-subtype (Brown et al., 2014). Studies have shown that when used in combination with rituximab, it is effective in patients with CLL and can improve lymphadenopathy and splenomegaly. When combined with rituximab in a phase 1 clinical studies, it showed good activity in patients with relapsed and refractory CLL (Furman et al., 2014).

It is well known that the BCR signalling pathway plays a key role in the pathogenesis of CLL (Chiorazzi et al., 2005, Bernal et al., 2001, Chen et al., 2002, Herishanu et al., 2011). Activation of δ isoform of phosphatidylinositol 3-kinase (PI3Kδ) is part of BCR signalling, where PI3Kδ is highly expressed in lymphoid cells (Okkenhaug and Vanhaesebroeck, 2003). In CLL cells signalling transduction as a result of activation of other surface receptors, such as CXCR4, CD40 and CD49d, also converges on PI3Kδ (Burger et al., 1999, Furman et al., 2000, Gattei et al., 2008).

Idelalisib is a competitive inhibitor that binds to the ATP-binding site in the PI3Kδ catalytic domain. Since PI3Kδ kinase is expressed in normal and malignant B-cell, it can prevent tumour proliferation in malignant B cell lines. It also inhibits several PI3Kδ kinase-dependent cellular signalling pathways, including BCR signalling, CXCR4 and CXCR5 signalling, which are involved in B cell migration and homing to lymph nodes and bone marrow (Wolford and Tewari, 2015, Burger and Gribben, 2014).

In 2014, the FDA approved idelalisib for the treatment of CLL (Food and Administration, 2014). The addition of idelalisib to rituximab was found to be superior to rituximab monotherapy in refractory patients, such as 17p deletion or TP53 mutations or unmutated IGHV (Furman et al., 2014).

1.1.5.4.2 BCL-2 inhibitor

Multiple studies have shown that the overexpression of anti-apoptotic BCL-2 protein in CLL can lead to the survival and accumulation of leukaemia cells *in vivo*. Excessive

BCL-2 protein expression is also related to chemotherapy resistance, which shortens overall survival in CLL patients (Robertson et al., 1996, Faderl et al., 2002). Therefore, BCL-2 has become a reasonable target for new therapeutic agents. One of the main methods for targeting BCL-2 is to develop BH3 mimics. The small molecule BH3 mimetic can tightly bind to BH3 domain of BCL-2 and interfere with its sequestration of pro-apoptotic BH3-only proteins, resulting in liberation of the BH3-only proteins (Merino et al., 2018).

The first generation of BH3 mimics is called ABT-737 and navitoclax (ABT-263). ABT-737 can simultaneously target BCL-2 and BCL-XL, both of which belong to the anti-apoptotic BCL-2 family proteins and share similar amino acid sequences. Because the inhibition of BCL-XL caused thrombocytopenia, therapeutic use of ABT-737 and ABT-263 is severely restricted. A new generation of BH3 mimics called venetoclax (ABT-199) has thus been developed, which can selectively target BCL-2. It can effectively induce CLL cell apoptosis and avoid the occurrence of thrombocytopenia (Souers et al., 2013). Venetoclax is highly cytotoxic against CLL cells *in vitro* irrespective of functional status of *TP53* in these cells (Anderson et al., 2016).

Clinical studies have shown that venetoclax has impressive clinical activity in CLL, especially in treating patients with refractory CLL (Roberts et al., 2016). However, despite significant improvement of CLL treatment with the novel drug, development of venatoclax resistance has recently been reported among CLL patients receiving the treatment (Herling et al., 2018b). For example, the resistance to venetoclax has been shown to be caused by an acquired point mutation in BCL-2 (G101V), albeit in some but not all cases (Blombery et al., 2019). *In-vitro* studies have also shown that CLL cells can develop resistance to ABT-199 by activation BCR signalling pathway (Bojarczuk et al., 2016) or via CD40 stimulation (Thijssen et al., 2015, Oppermann et al., 2016). Over-expression of other anti-apoptotic BCL-2 family proteins such as BCL-XL and MCL-1 in these cells has been implicated for the resistance to ABT-199 (Bojarczuk et al., 2016, Thijssen et al., 2015, Oppermann et al., 2016).

1.1.6 Drug resistance remains a challenger in CLL management

Despite significant advances in our understanding of CLL biology and progress in the treatment of the disease especially after the introduction of novel, small molecule inhibitors targeting BCR or anti-apoptotic protein into the CLL clinic in recent years, the disease is still incurable (Sharma and Rai, 2019). A major challenge in CLL management is that most patients receiving drug therapies including the novel, molecularly targeted agents eventually develop resistance, leading to relapsed disease (Kipps et al., 2017, Sharma and Rai, 2019). Whilst *TP53* mutations/deletions remain the most important adverse prognostic factor predicting poor response to drug therapies, tumour microenvironment-mediated protection of drug-induced apoptosis is also considered as an important cause of drug resistance in CLL given the high dependence of leukemic cells on external factors for proliferation and survival (Burger

and Gribben, 2014, Shain et al., 2015). The microenvironment-mediated drug resistance is now recognised as a form of *de novo* drug resistance where leukemic cells are protected from the initial effects of drug therapy. It is believed that particular niches within the microenvironment provide a safe haven for subpopulations of leukemic cells. This in turn confers leukemic cells a survival advantage, allowing them to survive the assault of drug, resulting in minimal residual disease (MRD) (Meads et al., 2009). Subsequently, these tissue residual leukemic cells are able to expand and evolve through either acquisition of additional genetic mutations or selection of pre-existing clones that have survived therapy and developed more complex phenotypes including acquired drug-resistance. Persistent expansion of such leukemic cells eventually results in relapsed disease, which is much less likely to respond to subsequent therapy after developing acquired resistance. Therefore, studying into how the microenvironment mediates the drug resistance in CLL has been an area of intensive basic and translational research over the years.

As a result of the better understanding the role of BCR (a key component of the CLL microenvironment) in pathogenesis of the disease, therapeutic options for CLL patients have improved significantly with the discovery of BCR signalling inhibitors, such as ibrutinib and idelalisib, with both agents capable of inducing rapid and durable remission, as described earlier. Despite their impressive clinical activity, patients who fail to respond or who relapse even after long periods of remission have now been reported (Woyach and Johnson, 2015). Similarly, introduction of venetoclax, a small molecule inhibitor of BCL-2, into the clinics as a novel therapeutic agent has improved the management of difficult-to-treat, high risk patients with CLL. Nevertheless, resistance to venetoclax has recently been reported (Herling et al., 2018b, Blombery et al., 2019). Evidence from laboratory studies suggests that CLL cells can develop resistance to venetoclax after receiving survival signals that are relevant to the CLL microenvironment, as previously discussed. Therefore, research into the mechanisms of drug resistance is still urgently required in order to inform the therapeutic strategy to overcome such resistance.

1.1.7. Research hypothesis

Based on the observation that complete response rate to venetoclax treatment is lower in CLL patients with bulky lymph nodes (8%) comparing to those without (38%) and the fact that disease progression occurred in 41 venetoclax-treated patients with CLL (35%) (Roberts et al., 2016), it indicates that full potential of venetoclax is likely to be achieved through combination with other drugs. Coupled with the fact that survival of CLL cells depends on the signals from the microenvironment, venetoclax in combination with a drug that inhibit the survival signal from the microenvironment could be a rational strategy for therapeutic approach. It has also been shown that CLL cells can develop resistance to ABT-199 via CD40 stimulation and this resistance is associated with up-regulated expression of BCL-XL and MCL-1 in CD40-stimulated CLL cells (Thijssen et al., 2015, Oppermann et al., 2016). Therefore,

the hypothesis of my research project is that a combination therapy involving BCL-2 inhibitor ABT-199 and inhibitor of BCL-XL or MCL-1 can induce apoptosis in CD40-stimulated CLL cells, thus overcoming CD40 stimulation-mediated resistance to ABT-199.

1.1.7.1 Research questions

To test the above hypothesis, I wanted to use an established co-culture system where CLL cells are cultured with transfected mouse embryonic fibroblasts expressing human CD154 to mimic the CD40-CD154 interaction between T cells and CLL cells in the lymph node microenvironment. Once the CD40 stimulation condition is optimised, I want to address the following research questions:

(1) How does CD40-stimulated CLL cells respond to ABT-199, in comparison with unstimulated cells?
(2) Whether MCL-1 and BCL-XL are upregulated in CD40-stimulated CLL cells?
(3) Whether pre-treatment of CD40-stimulated CLL cells with an inhibitor of MCL-1 or BCL-XL can restore their sensitivity to ABT-199?
(4) If the answer to the question 3 is yes, what are the underlying mechanisms mediating sensitisation effect by the MCL-1 or BCL-XL inhibitor?

1.1.7.2 Aims of the study

Therefore, the aim of my study is to identify optimal combination partners for ABT-199, which would be effective in eliminating ABT-199-resistant population of CLL cells. In doing so, my study would provide a proof-of-concept that resistance to venetoclax seen in patients with CLL can be overcome through a combination therapy involving inhibition of BCL-XL or MCL-1 and the BCL-2 inhibitor.

Chapter 2. Methodology

2.1 Methods

2.1.1 CLL cell preparation

Primary CLL cells from patients were used in my study. All patient samples were obtained with informed consent and the approval of the Liverpool (Adult) Local Research Ethics Committee. Clinical information of the CLL samples used in the study are provided in Table 1. Peripheral blood mononuclear cells (PMBCs) were isolated from venous blood donated by CLL patients by centrifugation of blood over Lymphoprep (Catalogue number: 1114544, Axis-Shield PoC AS, Oslo, Norway). PBMCs were routinely stored in a -150^{o}C freezer in the University of Liverpool Leukaemia Biobank.

Table 1. Clinical information of the CLL samples used in the study.

Sample no.	Gender	Age at diagnosis	Staging (Binet)	WBC (10^9/L)	IGHV mutation¶	Chromosomal abnormalities	Prior Treatment*
3379	M	69		228.5			
3605	M	60	B	252.8	M	13q-	no
3620	F	69	C	145.9	M	None	no
3642	M	67	C	129.4	UM	None	yes
3645	M	68	C	264.1	UM	None	no
3647	F	66	A	145.9	M	None	no
3650	F	63	B	119.6	M	None	no
3684	M	75	A	230.1	M	13q-	no
3694	M	55	C	119.8		None	yes
3702	M	61		33.1	UM	13q-	yes
3711	M	53		179.3	M	None	no

¶ IGHV mutation status refers to somatic mutation in IGHV gene of CLL cells as compared with the gene sequence of germ-line using 2% as a cut-off. M: mutated; UM: unmutated.
*Prior treatment consists of the therapeutic administration of various combinations of steroid, chlorambucil, fludarabine, or fludarabine plus cyclophosphamide and rituximab or ibrutinib.

2.1.2 Culturing primary CLL cells under standard conditions

Cryopreserved CLL cells were first thawed in ice-cold RPMI-1640 medium (catalogue number: R0883, Sigma-Aldrich, Poole, UK) which was supplemented with 10% heat-

inactivated fetal bovine serum (FBS, catalogue number: 10270106, Life Technologies/ThermoFisher Scientific, Paisley, UK), 2 mM L-glutamine (catalogue number: G4513, Sigma-Aldrich) and 1% penicillin/streptomycin (catalogue number: P0781, Sigma-Aldrich). Next, thawed cells were spun at 550g for 5 min at 4 °C, washed in 10 ml of the above RPMI medium, and finally re-suspended in 2-5 ml of the RPMI medium and kept in a 5 % CO_2 incubator at 37°C for 1 hour to recover.

After recovery, the viability of primary CLL cells was determined using an automated cell counter (Cellometer Auto T4 Cell Viability Counter, Nexcelom Bioscience, MA, USA) after staining the cells with viability dye (0.1 % trypan blue). The viability of primary CLL cells after thawing varies from sample to sample and the average viability of CLL cells was 78.6%. The density of viable CLL cells was then adjusted to 4 x 10^6 cells/ml with fresh RPMI medium and cultured in a 37°C humidified incubator containing 5 % CO_2.

2.1.3 Co-culture of CLL cells

To mimic the *in-vivo* cross-talk between CLL cells and T cells via CD40-CD154 interaction, primary CLL cells were co-cultured with transfected mouse embryonic fibroblasts expressing human CD154, as previously described (Vogler et al., 2009b).

2.1.3.1 Culturing parental and CD154-expressing mouse embryonic fibroblasts

Stably transfected mouse embryonic fibroblasts, both parental cells which were transfected with an empty vector and hence used as control, and those that were transfected with an expression vector containing human CD154 cDNA were provided by Professor Gerry Cohen in the Department and maintained as described (Zhuang et al., 2014, Vogler et al., 2009a). Briefly, both types of fibroblasts were maintained in DMEM medium (catalogue number: 32430100, Life Technologies/ThermoFisher Scientific) supplemented with 10% FBS, 1% Penicillin/Streptomycin and 2 mM L-glutamine and incubated at a 37°C, humidified incubator containing 5% CO_2. Before co-culture experiments, both fibroblasts were irradiated at a dose of 75 Gy to stop them dividing, and then seeded at a density of 3 x 10^5 cells/ml into multi-well plates and left in the 37°C incubator for 24 hours for the irradiated fibroblasts to form the adherent monolayer.

2.1.3.2 Co-culture of CLL cells with parental and CD154-expressing fibroblasts

Previous work from our laboratory has shown that co-culture of CLL cells with CD154-expressing fibroblasts for 24 h has resulted in significant up-regulation in expression of anti-apoptotic proteins including BCL-XL and MCL-1 and that the co-cultured CLL cells were protected from spontaneous apoptosis and cell death induced by fludarabine and bendamustine (Zhuang et al., 2014; Chapman et al., 2017). In this

study, thawed primary CLL cells (3 x 10^6 cells/ml) were seeded on top of the adherent fibroblasts at a 10:1 ratio and co-cultured for 24 hours. Co-cultured CLL cells were then harvested by gentle pipetting (to avoid disrupting the monolayer of fibroblasts, hence minimising the risk of contamination of the fibroblasts) for subsequent experiments (e.g. induction of apoptosis by ABT-199, Western blotting or FACS analysis).

2.1.4 Western blotting

2.1.4.1 Principle

Western blotting is a commonly used method in molecular biology, biochemistry and immunological studies. The earliest Western blotting was developed in 1979 by Harry Towbin from the Swiss Friedrich Miescher Institute (Towbin et al., 1979). In broad terms the method involves three stages: firstly, cellular proteins are separated based on their masses and charges by polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS). Secondly, proteins in the gel are transferred and immobilized onto a membrane (such as nitrocellulose or polyvinylidene difluoride (PVDF) membrane with high protein-binding affinity). Finally, proteins on the membrane are detected using either horseradish peroxidase (HRP) conjugated primary antibodies or two-step procedure involving primary antibodies and HRP conjugated secondary-antibodies, together with enhanced chemiluminescence (ECL) substrates (Mahmood and Yang, 2012).

Before electrophoresis, protein samples are usually heated to denature the proteins. This ensures that individual proteins can be separated in the gel based on size during electrophoresis. The most common type of gel electrophoresis is the polyacrylamide gels with addition of sodium dodecyl sulfate (SDS). In addition, strong reducing agents such as β-mercaptoethanol are used to remove secondary and tertiary structure (e.g. disulfide bonds [S-S] to sulfhydryl groups [SH and SH]), thus allowing separation of proteins based on their molecular mass during SDS-PAGE. Proteins are covered by the negatively charged SDS, effectively becoming anionic and migrating towards the positively charged anode through the acrylamide mesh of the gel. Smaller molecules move faster through this mesh and the proteins are separated based on their size (often described in kilodaltons, kDa). The concentration of acrylamide corresponds to resolution of the gel, i.e. the higher concentration of the acrylamide gives rise to the better resolution for proteins with lower molecular weight. Conversely, the lower concentration of acrylamide produces the better resolution for proteins with higher molecular weight.

2.1.4.2 Protein lysate preparation

Before lysis, CLL cells were collected into 1.5ml tubes and washed once in ice-cold

phosphate buffered saline (PBS). Approximately 4 x10^6 cells were lysed in 100 µl of RIPA buffer containing 50 mM Tris/HCL (pH 7.5), 150 mM NaCl, 1% (w/v) Triton-X100, 0.1 % (w/v) SDS, 0.5% (w/v) Na Deoxycholate supplemented with protease and phosphatase inhibitor cocktails. Protease inhibitor cocktail (catalogue number: P8340, Sigma-Aldrich) and phosphatase inhibitor cocktail (catalogue number; 524625, Calbiochem/Merck Millipore (UK), Watford, UK) were obtained commercially. Cells were vortexed and left on ice for 10 mins. To ensure that cells are completely lysed samples were then sonicated (5 cycles, 30 seconds ON/30 seconds OFF/cycle on "high setting") using the Diagenode Bioruptor (Seraing, Belgium). After sonication, cell lysates were clarified by centrifuging at 10,000g for 20 min at 4°C and supernatant (protein lysate) was collected into a new tube.

2.1.4.3 Protein concentration determination

Protein concentration was then determined using the Bio-Rad DC™ (detergent compatible) protein assay Kit (catalogue number: 500-0116, Bio-Rad laboratories, Hertfordshire, UK). The Bio-Rad DC™ protein assay is derived from the Lowry assay, which is based on two chemical reactions causing colour change. Firstly, protein reacts with copper in an alkaline solution, then the copper-bound protein can reduce folin phenol reagent via loss of oxygen atoms, resulting in reduced species which display a blue colour (Lowry et al., 1951). Thus, intensity of the blue colour closely correlates with concentration of cellular proteins.

2.1.4.4 SDS-PAGE and protein transfer

Next, fixed amount (e.g. 10 µg) of cellular protein was mixed with equal volume of 2 x Laemilli buffer containing 125 mM Tris/HCL (pH 6.8), 30% glycerol, 4% SDS, 0.1% bromophenol blue, 20 mM ethylenediaminetetraacetic acid (EDTA) and reducing agent β-mercaptoethanol. Protein samples were denatured by boiling at 95°C for 5 minutes before being loaded onto a pre-set polyacrylamide gel. Electrophoresis was performed under constant voltage of 115V for about 1.5 hour to separate proteins on the gel. At the end of SDS-PAGE, proteins on the gel were transferred to PVDF membrane (Catalogue no. 03010040001, Roche Diagnostics GmbH, Mannheim, Germany) through electrophoresis under constant current at 400 mA for 1 hour.

2.1.4.5 Immunoblotting and image visualization

After proteins have been transferred to PVDF membrane, the membrane was rinsed briefly in Tris-buffered saline plus Tween-20 solution (TBST) containing 20 mM Tris/HCL (pH 7.5), 150 mM NaCl and 0.1% Tween-20 for 15 minutes. It was then incubated with blocking solution (5% milk in TBST) for 30 minutes before incubation with primary antibody in 5% milk/TBST for 1 hour at room temperature or overnight at 4°C. The membrane was then washed with TBST and incubated with 5% milk/TBST for 30

minutes before incubation with appropriate secondary antibody conjugated with HRP in 5 % milk/TBST for 1 hour at room temperature. Finally, the membrane was washed 3 time in TBST before being exposed to ECL substrate solution (made of equal volume of peroxide solution and Luminol/enhancer solution, both obtained from Merck Millipore, UK) for 1 minute. The immunoreactive bands on the membrane were visualized on an Image Reader LAS-1000 System (Fujifilm, Tokyo, Japan).

2.1.5 Flow cytometry

2.1.5.1 Principle

Flow cytometry is a method commonly used in biomedical science research. A flow cytometer consists of five major parts: a flow cell, a measuring system, a detector, an amplification system, and a computer for data analysis (Picot et al., 2012). The flow cell has a liquid stream (sheath fluid), which helps the cells flow in singlet so that they pass single file through the light beam for sensing. The measuring system uses measurement of impedance (or conductivity) and optical systems such as lamps (e.g. mercury, xenon) or high-power water-cooled lasers (e.g. argon, krypton) or low-power air-cooled lasers such as argon (488 nm). The detector and analog-to-digital conversion system converts analogue determinants of forward-scattered light (FSC) and side-scattered light (SSC), and fluorescence signals from fluorochrome probes into digital signals which can be further amplified (in linear or logarithmic scale) and analysed by a computer (Givan, 2011).

Because the data generated by flow-cytometers can be displayed in a single dimension to produce a histogram, or in two-dimensional dot plots or even in three dimensions, the selected regions on these plots can be sequentially divided, based on fluorescence intensity, using a series of subset extractions, also known as gates. These data can be quantitatively analysed using relevant software (Sharpless et al., 1975).

2.1.5.2 Flow cytometry analysis of cell death

During apoptosis, residues of phosphatidylserine (PS) are flipped from the interior of the plasma membrane to the outside of cell surface (Fadok et al., 1992). Annexin V protein secreted by endothelial cells, in the presence of Ca^{2+} ions, binds to the PS residue with high affinity (Koopman et al., 1994, Vermes et al., 1995). It is because of this property that recombinant human annexin V conjugated with fluorochromes such as fluorescein isothiocyanate (FITC) have been developed to detect apoptotic cells on flow cytometry. Since PS exposure occurs in the early stages of apoptosis (Fadok et al., 1992), fluorescein-labelled annexin V staining can detect apoptotic cells in the early stage of cell death.

When using this method to measure apoptotic cells, another fluorescent dye is usually

added, which can distinguish between apoptotic cells with intact plasma membrane and cells with permeable membrane. Cells with permeable membrane is a feature of late stage of apoptosis which often leads to necrosis. Propidium iodide (PI) is a fluorescent DNA intercalating reagent, which is commonly used in flow cytometry. It can freely enter the cells with broken membrane and irreversibly bind to DNA. Therefore, PI, together with FITC-labelled annexin V, can be used to differentiate apoptotic cells (single positive for annexin V only), necrotic cells (double positive for annexin V and PI) and healthy cells (negative for both) on flow cytometry.

Thus, following treatment with various cytotoxic agents, CLL cells were harvested and washed in PBS. Cells were then re-suspended in annexin V-binding buffer containing 10 mM HEPES (pH7.4), 140 mM NaCl, 2.5 mM $CaCl_2$, and stained with FITC-labelled annexin V (catalogue number: 556419, BD Biosciences, Oxford, UK) and PI (catalogue number: P4864, Sigma-Aldrich). Cell death were analysed using an Attune NxT Acoustic Focusing Cytometer (Life Technologies/ThermoFisher Scientific), with excitation wavelength set at 488 nm and use of emission filters of 530 nm for FITC and 585 nm for PI, respectively.

2.1.5.3 Flow cytometry analysis of CD154 expression in transfected mouse embryonic fibroblasts

To monitor the surface expression of human CD154 on transfected mouse embryonic fibroblasts that were used for co-culture experiments, flow cytometry analysis was performed on a monthly basis on both parental and CD154-expressing fibroblasts. In brief, the culture medium of fibroblast monolayers was first removed and the cells were washed with pre-warmed PBS. Fibroblasts were then removed from the culture flask by incubating with Cell Dissociation Solution (cat. no. C5789, Sigma-Aldrich).

Next, cell number was counted on Cellometer (Nexcelom Bioscience) as described earlier. 5 x 10^5 cells from parental and CD154-expressing fibroblasts were aliquoted into a 1.5 ml tubes containing 0.5 ml of PBS containing 0.1% bovine serum albumin (BSA) and incubated with 10 µL of FITC-labelled mouse anti-human CD154 (cat.no. 555699, BD Biosciences,) or FITC-labelled mouse IgG1κ isotype control antibody (cat. no. 555748, BD Biosciences,) at room temperature for 10 mins in the dark. Finally, cells were analysed for the expression of CD154 on Attune NxT Acoustic Focusing Cytometer with excitation wavelength at 488 nm and emission at 530 nm.

2.1.6 Co-immunoprecipitation

2.1.6.1 Principle

Co-immunoprecipitation (Co-IP) is one of the commonly used methods to detect

protein-protein interaction (Berggård et al., 2007, Markham et al., 2007). In general, a protein of interest is pulled down using a specific antibody. Any proteins bound to the protein of interest which is bound to antibody is then co-precipitated by using protein A or G-coated sepharose beads. Any proteins that are not bound to the protein of interest are eliminated from the sample through a series of washes. The pulled-down immunocomplexes are then analysed by Western blotting.

In practice, Co-IP method consists of several steps, including preparation of protein extract (usually a cell lysate), incubation of protein lysate with a specific antibody, adding protein A or G coated beads to pull down the antibody and associated protein complex, purification of protein complexes, and detection of protein of interest in the immunoprecipitated complex by performing SDS-PAGE and immunoblotting. Depending on the specificity and quality of antibody and experimental conditions, Co-IP experiments may generate significant background noise due to nonspecific binding of proteins to the antibody or beads. Thus, use of an isotypic, non-specific antibody as a control for the same samples is critical to identifying specific interacting proteins.

2.1.6.2 Co-IP procedure

To detect protein interaction between BCL-XL and BIM in CD40-stimulated CLL cells, CLL cells co-cultured with CD154-expressing fibroblasts were harvested 24 hours after co-culture and cell number counted on Cellometer (Nexcelom Bioscience). 1 x 10^7 cells were collected into each tube and washed with ice-cold PBS. Cell pellet was re-suspended in 100 µL Co-IP lysis buffer containing 1% CHAPS, 10 mM HEPES (pH 7.4), 150 mM NaCl, 2 mM EDTA, and a cocktail of protease inhibitors (cat. no. P8340, Sigma-Aldrich) and incubated for 1 hour at 4°C on a rotating device. The cell lysate was spun by centrifugation at 13,000 g for 20 minutes at 4 °C and resulting supernatant was collected into a new tube and used as a starting material for Co-IP. Protein G sepharose beads (cat. no. TB259624, Life Technologies/ThermoFisher Scientific) were added to the lysate to pre-clear proteins that will bind to protein G or beads and incubated for 1 hour at 4 °C. The protein concentration was then determined using the Bradford assay kit (Bio-Rad laboratories). For each Co-IP, 100 µg of protein lysates was incubated with 1 µg of rabbit anti-BCL-XL antibody or an equal amount of control rabbit IgG overnight at 4°C. Immunoprecipitated complexes were then captured using 50ul of protein G sepharose beads following incubation for 1 hour at 4 °C. The beads bound immuno-complexes were collected following centrifugation and washed 3 times with ice-cold wash buffer containing 10 mM HEPES (pH 7.4), 150 mM NaCl, 2 mM EDTA and 0.2% CHAPS.). The proteins that were bound to the beads are then analysed by Western Blotting, as described earlier.

2.1.7 siRNA knockdown experiment

2.1.7.1 Principle

Small interfering RNA (siRNA) is a type of double-stranded RNA (dsRNA) molecule, typically 20-25 bases in length. Some are similar to microRNA (miRNA). It can interrupt the expression of specific genes by degrading mRNA after transcription, thus inhibiting the translation of specific mRNAs into proteins (Bernstein et al., 2001).

Long dsRNA is cleaved by an endo-ribonuclease called DICER (Bernstein et al., 2001). DICER cuts long dsRNA into short interfering RNA or siRNA, which then form the RNA-Induced Silencing Complex (RISC) (Tijsterman and Plasterk, 2004)

The siRNA-induced post transcriptional gene silencing begins with the formation of the RNA-induced silencing complex (RISC) (Tijsterman and Plasterk, 2004). The complex shuts down gene expression by cleaving the mRNA molecules. To begin the process, the guide strand of the two siRNA strands (also known as anti-sense strand) is loaded into the RISC while the passenger strand (also called sense strand) is degraded (Lee et al., 2004). Then, the siRNA searches and directs RISC to perfectly matched sequence on the target mRNA molecules (Carthew and Sontheimer, 2009). The cleavage of the mRNA molecules is mediated by the Piwi domain of Argonaute proteins of the RISC (Ender and Meister, 2010). The mRNA molecule is then cleaved at the phosphodiester bond between the target nucleotides which are paired to siRNA residues 10 and 11 at the 5' end (Tomari and Zamore, 2005). This cleavage results in mRNA fragments being further digested by cellular exonucleases. Detachment of the target mRNA strand from RISC after the cleavage makes room for more mRNA to be silenced (Tomari and Zamore, 2005).

After siRNA is constructed against the target gene, it can be effectively delivered into a cell through a transfection procedure. Delivery methods include the use of cationic liposomes, polymer nanoparticles, and lipid conjugation (Zhang et al., 2007). Electrical pulses can also be used to deliver siRNA into cells through electroporation (Luft and Ketteler, 2015). Under quick but powerful electrical shock, the phospholipid molecules on the cell membrane rearrange themselves, resulting in the formation of hydrophilic pores and temporary permeability of the membrane. This allows for entry of siRNA into the cells. Electroporation method is particularly useful for cells that are difficult to transfect. However, cell death is much higher with this technique.

2.1.7.2 BIM siRNA knockdown experiment in CLL cells.

To knockdown the expression of BIM protein in CLL cells, cryopreserved CLL cells were thawed and cell number counted. 10 x 10^6 cells were collected into each of 2 x 1.5mL tubes and spun at 550 g at room temperature. Cells were then re-suspended in 100 µL

transfection solution from a Human B cell Nucleofector Kit (cat. no. VPA-1001, Lonza Biologics plc. Manchester, UK). 0.5 nmol of BIM siRNA duplexes or 0.5 nmol of non-specific control siRNA (both commercially obtained from Qiagen) were added to the cell suspension and mixed well before transferring to a cuvette (provided within Nucleofector kit) and placed inside the Nucleofector device (Amaxa AG/Lonza). Electroporation was performed using program X-03 on the Nucleofector device. Electroporated cells were then immediately mixed with 0.9 ml of pre-warmed RPMI medium and transferred to a 1.5 ml Eppendorf tube and incubated at 37^{o}C for 30 minutes. Cells were then adjusted to a cell density of 3 x 10^{6} cells/ml in pre-warmed fresh medium and seed the CLL cells on the monolayer of CD154-expressing fibroblasts. 24h later, transfected CLL cells on co-culture were harvested and cell number adjusted to 4 x 10^{6} cells/ml. The expression of BIM in transfected CLL cells were then determined by Western blotting, as described.

2.2 Materials

2.2.1 Reagents/chemicals

All chemicals, unless otherwise stated, were obtained from Sigma-Aldrich Company.

2.2.1.1 BCL-2 inhibitor ABT-199

ABT-199 was purchased from Selleck Chemicals (Houston, USA) via Stratech Scientific Ltd, Sulfolk, UK (catalogue number: S8048-SEL) and prepared in DMSO as 10 mM stocks and stored in a -20^{o}C freezer.

2.2.1.2 Non-selective CDK9 inhibitor Voruciclib

Voruciclib was directly purchased from ChemieTek, Indianapolis, USA (catalogue number: CT-VORU, www.chemietek.com) and prepared in DMSO as 10 mM stocks and kept in a -20^{o}C freezer.

2.2.1.3 BCL-XL inhibitor A-1331852

A-1331852 was purchased from MedChemExpress (New Jersey, USA) via Insight Biotechnology Ltd, Middlesex, UK (catalogue number: HY-19741) and prepared in DMSO as 10 mM stocks that were kept in -20^{o}C freezer.

2.2.1.4 BIM siRNA

Small interfering RNA (siRNA) targeting human *BCL2L11* gene encoding BIM protein was purchased from QIAGEN, Manchester, UK (catalogue number: SI02655359),

together with the negative control siRNA from the same supplier (catalogue number: 1027310).

2.2.2 Antibodies

Details of primary antibodies used for Western blotting are provided as follows: rabbit polyclonal MCL-1 antibody (S-19) from Santa Cruz Biotechnology Inc, California, USA via Insight Biotechnology Ltd (catalogue number: sc-819); rabbit monoclonal antibodies against BCL-2 (D55G8), BCL-XL (54H6) and BIM (C34C5) from Cell Signaling Technology, London, UK (catalogue numbers of 4223, 2764 and 2933, respectively) and mouse monoclonal antibody specific to β-actin (AC-74) from Sigma-Aldrich Company, Pool, UK (catalogue number: A5316). Secondary goat anti-rabbit antibody conjugated with horse-radish peroxidase (HRP) and goat anti-mouse antibody with HRP were purchased from Santa Cruz Biotechnology (catalogue numbers of sc-2004 and sc-2055, respectively). Rabbit antibodies to BCL-XL (54H6) was also used for co-immunoprecipitation experiments.

2.3 Statistical analysis

2.3.1 Statistical tests used

Two-tailed, paired Student t-test was used and p values calculated using Microsoft Office Excel 2010 to determine if the difference between the two groups is significant. p value of <0.05 is considered to be statistically significant.

2.3.2 ImageJ software

ImageJ is a Java-based image processing software (Collins, 2007). ImageJ can be used to calculate pixel value in a defined area. In my study, it has been used for the densitometry analysis of the intensity of signals of proteins of interest on the membranes from Western blotting.

Chapter 3. Characterisation of sensitivity of CD40-stimulated CLL cells to ABT-199

3.1 Background

Although there are several effective conventional and novel therapies available to treat patients with CLL, one important problem yet to be resolved is the recurrence of relapsed disease. The tumour microenvironment (TME) of the lymph nodes and to a lesser degree the bone marrow is the major location for CLL cell proliferation (Burger, 2011). TME regulates the survival and apoptosis of CLL cells through activating pro-survival and anti-apoptosis signalling pathways. The fact that CLL cells overexpress anti-apoptotic protein BCL-2 make this protein an ideal target for therapeutic intervention. Indeed, BCL-2 inhibitor ABT-199 has been successfully developed and shows impressive clinical activity in CLL (Souers et al., 2013). However, resistance to ABT-199 has recently been reported among patients with CLL receiving treatment (Herling et al., 2018b, Blombery et al., 2019). Laboratory studies have also shown that CLL cells can develop resistance to ABT-199 via activation BCR signalling pathway (Bojarczuk et al., 2016) or via CD40 stimulation (Thijssen et al., 2015, Oppermann et al., 2016). BCR signalling and CD40 stimulation are the major pro-survival signalling pathways that are specifically activated in CLL cells residing in the lymph nodes (ten Hacken and Burger, 2016). Activation of these pathways is thought to be mediated via the interaction of CLL cells with the neighbouring T cells, nurse-like cells and stromal cells (Burger, 2011). As a result, CLL cells acquire additional pro-survival signals and become less sensitive to drug therapy, which may be responsible for residual disease after conventional treatment (Burger, 2011). CLL cells are known to depend on microenvironmental stimuli for survival.

Engagement of CLL B cells by T cells via ligation of CD40 with CD154 (CD40 ligand) is a key molecular mechanism mediating interaction between CLL cells and T cells (Schattner, 2000). Activation of CD40 signalling has been shown to be sufficient to rescue CLL cells from spontaneous apoptosis in vitro (Purroy et al., 2015). Stimulation of CD40 receptor in CLL B cells leads to activation of transcription factor NF-κB through both canonical and non-canonical pathways (Bishop et al., 2007, Elgueta et al., 2009, Rickert et al., 2011), but also via a PI3K/AKT-mediated signalling mechanism (Cuni et al., 2004). The balance in the levels of expression between the pro- and anti-apoptotic members of Bcl-2 family of proteins is clearly important in determining the sensitivity of cells to apoptosis. For example, circulating CLL cells not only express high levels of Bcl-2, but also express high levels of Bim (Moore et al., 2007, Mason et al., 2009). However, it was also shown that almost all BIM was constitutively bound to BCL-2 and thus kept inactive (Moore et al., 2007, Melarangi et al., 2012), potentially explaining why CLL cells are long-lived *in vivo*.

ABT-199 is a novel BCL-2 inhibitor that has shown excellent results in numerous clinical

studies. As described earlier, BCL-2 inhibitor ABT-199 is a BH3 mimetic that is very effective in inducing apoptosis of circulating CLL cells. However, it is not very effective in inducing apoptosis of CLL cells that are activated by the stimuli relevant to CLL microenvironment, such as CD40 stimulation (Thijssen et al., 2015, Oppermann et al., 2016). CD40 stimulation of CLL cells leads to increased expression of anti-apoptotic proteins such as MCL-1, BCL-XL, A1/BFL1 and c-FLIP (Vogler et al., 2009b, Zhuang et al., 2014).

Whilst the overall aim of my study is to develop a strategy of combination therapy to overcome resistance to ABT-199 in CD40-stimulated CLL cells, in this part of the study I want to first characterise the responsiveness of CD40-stimulated CLL cells to induction of apoptosis by ABT-199, in comparison with unstimulated cells. For CD40 stimulation, I used an established co-culture system where primary CLL cells were co-incubated with monolayers of stably transfected mouse fibroblasts expressing human CD154 (Vogler et al., 2009b, Zhuang et al., 2014). CLL cells co-cultured with monolayers of parental mouse fibroblasts not expressing human CD154 were used as a control. After co-culture for 24 h, CLL cells were collected from the respective monolayers and incubated with a range of concentration of ABT-199. Cell death was measured by flow cytometry following staining the cells with FITC-labelled annexin V and PI, as described in the Method. The expression of anti-apoptotic BCL-2 family of proteins of CLL cells co-cultured with the respective monolayers of fibroblasts was also examined using Western blotting method.

3.2 Methods

3.2.1 CLL samples

Cryopreserved primary CLL samples stored in the University of Liverpool Leukaemia Biobank were used in my study. The clinical information of the CLL samples used in this part of the study is provided in the Methodology chapter. After thawing, CLL cells were maintained in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin, as described in the Methodology chapter.

3.2.2 Coculture of CLL cells with transfected CD154-expressing mouse fibroblasts

Stably transfected mouse fibroblasts expressing human CD154 (CD40L) and control fibroblasts transfected with empty plasmid were maintained as described in the Methodology chapter. For CD40 stimulation, CLL cells were seeded on an adherent monolayer of fibroblasts expressing CD154 or control fibroblasts at a ratio of 10:1 and cultured at 37°C for 24 h. To determine the killing effect of ABT-199 on CD40-stimulated cells, CLL cells were collected from the co-culture with the respective monolayers and incubated under standard culture conditions at a density of 4 x 10^6

cells/mL in the presence or absence of ABT-199 for 4 h. The incubation time of 4 h with ABT-199 was chosen because ABT-199 has been shown to be very effective in inducing apoptosis of primary CLL cells with an effective concentration that kills 50% of the treated cells (EC_{50}) determined at 7.6nM after only 4h of exposure (Vogler et al., 2013).

3.2.3 Flow cytometric analysis of cell death

At the end of the incubation with cytotoxic drugs, CLL cells were harvested and cell death measured using a flow cytometry method employing dual staining with FITC labelled annexin-V and propidium iodide as described in the Methodology section. Aliquots of cells from the same experiment were analysed by Western blotting for protein expression.

3.2.4 Western blotting analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were performed essentially as described in the Methodology chapter. Briefly, cellular proteins were separated on an SDS-polyacrylamide gel and transferred to polyvinilidene difluoride (PVDF) membranes (Roche/Sigma-Aldrich Company, Dorset, UK), which were then incubated with the appropriate primary antibodies. Immunoreactivity was detected with the relevant horse-radish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology), which in turn were visualized on an Image Reader LAS- 1000 (Fujifilm, Tokyo, Japan) using an enhanced chemiluminescence (ECL) kit (GE Healthcare Life Sciences, Buckinghamshire, UK). For quantification of the signals, the images were further analysed on the same instrument using 2D Densitometry Aida Image Analyzer software (Fujifilm).

3.3 Results

3.3.1 Phenotyping of transfected mouse fibroblasts

First, flow cytometry was used to confirm the phenotype of the two transfected mouse fibroblasts by staining the cells with FITC-labelled anti-CD154 antibody. Isotype antibody was used as a control. As shown in Figure 3(left panel), the percentage of CD154+ve cells was less than 1 % in control parental fibroblasts. In contrast, over 93% of CD154-expressing fibroblasts were positive when stained with anti-CD154 antibody (Figure 3, right panel). Therefore, I confirmed that the two groups of fibroblasts expressed the correct phenotypes and CLL cells co-cultured with CD154-expressing fibroblasts would receive the CD40 stimulation. In order to ensure that correct types of fibroblasts were used throughout my study, I tested the expression of CD154 on both fibroblasts once every month. When the positivity of CD154 expression was above 90 % in CD154-expressing fibroblasts, but less than 1 % in parental fibroblasts I continued studies for subsequent co-culture experiments.

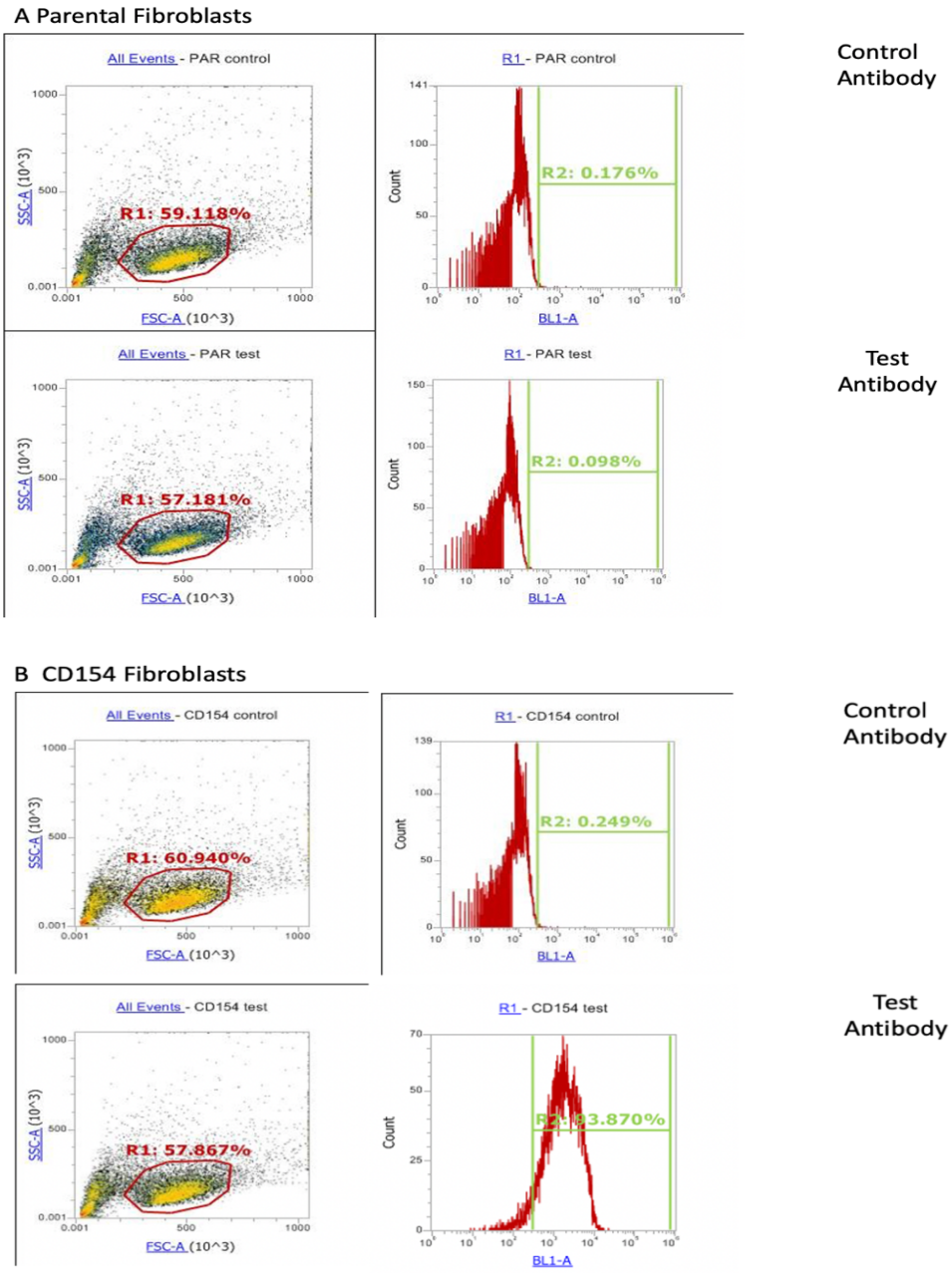


Figure 3. Confirmation of the phenotype of parental and CD154-expressing mouse fibroblasts used for co-culture experiments. (A) Parental and (B) CD154-expressing fibroblasts were incubated with 10µl FITC-labelled mouse IgG1κ isotype control antibody (top panels) and 10µl FITC-labelled mouse anti-human CD154 antibody (bottom panels). CD154 expression on both types of fibroblasts was analysed using flow cytometry, as described in Methods.

3.3.2 Induction of cell death by ABT-199 in CD40-stimulated CLL cells

3.3.2.1 Result from CLL sample #3650

I first co-cultured CLL cells from sample #3650 with control parental or CD154-expressing fibroblasts for 24 hours. Co-cultured CLL cells were then harvested by gentle pipetting and incubated under standard culture conditions at a density of 4 x 10^6 cells/mL with or without ABT-199 for 4 h. As shown in Figure 4, in the absence of ABT-199 CLL cells co-cultured with parental fibroblasts (i.e. unstimulated) were subjected to higher level of spontaneous apoptosis with the death rate of 15 % detected, whereas in CLL cells co-cultured with CD154-expressing fibroblasts (i.e. CD40-stimulated) cell death rate of only 6.4 % detected. With the increasing concentration of ABT-199, the cell death rate of unstimulated CLL cells increased in a concentration-dependent fashion and reached to 93.2 % with 10 nM ABT-199 (Figure 4, blue line). In contrast, the cell death rate of CD40-stimulated CLL cells was lower at all concentrations of ABT-199 tested and reached only 20.9% at the corresponding 10 nM ABT-199 (Figure 4, orange line). Even at 1000 nM, ABT-199 induced only about 45 % of cell death in CD40-stimulated CLL cells whereas it killed almost all unstimulated cells (Figure 4). The above result showed that CD40 stimulation could reduce both spontaneous and ABT-199-induced cell death, in agreement with previous findings (Thijssen et al., 2015).

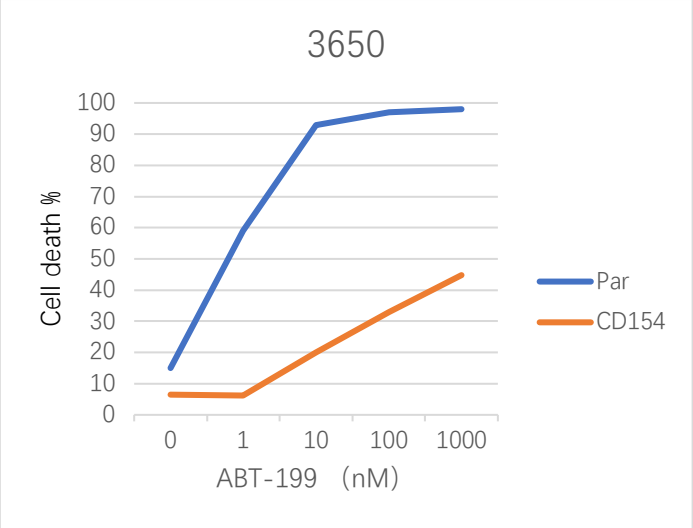


Figure 4. Induction of cell death by ABT-199 in co-cultured CLL cells (sample #3650). The CLL cells were cocultured for 24 h with either the parental control fibroblasts (Par) or CD154-expressing fibroblasts (CD154). The CLL cells were then harvested and incubated under standard culture conditions with varying concentrations of ABT-199 for 4 hours. At the end of incubation, cells were collected and stained with Annexin V and PI before cell death analysis by flow cytometry.

3.3.2.2 Result from CLL sample #3642.

I next repeated the same experiment using CLL cells from second patient sample (#3642). As shown in Figure 5, in the absence of ABT-199, the unstimulated cells undergo spontaneous apoptosis with14 % of cells double positive for annexin V and PI. However, apoptotic rate of CD40-stimulated CLL cells was only 2.4 %, much lower than that in unstimulated cells. Again, with an increase in concentrations of ABT-199, cell death was induced in a concentration-dependent fashion in unstimulated CLL cells. The cell death rate was 36% at 1 nM ABT-199 reached 93% at 10 nM and above of ABT-199 (Figure 5, blue line). The CD40-stimulated CLL cells, however, had a cell death rate of 4.5 % at 1 nM; 21.3 % at 10 nM; 38.6 % at 100 nM and 52.2 % at 1000 nM ABT-199, respectively (Figure 5, orange line). The above results again demonstrated that CD40 stimulation clearly had a protective effect on CLL cells against ABT-199-induced cell death.

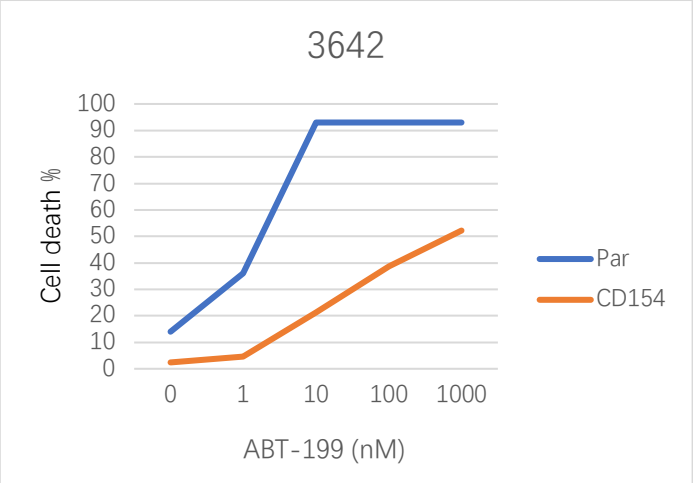


Figure 5. Induction of cell death by ABT-199 in co-cultured CLL cells (sample #3642). The CLL cells were cocultured for 24 h with either the parental control fibroblasts (Par) or CD154-expressing fibroblasts (CD154). The CLL cells were then harvested and incubated under standard culture conditions with varying concentrations of ABT-199 for 4 hours. At the end of incubation, cells were collected and stained with Annexin V and PI before cell death analysis by flow cytometry.

3.3.2.3 Result from CLL sample #3645

I then used CLL cells from the third sample (#3645) and performed the same experiment. As shown in Figure 6, in the absence of ABT-199, unstimulated CLL cells underwent spontaneous apoptosis with cell death rate of 25 %. However, CD40-stimulated CLL cells had a much less spontaneous cell death (8.4 %). With the increasing concentrations of ABT-199, unstimulated CLL cells underwent cell death in a concentration-dependent manner. At 10 nM ABT-177, 95 % of unstimulated CLL cells was killed (Figure 6, blue line). In contrast, in CD40-stimulated CLL cells only 29.8 % of

cell death was detected at the corresponding 10 nM ABT-199 (Figure 6, orange line). When the concentration of ABT-199 was increased to 100 nM, the cell death rate of unstimulated CLL cells reached 94.9 %, whereas only 40.9 % of CD40-stimulated CLL cells were dead (Figure 6). Again, ABT-199 was very effective in killing unstimulated CLL cells, but less so to CD40-stimulated CLL cells.

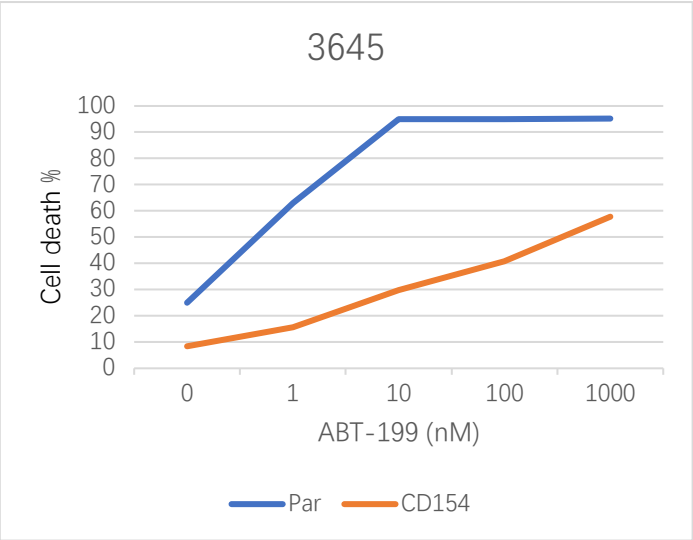


Figure 6. Induction of apoptosis by ABT-199 in co-cultured CLL cells (sample #3645). The CLL cells were cocultured for 24 h with either the parental control fibroblasts (Par) or CD154-expressing fibroblasts (CD154). The CLL cells were then harvested and incubated under standard culture conditions with varying concentrations of ABT-199 for 4 hours. At the end of incubation, cells were collected and stained with Annexin V and PI before cell death analysis by flow cytometry.

3.3.2.4 Result from CLL sample #3647

I next repeated the same experiment using CLL cells from fourth patient sample (#3647). As shown in Figure 7, in the absence of ABT-199, the unstimulated cells undergo spontaneous apoptosis with 9.5 % of cells double positive for annexin V and PI. However, cell death rate of CD40-stimulated CLL cells was 6 %, lower than that in unstimulated cells. Again, with an increase in concentrations of ABT-199, cell death was induced in a concentration-dependent fashion in unstimulated CLL cells. The cell death rate was 60.6 % at 1 nM, 93.7 % at 100 nM and 94.1 % at 1,000 nM ABT-199, respectively (Figure 7, blue line). In contrast, the CD40-stimulated CLL cells had a cell death rate of 9. 9% at 1 nM; 29.4 % at 10 nM; 41.9 % at 100 nM and 48.5 % at 1,000 nM ABT-199, respectively (Figure 7, orange line). The results demonstrated that CD40 stimulation clearly had a protective effect on CLL cells.

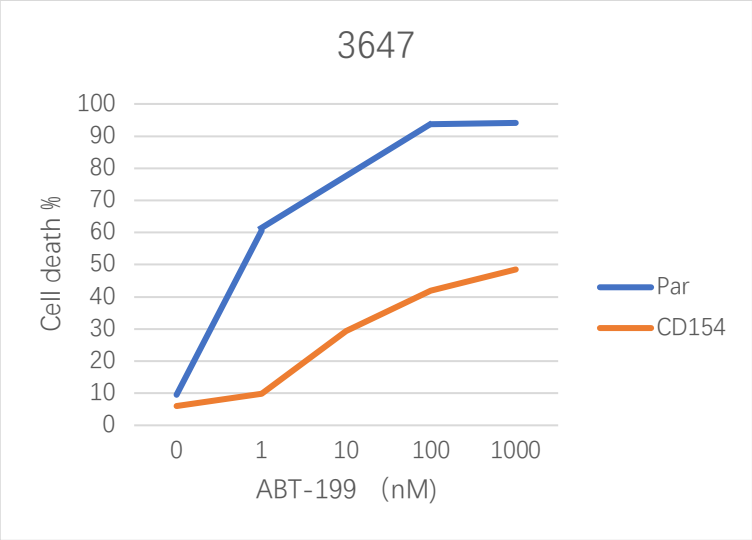


Figure 7. Induction of cell death by ABT-199 in co-cultured CLL cells (sample #3647). The CLL cells were cocultured for 24 h with either the parental control fibroblasts (Par) or CD154-expressing fibroblasts (CD154). The CLL cells were then harvested and incubated under standard culture conditions with varying concentrations of ABT-199 for 4 hours. At the end of incubation, cells were collected and stained with Annexin V and PI before cell death analysis by flow cytometry.

3.3.2.5 Pooled data analysis of induce of cell death by ABT-199.

I then pooled cell death data from the above four CLL samples and showed that in the absence of ABT-199, unstimulated CLL cells experienced an average of 16 % of spontaneous cell death, as compared to an average of 6 % in CD40-stimulated CLL cells (Figure 8). I performed statistical analysis to determine if the difference in cell death between stimulated versus unstimulated cells was significant. A two-tailed, paired Students' t-test was used for the analysis, which showed that p value was 0.035 (i.e. less than 0.05). Therefore, CD40 stimulation significantly reduced spontaneous cell death of CLL cells.

I next performed similar statistical test to see if the difference in ABT-199-induced cell death was significant between the two group of CLL cells. At a concentration of 1 nM, ABT-199-induced an average of 9 % cell death in CD40-stimulated CLL cells whereas cell death was increased to 55 % in unstimulated CLL ($p = 0.025$). Similarly, at 10 nM, ABT-199-induced cell death in 25 % of CD40-stimulated CLL cells, comparing to 94 % in unstimulated CLL cells ($p = 0.001$). At 100 nM, ABT-199 induced cell death of 95 % in unstimulated CLL cells, whereas it only killed 39 % of CD40-stimulated CLL cells ($p = 0.002$). Finally, at a concentration of 1000 nM, ABT-199 induced cell death in 95 % of unstimulated CLL cells, but only 51 % in CD40-stimulated CLL cells ($p = 0.001$).

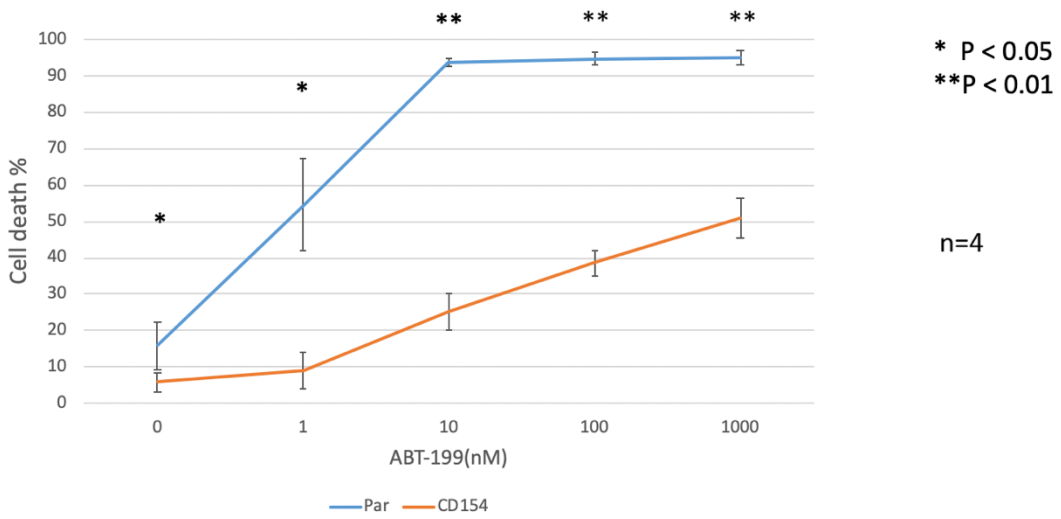


Figure 8. Pooled data analysis of induction of apoptosis by ABT-199 in co-cultured CLL cells (n=4). The CLL cells were cocultured for 24 h with either the parental control fibroblasts (Par) or CD154-expressing fibroblasts (CD154). The CLL cells were then harvested and incubated under standard culture conditions with varying concentrations of ABT-199 for 4 hours. At the end of incubation, cells were collected and stained with Annexin V and PI before cell death analysis by FACS. A two-tailed, paired Students' t-test was used to determine the significance of difference in cell death between CD40-stimulated and unstimulated cells (* refers to p value of <0.05 and ** p value <0.01). Each data point represents mean ± standard error of results from independent experiments using 4 different CLL cases.

Based on the above results, it was clear that, although the addition of ABT-199 resulted in a concentration-dependent increase in cell death in both CD40-stimulated and unstimulated CLL cells, ABT-199 is significantly more effective in killing unstimulated than CD40-stimulated CLL cells. Therefore, data from my study showed that CD40 stimulation clearly protected CLL cells from ABT-199-induced cell death.

3.3.3 Expression of BCL-2, MCL-1 and BCL-XL in CD40-stimulated CLL cells

Since previous studies from our group and others have shown that CD40 stimulation leads to increased expression of anti-apoptotic BCL-2 family proteins such as MCL-1 and BCL-XL (Vogler et al., 2009a, Zhuang et al., 2014), I wanted to confirm these findings in my study by performing Western blotting to examine the expression of these proteins in CD40-stimulated and unstimulated cells.

Primary CLL cells from three individual patients with CLL (cases #3642, #3647 and #3650) were used for this part of the work. CLL cells were first co-cultured with parental or CD154-expressing mouse fibroblasts for 24 h. As shown in Figure 9, level of expression of MCL-1 was low in unstimulated CLL cells from all three samples. CD40 stimulation induced increased expression of MCL-1 in all three samples. Similarly, level

of BCL-XL expression was low in unstimulated CLL cells from two of the three sample examined (Figure 9, #3642 and #3650). Unstimulated CLL cells from sample #3647 appeared to express BCL-XL. However, upon CD40 stimulation, CLL cells from all three samples expressed increased amount of BCL-XL. In contrast, the level of BCL-2 remained unchanged in all CLL cells regardless of CD40 stimulation (Figure 9).

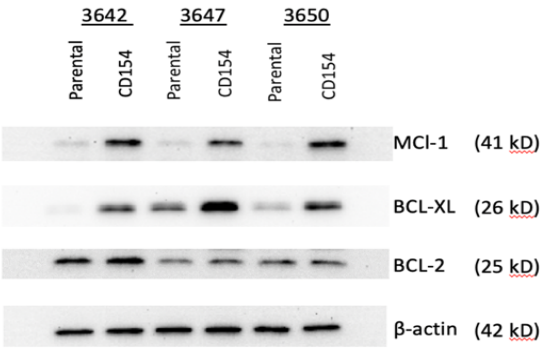
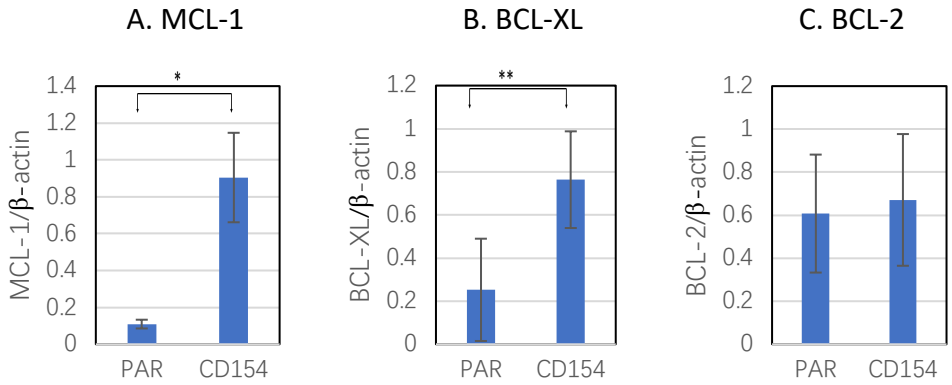


Figure 9. Anti-apoptotic proteins MCL-1 and BCL-XL are upregulated in response to CD40 stimulation. Primary CLL cells from three patients (sample #3642, #3647 and #3650) were co-cultured with parental or CD154-expressing mouse fibroblasts for 24 hours. Cellular proteins were separated by SDS-PAGE and transferred to PVDF membrane, which was probed with antibodies against MCL-1, BCL-XL or BCL-2 individually. β-actin was probed for protein loading control.

Next, I performed densitometry analysis to quantify the signals on the membrane that corresponded to MCL-1, BCL-XL and BCL-2. As shown in Figure 10A, comparing to unstimulated cells the increase in MCL-1 in CD40-stimulated CLL cells was statistically significant ($p < 0.05$). Likewise, the increase in BCL-XL was also significant ($p < 0.01$) (Figure 10B). However, no significant difference in level of BCL-2 expression between CD40-stimulated and unstimulated cells was observed (Figure 10C).

In summary, the data I obtained so far showed that CD40 stimulation protected CLL cells from spontaneous and ABT-199-induced cell death. Furthermore, this protection was associated with increased expression of anti-apoptotic proteins MCL-1 and BCL-XL in CD40-stimulated CLL cells.



*p<0.05, **p<0.01

Figure 10. Quantification of signals corresponding to MCL-1, BCL-XL and BCL-2 in CD40-stimulated and unstimulated CLL cells by densitometry analysis (n=3). (A) Ratio of relative expression of MCL-1 in relation to β-actin in unstimulated (PAR) and CD40-stimulated (CD154) CLL cells. (B) Ratio of relative expression of BCL-XL in relation to β-actin in unstimulated (PAR) and CD40-stimulated (CD154) CLL cells. (C) Ratio of relative expression of BCL-2 in relation to β-actin in unstimulated (PAR) and CD40-stimulated (CD154) CLL cells. A two-tailed, paired Students' t-test was used to determine the significance of difference in ratio of protein of interest over β-actin in CD40-stimulated versus unstimulated cells (* refers to p value of <0.05 and ** p value <0.01). Each bar represents mean ± standard deviation of the results using three different CLL cases.

3.4 Discussion

The aim of this part of my study was to independently examine how CD40-stimulated CLL cells responded to ABT-199-induced apoptosis, in comparison with unstimulated CLL cells. I used 4 CLL samples from patients with CLL for the study. The results clearly showed that CD40-stimulated CLL cells were not as sensitive to ABT-199 as the unstimulated cells. ABT-199 was less effective in killing CD40-stimulated cells at all concentrations tested, when compared to unstimulated CLL cells. This was the case in all 4 CLL samples examined. Due to small number of CLL samples used in the study, it was not possible to determine whether the sensitivity to ABT-199 was correlated to the clinical features (e.g. disease stage, cytogenetics and treatment history) of the CLL patients.

Next, I used Western Blotting analysis to explore the changes in the expression of anti-apoptotic BCL-2 family proteins. The results showed that the expression of MCL-1 and BCL-XL proteins was increased in CD40-stimulated CLL cells, whereas level of BCl-2 expression remained unchanged. Upregulation of MCL-1 and BCL-XL in CD40-stimulated cells correlated to reduction in ABT-199-induced apoptosis in these cells.

In CLL clinics, the recurrence of the relapsed disease after initial remission following treatment represents an important challenge for the clinicians. Understanding how drug resistance develops has thus been the focus of the intense scientific investigation in CLL research communities over the years. One of the causes for relapsed disease is that chemotherapy drugs do not effectively kill CLL cells residing in the bone marrow and lymph nodes, resulting in minimal residual disease (Burger and Gribben, 2014, Shain et al., 2015).

In lymph nodes (LN), CLL cells receive survival signals from the microenvironment, which could result in resistance to cytotoxic agents (Burger and Gandhi, 2009, Caligaris-Cappio, 2003). Compared with CLL cells from peripheral blood, CLL cells derived from LNs showed altered expression of apoptotic genes, including increased expression of BCL-XL and MCL-1 and decreased levels of pro-apoptotic BH3-only protein Noxa (Smit et al., 2007, Tromp et al., 2012). CD40 stimulation of CLL cells also leads to upregulation of BCL-XL and Mcl-1 and downregulation of Noxa (Tromp et al., 2010, Kater et al., 2004, Willimott et al., 2007) and lead to resistance to apoptosis induced by multiple drugs (Romano et al., 1998, Hallaert et al., 2008). Targeting anti-apoptotic proteins (e.g. BCL-XL, MCL-1, and A1/Bfl-1) may provide important new therapeutic opportunities to overcome chemotherapy resistance in the LN microenvironment.

In my study, using an established in vitro model of CD40 stimulation to mimic the *in vivo* interaction of CLL cells with activated T cells in the lymph node microenvironment, I demonstrated that CD40 stimulation induced upregulation of anti-apoptotic proteins MCL-1 and BCL-XL. The elevation of MCL-1 and BCL-XL also corresponds to a decrease in the cell death rate of CLL cells after CD40 stimulation. Thus, my results were consistent with the findings from previous studies where CD40-stimulation has been shown to protect CLL cells from cell death induced by a variety of drugs (Kitada et al., 1999, Kater et al., 2004, Hallaert et al., 2008, Vogler et al., 2009b, Tromp et al., 2012, Dietrich et al., 2012, Zhuang et al., 2014). However, due to the small number of CLL samples used, there are limitations in interpreting the results. If there was a follow-up experiment, I would increase the sample number so that the results obtained would be more persuasive. In addition, I would choose samples from patients with different clinical features such as different cytogenetic defects and the treatment history. In doing so, it would provide insight into whether the sensitivity to ABT-199 is also influenced by the clinical features of the samples.

Recently, it has been shown that CD40 stimulation using the co-culture systems can also leads to resistance to ABT-199-induced apoptosis in CLL cells (Thijssen et al., 2015, Oppermann et al., 2016). This resistance is associated with increased expression of MCL-1, BCL-XL and A1/BFL1. It has also been shown that CLL cells co-cultured with autologous activated T cells were also protected against ABT-199-induced apoptosis (Elías et al., 2018). Again, the protection has been shown to correlate with upregulation of MCL-1 and BCL-XL in CLL cells. Since the activated T cells has been

shown to be in close contact with CLL cells in the lymph nodes (Granziero et al., 2001, Ghia et al., 2002) and to activate CLL cells via CD40 signalling pathway (Pascutti et al., 2013), it is plausible that the above observations from the *in vitro* studies of mine and others may reflect, to a degree, what happens to CLL cells in the lymph node microenvironment. Indeed, comparing to their counterparts in the blood, CLL cells localised in the lymph nodes have been shown to express higher levels of MCL-1 and BCL-XL proteins (Smit et al., 2007, Thijssen et al., 2015).

This led me to test the idea that pharmacological inhibition of BCL-XL or MCL-1 may be useful to increase ABT-199-induced apoptosis of CD40-stimulated CLL cells. In the next part of the study, I will thus describe the results of using an MCL-1 or BCL-XL inhibitor in combination with ABT-199 to see if I could increase the sensitivity of CD40-stimulated CLL cells to induction of apoptosis by ABT-199.

Chapter 4. Inhibition of BCL-XL significantly increased sensitivity of CD40-stimulated CLL cells to ABT-199

4.1. Background

In the previous chapter, I demonstrated that expression of MCL-1 and BCL-XL was up-regulated in CD40-stimulated CLL cells (via co-culture with CD154-expressing fibroblasts), which was associated with reduced sensitivity to ABT-199. In this part of the research project, I will explore whether use of an inhibitor of MCL-1 or BCL-XL could restore the sensitivity of CD40-stimulated CLL cells to ABT-199. If the answer is yes, I will then study the molecular mechanism underlying the restoration of the sensitivity.

Over the past 20 years or so, intensive research on apoptotic pathways regulated by BCL-2 family of proteins have led to the discovery of small molecule inhibitors, so called "BH3-mimetics", which target the pro-survival BCL-2 family of proteins and directly activate apoptosis (Delbridge et al., 2016b). ABT-263 (also known as navitoclax) is the first BH3 mimetic that was developed almost ten years ago and has shown promising clinical activity in lymphoid malignancies such as CLL. However, because ABT-263 inhibited BCL-2 as well as BCL-XL, its efficacy was limited by the thrombocytopenia occurring in patients receiving treatment as a result of BCL-XL inhibition. This has prompted the development of a BCL-2 selective inhibitor, ABT-199 (also known as venetoclax), which shows potent anti-cancer activity in lymphoid malignancies including CLL, but avoids causing thrombocytopenia (Souers et al., 2013).

Using similar structure-based targeting approach, BH3-mimetics specifically targeting BCL-XL have also been developed recently (Tao et al., 2014, Leverson et al., 2015b). Among them is A-1331852, a BCL-XL-selective inhibitor with oral bioavailability. A-1331852 binds to BCL-XL with high affinity (the inhibitory constant Ki being <0.01 nM) and disrupts the BCL-XL-BIM complex, inducing apoptosis in BCL-XL-dependent cancer cells (Leverson et al., 2015b). It has also been shown that A-1331852 has significantly enhanced the efficacy of bortezomib in a xenograft model of multiple myeloma that expresses high levels of BCL-XL (Punnoose et al., 2016). In addition, A-1331852 alone has also induced extensive apoptosis and, when used in combination, significantly enhanced tyrosine kinase inhibitor (TKI)-induced cell death in chronic myeloid leukaemia (CML) cell lines and primary $CD34^+$ cells from patients with CML (Lucas et al., 2016).

In contrast to the development of BH3-mimetics targeting BCL2 and BCL-XL, discovery of potent MCL-1 inhibitors has proven to be a difficult challenge, largely due to the key structural differences in the BH3-binding grooves of MCL-1 and BCL-2 proteins (Billard,

2013, Belmar and Fesik, 2015). Thus, for those putative MCL-1 inhibitors developed, most of them have been found to be either insufficiently potent or not selective enough in inducing apoptosis in MCL-1-dependent cancer cell lines (Varadarajan et al., 2013). Nevertheless, given the important role of MCL-1 in pathogenesis of cancers and in mediating drug resistance (Belmar and Fesik, 2015), research efforts remain unremitting in search for direct or indirect inhibitors of MCL-1. Voruciclib (previously known as P1446A-05), a small molecule flavone derivative, has been shown to potently block the activity of cyclin-dependent kinase (CDK) 9 and inhibit the expression of MCL-1 in cancer cells and, when combined with ABT-199, it induces tumor cell apoptosis and tumor growth inhibition in xenograft models of high-risk diffuse large B-cell lymphoma (DLBCL) (Dey et al, 2017). Under physiological conditions, CDK9, in complex with cyclin T and a transcription factor P-TEFb (positive transcription elongation factor b), promotes transcriptional elongation by phosphorylating the carboxy-terminal domain (CTD) of the RNA polymerase II (RNAPII) (Wang and Fischer, 2008). Inhibition of CDK9 by flavopiridol results in hypophosphorylation of RNAPII CTD, leading to reduced RNA synthesis and a decline of short-lived proteins such as MCL-1 (Wang and Fischer, 2008). However, unlike flavopiridol, voruciclib has been shown to have a higher affinity for CDK9/cyclin T complex with half-maximal inhibitory concentrations (IC_{50}) of 22 nM (Eliades et al., 2016). It is orally bioavailable with favorable safety profile and tolerability from early phase clinical studies (Joshi et al., 2012, Gupta et al., 2012).

Therefore, in the first part of this study, I used CDK-9 inhibitor voruciclib and BCL-XL inhibitor A-1331852 to test whether either inhibitor is useful to reduce the cyto-protective effect caused by CD40 stimulation and to restore the sensitivity of CD40-stimulated cells to ABT-199.

It is also known that the pro-apoptotic BH3-only proteins such as BIM are important determinants in committing lymphoid cells to apoptosis (Bouillet et al., 1999, Cory and Adams, 2002). BIM is a member of the BH3-only proteins in the BCL-2 family. BIM has three different splicing isoforms, namely BIM-EL (main isoform), BIM-L and BIM-S (O'Connor et al., 1998). In healthy cells, BIM is suppressed by complexing with dynein light chain 1 (encoded by *DYNLL1* gene), restricting it to microtubule-based dynein complexes (Puthalakath et al., 1999). When cells are exposed to apoptotic stimuli, BIM can be phosphorylated by JNK at T116, which causes BIM to dissociate from the dynein complex, enabling liberated BIM to activate apoptosis through mitochondria pathway (Lei and Davis, 2003).

In CLL, BIM has been shown to be consistently expressed across CLL samples (Moore et al., 2007). It is required for apoptosis of CLL cells induced by a variety of therapeutic agents including fludarabine (Sharma et al., 2013) and glucocorticoids (Sharma et al., 2013, Melarangi et al., 2012). It has also been shown that CLL cells express significantly more BCL-2 than BIM (Mason et al., 2009) and that almost all BIM are bound to BCL-2 in healthy CLL cells (Del Gaizo Moore et al, 2007; Melarangi et al, 2012), keeping BIM

in check from activating apoptosis.

In apoptosis of CLL cells induced by ABT-737 , it was shown that BIM was displaced by ABT-737 from the binding with BCL-2, thus enabling the released BIM to activate BAX/BAK, inducing mitochondrial apoptosis and rapidly killing CLL cells (Moore et al., 2007). Study also showed that level of BCL-2 expression alone does not determine sensitivity to ABT-737 (Mason et al., 2009). In contrast, disruption of BCL-2 complex with BIM is a key mode of action for ABT-737 in killing CLL cells (Moore et al., 2007). Since BIM are consistently expressed in CLL cells which may have already readied CLL cells to apoptosis. However, CLL cells can prolong their survival *in vivo* due to their expression of BCL-2 at higher level. Treatment of CLL cells with BCL-2 specific BH3 mimetic such as ABT-199 is thus predicted to be effective in inducing apoptosis as it lowers the threshold of apoptosis in CLL cells and renders the leukemic cells sensitive to BIM-induced apoptosis. This notion is consistent with observation that mitochondrial priming measured by BH3 profiling can determine initial response to cytotoxic chemotherapy in acute myelogenous leukemia (AML) (Vo et al., 2012).

Therefore, in the second part of this study, if the inhibitor of MCL-1 or BCL-XL can increase the sensitivity of CD40-stimulated CLL cells to ABT-199, I will investigate the underlying mechanisms responsible for the sensitization effect. Finally, to determine if BIM is required for ABT-199-induced apoptosis of CLL cells, I will perform experiments to knock down BIM expression in CLL cells using siRNAs specific to BIM.

4.2 Methods

4.2.1 Treatment of CD40-stimulated CLL cells with MCL-1 or BCL-XL inhibitor

To mimic CD40 stimulation, primary CLL cells were cultured on an adherent monolayer of transfected mouse fibroblasts expressing human CD154 for 24 h, as described in the previous chapter. CLL cells co-cultured with control fibroblasts were used as a control. To determine the effect of the CDK-9 inhibitor (voruciclib) or BCL-XL (A-1331852) on CD40-stimulated cells, CLL cells were collected from the co-cultures and incubated under standard culture conditions at a density of 4 x 10^6 cells/mL in the presence or absence of the inhibitor at the indicated concentrations for 24 h. These CLL cells were then further incubated with ABT-199 at the indicated concentration for 4 h. Cell death was measured by flow cytometry as described in the previous chapter.

4.2.2 Co-immunoprecipitation (Co-IP)

To detect protein interaction between BCL-XL and BIM in CD40-stimulated CLL cells, CLL cells co-cultured with CD154-expressing fibroblasts were harvested 24 hours after co-culture and cell number counted on Cellometer (Nexcelom Bioscience). The detailed steps of the Co-IP were described in the Methodology chapter (Chapter 2).

The proteins that were bound to the beads are then analysed by Western Blotting, which was also described in Methodology chapter.

4.2.3 BIM siRNA knockdown experiment in CLL cells.

To knockdown the expression of BIM protein in CD40-stimulated CLL cells, cryopreserved CLL cells were thawed and recovered before being transfected with BIM siRNA (see Methodology in chapter 2 for detailed information of the experiment). The transfected CLL cells were then co-cultured with CD154-expressing fibroblasts for 24 h. CLL cells were then collected from the co-cultures and incubated under standard culture conditions at a density of 4 x 10^6 cells/mL in the presence or absence of ABT-199 at the indicated concentrations for 4 h before harvest. The expression of BIM in transfected CLL cells were then determined by Western blotting, as described in the Methodology chapter.

4.3 Results

4.3.1 The CDK-9 inhibitor voruciclib did not increase sensitivity of CD40 stimulated CLL cells to ABT-199.

4.3.1.1 Induction of apoptosis by voruciclib with or without ABT-199 (CLL sample #3642)

I first co-cultured primary CLL cells from sample #3642 with control parental or CD154-expressing fibroblasts for 24 hours. Co-cultured CLL cells were then harvested by gentle pipetting and incubated under standard culture conditions at a density of 4 x 10^6 cells/mL with voruciclib at a range of concentrations for 24 h. As shown in Figure 11A, CLL cells co-cultured with parental fibroblasts (i.e. unstimulated) were subjected to spontaneous apoptosis with the death rate of 10 % detected, whereas CLL cells co-cultured with CD154-expressing fibroblasts (i.e. CD40-stimulated) had a very low death rate (approximately 5 %). With the increasing concentration of voruciclib, the cell death rate of unstimulated CLL cells was slightly increased (Figure 11A). In contrast, the cell death rate of CD40-stimulated CL cells was lower at the concentrations (up to 1000 nM) of voruciclib tested. Next, I used voruciclib in combination with ABT-199 to investigate whether voruciclib could increase the ABT-199-induced apoptosis in CD40-stimulated CLL cells. As shown in Figure 11B, CLL cells co-cultured with parental fibroblasts have reached a death rate of approximately 97 % with a combined treatment of 10 nM ABT-199 and 10 nM voruciclib. At the corresponding concentrations of both inhibitors, the death rate of CD40-stimulated CLL cells is only at 3 %. This remained unchanged with the treatment of up to 1000 nM voruciclib in the presence of 10 nM ABT-199 (Figure 11B). At 10,000 nM, voruciclib caused cell

death in both CD40-stimulated and unstimulated CLL cells (~70 % and ~80%, respectively) (Figure 11A) and addition of 10 nM ABT-199 did not increase the level of cell death in CD40-stimulated CLL cells (Figure 11B). To determine if voruciclib had any effect on the expression of MCL-1, I examined its expression in co-cultured CLL cells by Western blotting analysis. As shown in Figure 11C, voruciclib at the concentration up to 1000 nM did not reduce the expression of MCL-1, suggesting that the drug at these concentrations may not be effective. Thus, the above result showed that voruciclib at the range of concentrations tested could not increase ABT-199-induced cell death in CD40-stimulated CLL cells. It is noted that 10 nM ABT-199 alone induced less amount of apoptosis this time (Figure 11B) than that from the previous experiment (Figure 5, Chapter 3) in both CD40-stimulated and unstimulated cells even though the CLL cells used were from the same case (#3642). Although the exact reason is unclear, it is possible that sample preparation on different days of experiment may contribute to the variation seen.

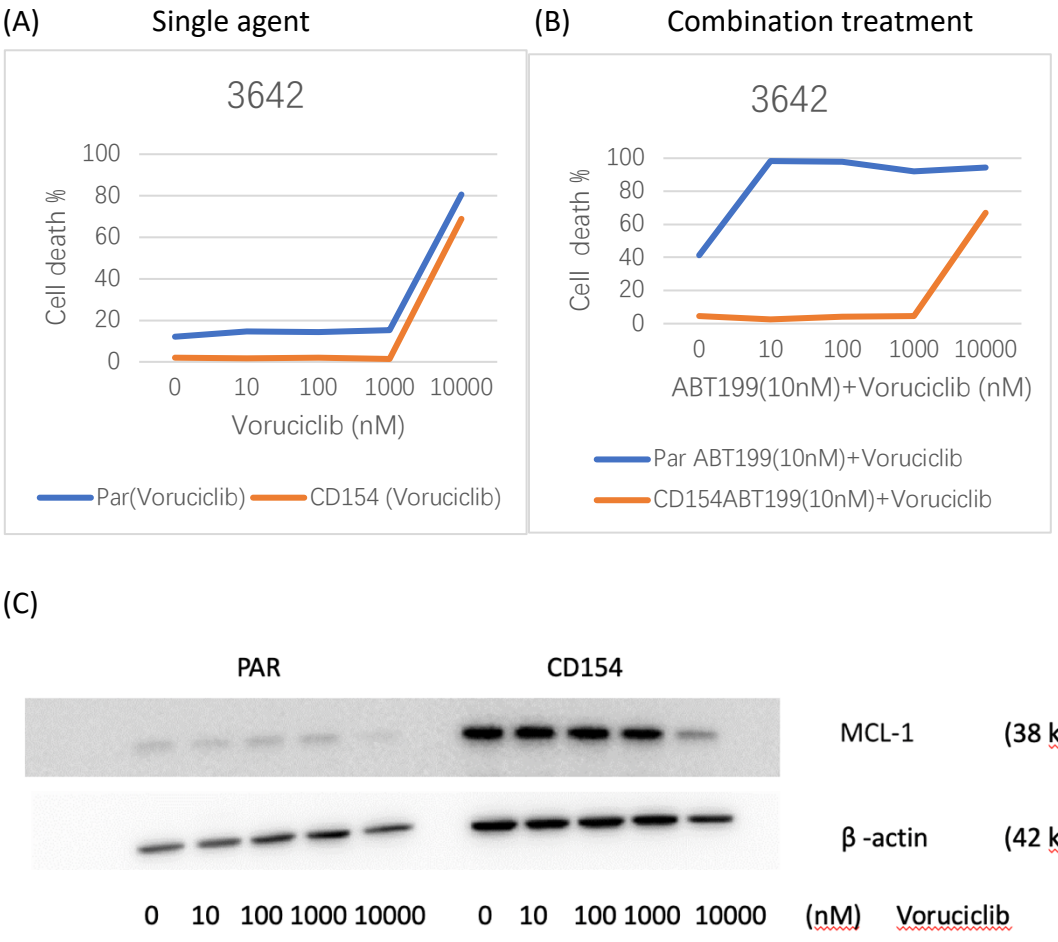
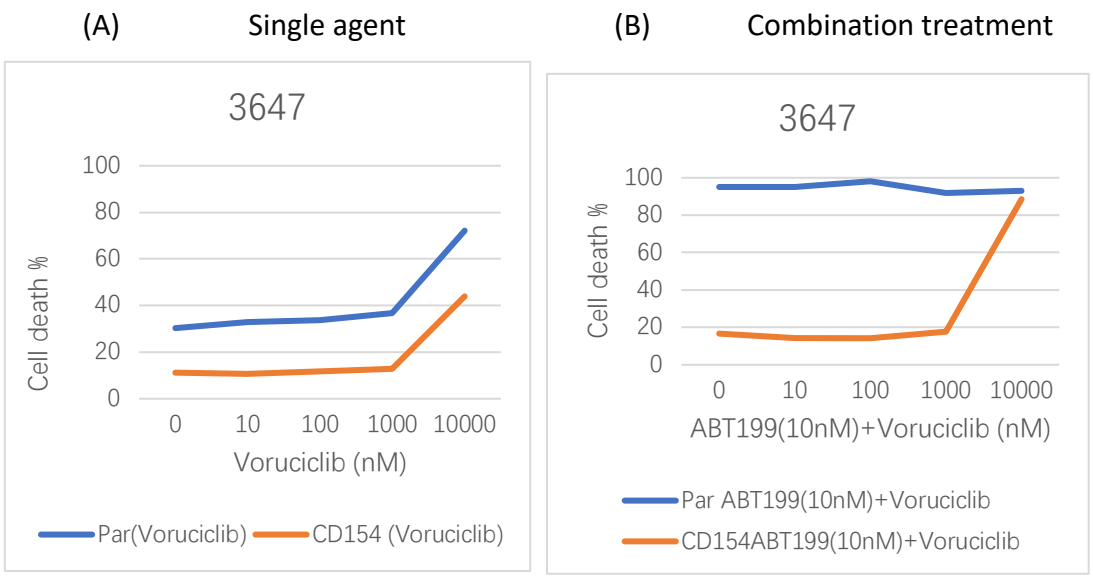


Figure 11. Induction of cell death by voruciclib alone and in combination with ABT-199 (CLL sample #3642). (A) Primary CLL cells (sample 3642) were co-cultured with CD154-expressing or parental (PAR) fibroblasts for 24 h. Co-cultured CLL cells were then collected and incubated with voruciclib at the indicated concentrations for 24 h. Cell death was measured by flow cytometry as previously described. (B) Following

incubation of co-cultured CLL cells with voruciclib for 24 h as in (A), cells were further incubated with ABT-199 (10 nM) for 4 h before cell death analysis by flow cytometry. (C) Expression of MCL-1 in respectively co-cultured CLL cells treated with voruciclib was detected by Western blotting, as previously described.

4.3.1.2 Induction of apoptosis by voruciclib with or without ABT-199 (CLL sample #3647)

I then repeated the above experiments using primary CLL cells from sample # 3647. As shown in Figure 12A, the level of spontaneous apoptosis of CLL cells co-cultured with parental fibroblasts (i.e., unstimulated) is higher (~30%). Level of spontaneous cell death of CLL cells co-cultured with CD40-expressing fibroblasts (i.e., CD40 stimulated) was detected at only approximately 11%. Addition of voruciclib did not increase cell death in both stimulated and unstimulated CLL cells. When the voruciclib concentration increased to 1000 nM, the cell death of unstimulated CLL cells did not increase significantly. At the concentration of 10,000 nM, voruciclib increased the rate of cell death to over 70% in unstimulated cells. In comparison, the cell death rate of CD40-stimulated CLL cells remained low in response to voruciclib (up to concentrations of 1,000 nM). At 10,000 nM, voruciclib induced cell death in about 45% of CD40-stimulated cells. Next, I combined voruciclib with ABT-199 to investigate whether the combination could kill the CD40-stimulated CLL cells. As shown in Figure 12B, CLL cells co-cultured with parental fibroblasts have reached a mortality rate of about 97% at a concentration of 10 nM of ABT-199. At the corresponding concentration, the death rate of CD154-expressing fibroblasts (i.e., stimulated by CD40) was only 3%. With the increase in concentrations of up to 1,000 nM, voruciclib did not increase the level of cell death in CD40-stimulated cells that were treated with 10 nM ABT-199 (Figure 12B). However, at 10,000 nM, voruciclib increased the rate of cell death to almost 90% in CD40-stimulated cells that were treated with 10 nM ABT-199 (Figure 12B). I therefore examined the expression of MCL-1 in voruciclib-treated cells by Western blotting. As shown in Figure 12C, at the concentrations of up to 1,000 nM, voruciclib did not induce the reduction of MCL-1 expression in CD40-stimulated CLL cells. However, at 10,000 nM, voruciclib induced moderate reduction in MCL-1 expression in CD40-stimulated CLL cells. Thus, the above results still showed that CD40 stimulation can reduce spontaneous cell death, but voruciclib at the concentrations of up to 1,000 nM cannot increase ABT-199-induced cell death in CD40-stimulated cells.



(C)

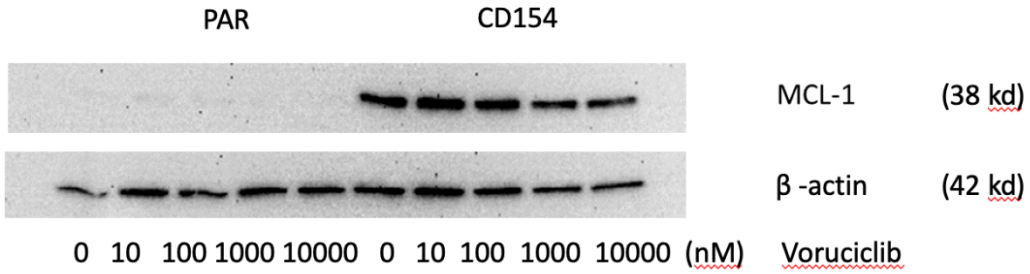


Figure 12. Induction of cell death by voruciclib alone and in combination with ABT-199 (CLL sample #3647). (A) Primary CLL cells (sample 3647) were co-cultured with CD154-expressing or parental (PAR) fibroblasts for 24 h. Co-cultured CLL cells were then collected and incubated with voruciclib at the indicated concentrations for 24 h. Cell death was measured by flow cytometry as previously described. (B) Following incubation of co-cultured CLL cells with voruciclib for 24 h as in (A), cells were further incubated with ABT-199 (10 nM) for 4 h before cell death analysis by flow cytometry. (C) Expression of MCL-1 in respectively co-cultured CLL cells treated with voruciclib was detected by Western blotting, as previously described.

4.3.1.3 Induction of apoptosis by voruciclib with or without ABT-199 (CLL sample #3650)

Because of the difference I observed in the induction of apoptosis by voruciclib at 10,000 nM plus 10 nM ABT-199 in the CD40-stimulated CLL cells from previous two CLL samples, I repeated the same experiments using the third CLL sample (case 3650). As shown in Figure 13A, CLL cells co-cultured with parental fibroblasts (i.e., unstimulated) experienced a higher level of spontaneous apoptosis, with a cell death

rate of approximately 10 %, compared with 2 % in CD40-stimulated cells. As the concentration of voruciclib increased to up to 1,000 nM, the cell death rate of unstimulated CLL cells did not increase accordingly. Again, at 10,000 nM, voruciclib induced significant amount of cell death in unstimulated CLL cells (~80 %). In contrast, voruciclib at the concentrations of up to 1,000 nM did not induce significant amount of cell death of CD40-stimulated CLL cells and even at 10,000 nM, it induced only about 25 % of cell death in these cells. Next, I combined voruciclib with ABT -199 to see whether the combination could increase the ABT-199-induced apoptosis in CD40-stimulated CLL cells. As shown in Figure 13B, unstimulated CLL cells experienced almost 90 % cell death when treated with 10 nM ABT-199. Interestingly, CD40-stimulated CLL cells treated in the same manner did not experience significantly increased cell death even at the concentrations of 1,000 and 10,000 nM voruciclib plus 10 nM ABT-199 (both at ~25%). Again, at the concentrations of up to 1,000 nM, voruciclib did not induce the reduction of MCL-1 expression in CD40-stimulated CLL cells and only at 10,000 nM did it induce moderate reduction in MCL-1 expression in these cells (Figure 13C).

Thus, the above results again showed that CD40 stimulation can reduce spontaneous cell death, but voluciclib at the concentrations of up to 1,000 nM cannot increase ABT-199-induced cell death in CD40-stimulated cells.

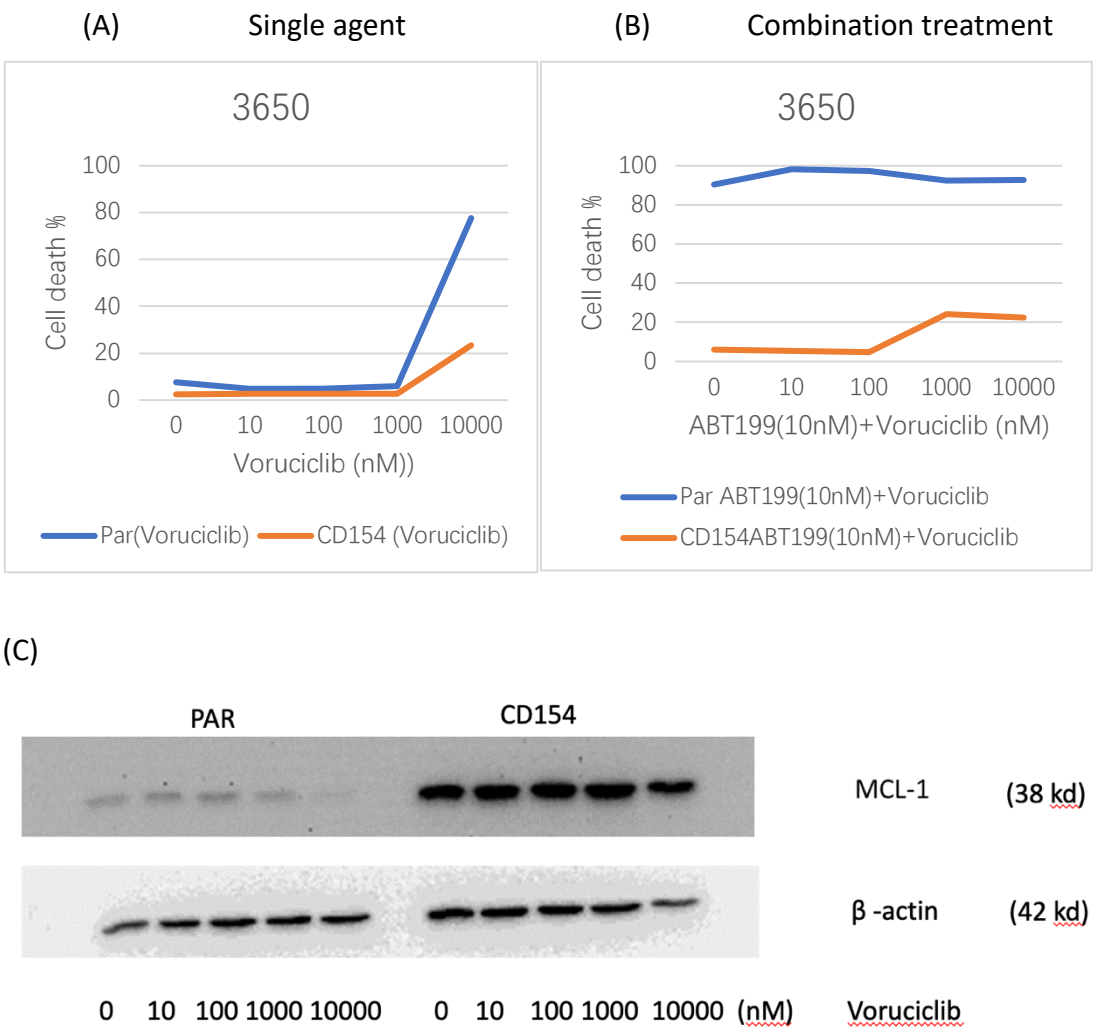


Figure 13. Induction of cell death by voruciclib alone and in combination with ABT-199 (CLL sample #3650). (A) Primary CLL cells (sample 3650) were co-cultured with CD154-expressing or parental (PAR) fibroblasts for 24 h. Co-cultured CLL cells were then collected and incubated with voruciclib at the indicated concentrations for 24 h. Cell death was measured by flow cytometry as previously described. (B) Following incubation of co-cultured CLL cells with voruciclib for 24 h as in (A), cells were further incubated with ABT-199 (10 nM) for 4 h before cell death analysis by flow cytometry. (C) Expression of MCL-1 in respectively co-cultured CLL cells treated with voruciclib was detected by Western blotting, as previously described.

4.3.1.4 Pooled data analysis of induction of apoptosis by voruciclib with or without ABT-199

Next, I pooled the cell death data from the three CLL samples mentioned above and performed statistical analysis to determine if the difference in cell death of CD40-stimulated CLL cells treated with voruciclib alone versus vorucilcib plus ABT-199 was significant.

(A) Single agent (B) Combination treatment

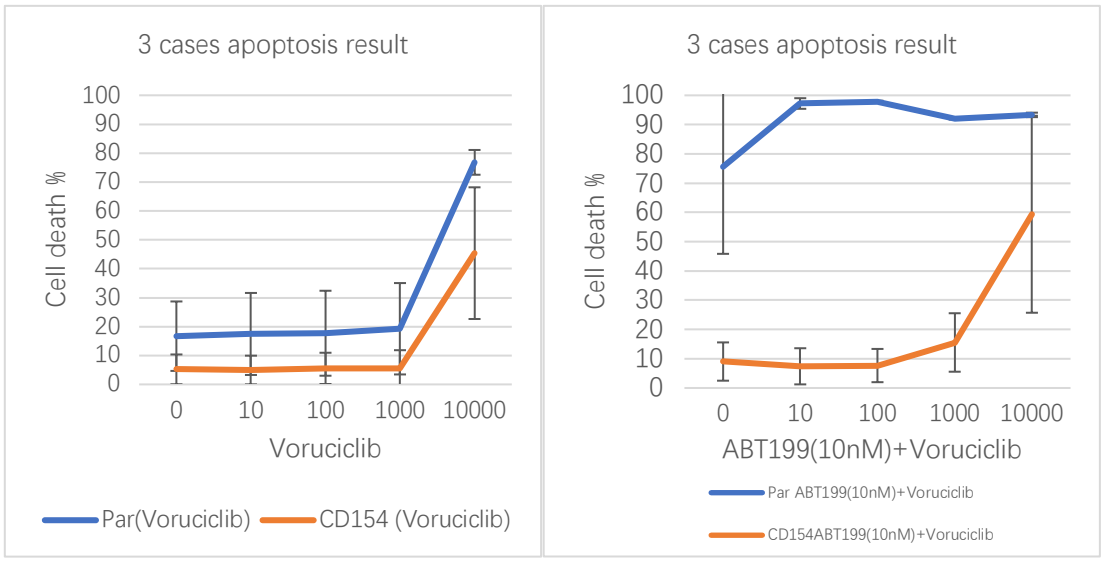


Figure 14. Induction of cell death by voruciclib alone and in combination ABT-199 (pooled data analysis, n=3). (A) Induction of cell death by voruciclib at the indicated concentrations in respectively cocultured CLL cells. (B) Induction of cell death by voruciclib in combination with ABT-199 in respectively cocultured CLL cells. Each data point represents mean ± standard deviation of results from independent experiments using 3 different CLL samples.

As shown in Figure 14 (A), treatment with voruciclib alone (up to 1000 nM) was unable to induce apoptosis in both unstimulated and CD40-stimulated CLL cells, suggesting that MCL-1 inhibition alone is not sufficient to initiate apoptosis. In addition, voruciclib was not able to increase ABT-199-induced apoptosis of CD40-stimulated CLL cells to the similar levels seen in the unstimulated CLL cells (Figure 14 B). At 10,000 nM, voruciclib becomes cytotoxic as it killed CLL cells with or without CD40 stimulation. I then used two-tailed, paired student's t-test to see if the difference in cell death of CD40-stimulated CLL cells treated with voruciclin plus or minus ABT-199 was significant. The results showed that the p values at each concentration of voruciclib ± ABT-199 were all greater than 0.05, indicating that the difference were not statistically significant. Taken together, voruciclib at the concentrations tested did not overcome the cyto-protective effects of CD40 stimulation against ABT-199-induced cell death. However, caution must be exercised as this conclusion was based on the assumption that the drug vorucilib was effective at inhibiting CDK9 in CLL cells. Further experiment is required to confirm whether this is the case before the ability of drug to alter the expression of MCL-1 can be dismissed.

Therefore, in the next step I explored whether use of the BCL-XL inhibitor can restore the sensitivity of CD40 stimulated CLL cells to ABT-199-induced apoptosis.

4.3.2 BCL-XL inhibitor A-1331852 significantly increased sensitivity of CD40-stimulated CLL cells to ABT-199

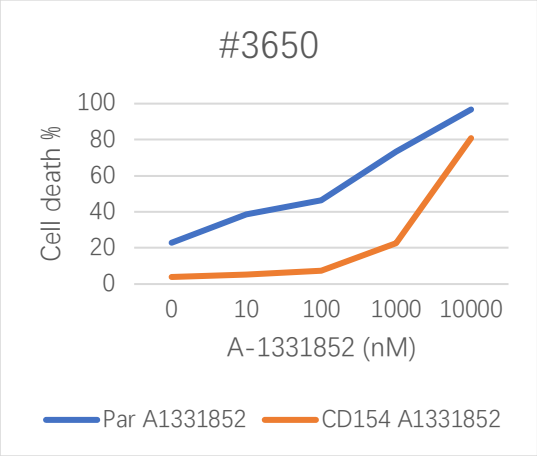
4.3.2.1 Induction of cell death by A-1331852 with or without 10 nM ABT-199

4.3.2.1.1 Result from CLL sample #3650

First, I used primary CLL cells from sample #3650 and co-cultured them with control parents or CD154-expressing fibroblasts for 24 hours. Co-cultured CLL cells were then collected by gentle pipetting and incubated at a density of 4×10^6 cells / mL with A-1331852 at a range of concentrations under standard culture conditions for further 24 hours. As shown in Figure 15A (blue line), with the increase of concentration, A-1331852 induced cell death in unstimulated CLL cells in a concentration-dependent manner, with cell death rate reaching 38.57 % at 10 nM, 46.35 % at 100 nM, 73.26 % at 1,000 nM and 96.75 % at 10,000 nM, respectively. In contrast, the cell death rate of CD40 stimulated CL cells was much lower at all A-1331852 concentrations tested, with 5.14 % at 10 nM, 7.34 % at 100 nM, 22.58 % at 1,000 nM and 80.88 % at 10,000 nM, respectively (Figure 15A, orange line).

Next, I used A-1331852 in combination with 10 nM ABT-199 to investigate whether the combination could increase the level of apoptosis of CD40 stimulated cells to that seen in unstimulated cells. As shown in Figure 15B (blue line), unstimulated CLL cells were undergoing high levels of apoptosis when treated with 10 nM ABT-199 alone, with cell death rate of 96.2 % detected. ABT-199, in combination with A-1331852 at the concentrations indicated, killed almost all unstimulated CLL cells. In contrast, the death rate of CD40-stimulated CLL cells treated with 10 nM ABT-199 plus different concentrations of A-1331852 was much lower with only 53.38 % at 10 nM, 65.81% at 100 nM, 70.74% at 1,000 nM and 90.42 % at 10,000 nM A-1331852, respectively (Figure 15B, orange line). Therefore, the above results showed that A-1331852 alone can induce a concentration-dependent cell death in unstimulated CLL cells, but less effective in CD40-stimulated CLL cells. More interestingly, A-1331852 also increased ABT-199-induced cell death in CD40-stimulated CLL cells.

(A) Single agent



(B) Combination treatment

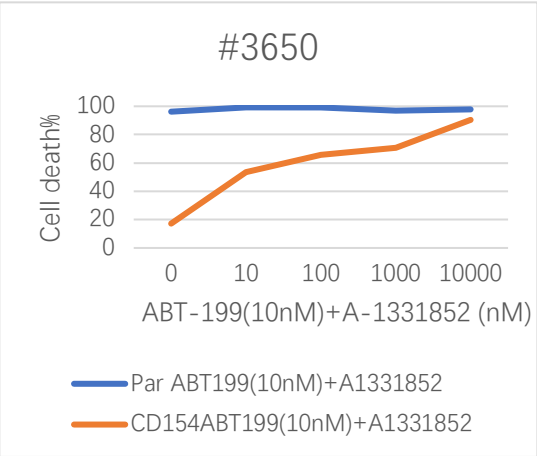


Figure 15. Induction of cell death by A-1331852 alone and in combination with 10 nM ABT-199 (CLL sample 3650). (A) Primary CLL cells (sample 3650) were co-cultured with CD154-expressing or parental (PAR) fibroblasts for 24 h. Co-cultured CLL cells were then collected and incubated with A-1331852 at the indicated concentrations for 24 h. Cell death was measured by flow cytometry as previously described. (B) Following incubation of co-cultured CLL cells with A-1331852 for 24 h as in (A), cells were further incubated with ABT-199 (10 nM) for 4 h before cell death analysis by flow cytometry.

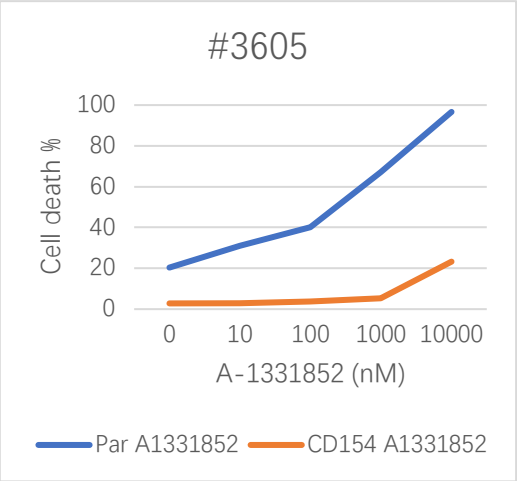
4.3.2.1.2 Result from CLL sample #3605

I next repeated the above experiments using primary CLL cells from sample #3605. As shown in Figure 16A (blue line), with the increasing concentration of A-1331852, the cell death rate of unstimulated CLL cells also increased in a concentration-dependent manner, reaching 31.17 % at 10 nM, 40.03 % at 100 nM, 67.12 % at 1,000 nM and 96.66 % at 10, 000 nM, respectively. In contrast, the cell death of CD40 stimulated CL cells was much lower when treated with the same concentrations of the inhibitor. A-1331852 induced <5 % cell death at 10 nM and 100 nM, just over 5 % at 1,000 nM and about 23 % at 10,000 nM, respectively (Figure 16A, orange line).

Next, I used A-1331852 in combination with ABT-199 to investigate whether the combination treatment could restore the sensitivity of CD40-stimulated CLL cells to 10 nM ABT-199. As shown in Figure 16B, 10 nM ABT-199 alone induced 95.53 % cell death in unstimulated cells, but only 4.78 % cell death in CD40-stimulated cells. Again, combination of A-1331852 and ABT-199 consistently killed almost all unstimulated CLL cells (Figure 16B, blue line). However, an increase in concentration of A-1331852 has only modestly increased ABT-199-induced cell death in CD40-stimulated cells, with 15.43 % cell death at 10 nM, 21.68 % at 100 nM, 20.37 % at 1,000 nM and 26.27 % at 10,000 nM, respectively (Figure 16B, orange line). Therefore, the above results were largely consistent with what was observed with CLL case 3650, as described previously.

A-1331852 alone can induce a concentration-dependent cell death in unstimulated CLL cells, but less effective in CD40-stimulated CLL cells. In addition, A-1331852 appeared to increase ABT-199-induced cell death in CD40-stimulated CLL cells.

(A) Single agent



(B) Combination treatment

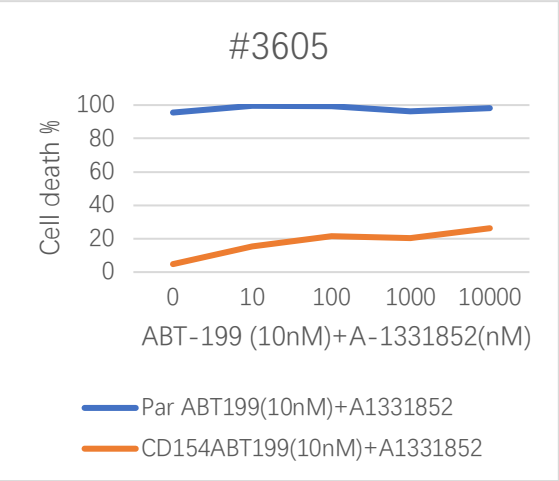


Figure 16. Induction of cell death byA-1331852 alone and in combination with 10 nM ABT-199 (CLL sample 3605). (A) Primary CLL cells (sample 3605) were co-cultured with CD154-expressing or parental (PAR) fibroblasts for 24 h. Co-cultured CLL cells were then collected and incubated with A-1331852 at the indicated concentrations for 24 h. Cell death was measured by flow cytometry as previously described. (B) Following incubation of co-cultured CLL cells with A-1331852 for 24 h as in (A), cells were further incubated with ABT-199 (10 nM) for 4 h before cell death analysis by flow cytometry.

4.3.2.1.3 Result from CLL sample #3620

To ensure that the results from the previous two cases can be reproducible, I used primary CLL cells from a third CLL sample (#3620) and repeated the experiments. As shown in Figure 17A (blue line), with the increase of concentrations, A-1331852 induced the cell death of unstimulated CLL cells in a concentration-dependent manner, reaching 14.37 % in 10 nM, 18.23 % at 100 nM, 45.25 % at 1,000 nM and 89.91 % at 10,000 nM, respectively. In contrast, at all concentrations of A-1331852 tested, the cell death rate of CD40-stimulated CL cells was much lower, with 3.36 % at 10 nM, 6.59 % at 100 nM, 4.53 at 1,000 nM and 21.46 % at 10,000 nM, respectively (Figure 17A, orange line).

Next, I used A-1331852 in combination with 10 nM ABT-199 to investigate whether the combination can increase cell death in CD40-stimulated cells. As shown in Figure 17B, 10 nM ABT-199 alone caused significant amounts of cell death, with a cell death rate of 85.81% detected. This cell death remained high with co-treatment of A-1331852 in unstimulated CLL cells (Figure 17B, blue line). However, increased concentrations of A-1331852 only modestly increased ABT-199-induced cell death in CD40-stimulated CLL

cells, reaching 10.52 % cell death at 10 nM, 11.7 % at 100 nM, 16.02 % at 1,000 nM and 26.84 % at 10,000 nM, respectively (Figure 17B, orange line). Thus, the results from the third CLL case (#3620) largely confirmed the observations from the previous two CLL samples.

(A) Single agent (B) Combination treatment

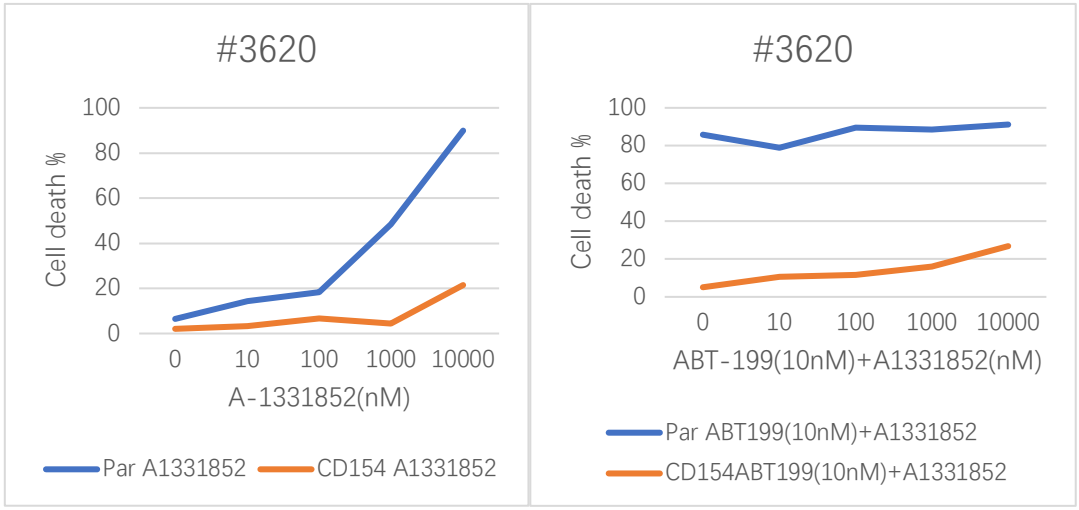


Figure 17. Induction of cell death byA-1331852 alone and in combination with 10 nM ABT-199 (CLL sample 3620). (A) Primary CLL cells (sample 3620) were co-cultured with CD154-expressing or parental (PAR) fibroblasts for 24 h. Co-cultured CLL cells were then collected and incubated with A-1331852 at the indicated concentrations for 24 h. Cell death was measured by flow cytometry as previously described. (B) Following incubation of co-cultured CLL cells with A-1331852 for 24 h as in (A), cells were further incubated with ABT-199 (10 nM) for 4 h before cell death analysis by flow cytometry.

4.3.2.1.4 Pooled data analysis of induction of apoptosis by A-1331852 with without 10 nM ABT-199

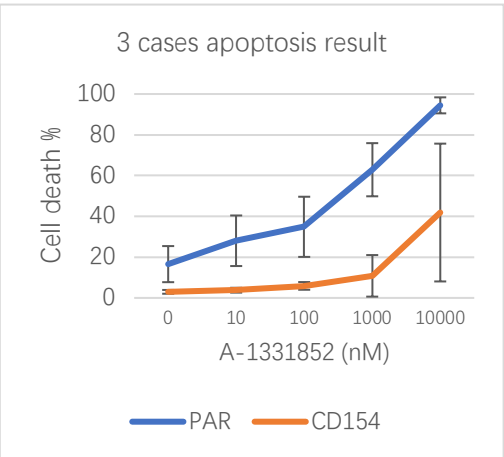
I next combined the cell death data of the above three CLL samples and performed statistical analysis. As shown in Figure 18A (blue line), A-1331852 alone induced a concentration-dependent cell death in unstimulated CLL cells, with an average cell death of 25.79 % at 10 nM, 31.27 % at 100 nM, 56.49 % at 1,000 nM and 91.16 % at 10, 000 nM, respectively. In contrast, A-1331852 alone only induced modest cell death in CD40-stimulated CLL cells, causing an average of cell death of 9.69 % at 10 nM, 11.81 % at 100 nM, 17.38 % at 1,000 nM and 51.71 % at 10,000 nM, respectively (Figure 18A, orange line).

Next, I analysed the cell death induced by combined treatment of A-1331852 with 10 nM ABT-199. As shown in Figure 18B, 10 nM ABT-199 alone caused an average of 87.19 % of cell death in unstimulated cells, but only ~10 % in CD40-stimulated CLL cells.

Addition of A-1331852 maintained high level of ABT-199-induced cell death in unstimulated cells (Figure 18B, blue line). In contrast, with the increased concentrations, A-1331852 only modestly increased cell death induced by 10 nM ABT-199 in CD40-stimulatde CLL cells, with an average of cell death of 10.96 % at 10 nM, 27.05 % at 100 nM, 34.92 % at 1,000 nM and 51.31 % at 10,000 nM, respectively (Figure 18B, orange line).

I then performed a statistical analysis to determine if the difference in cell death of CD40-stimulated CLL cells treated with A-1331852 alone versus A-1331852 plus ABT-199was significant. Two-tailed, paired Student's t test was used for analysis and the results showed that the p values were all greater than 0.05 at all concentrations of A-1331852 used in the study. Therefore, the A-1331852 did not significantly increase sensitivity of CD40-stimulated to cell death induced by 10 nM ABT-199. However, due to the small number of CLL samples used in the study and wide variation of the data observed, caution is needed in interpreting the results. Nevertheless, A-1331852 induced cell death in a concentration-dependent manner in unstimulated CLL cells and, to a lesser degree, also in CD40-stimulated CLL cells, suggesting that it is cytotoxic to CLL cells. I thus wanted to test if it can sensitise CD40-stimulated cells to cell death induced by ABT-199 at higher than 10 nM concentration.

(A) Single agent



(B) Combination treatment

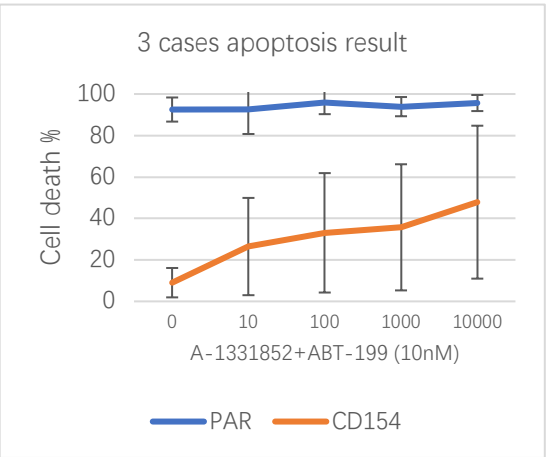


Figure 18. Induction of cell death by A-1331852 and in combination with 10nM ABT-199 (pooled data analysis, n=3). (A) Primary CLL cells were co-cultured with CD154-expressing or parental (PAR) fibroblasts for 24 h. Co-cultured CLL cells were then collected and incubated with A-1331852 at the indicated concentrations for 24 h. Cell death was measured by flow cytometry as previously described. (B) Following incubation of co-cultured CLL cells with A-1331852 for 24 h, as in (A), cells were further incubated with ABT-199 (10 nM) for 4 h before cell death analysis by flow cytometry. In both (A) and (B), data represent the mean ± SD from three independent experiments using three different CLL samples.

4.3.2.2 Induction of cell death by A-1331852 with or without 100 nM ABT-199

In the previous chapter, I showed that CD40-stimulated CLL cells developed resistance to ABT-199, whereas unstimulated CLL cells were very sensitive to cell death induced by the BCL-2 inhibitor. At 100 nM, ABT-199 only induced < 40 % cell death in CD40-stimulated CLL cells, whereas it potently killed almost all unstimulated CLL cells (Figure 8, Chapter 3). Therefore, I selected 100 nM ABT-199 for the following experiments of combination treatment with A-1331852 to see if the BCL-XL inhibitor can restore the sensitivity of CD40-stimulated cells to cell death induced by 100 nM ABT-199.

4.3.2.2.1 Results from CLL sample #3620

Cryopreserved primary CLL cells from sample #3620 were thawed and co-cultured with control parents or CD154 expressing fibroblasts for 24 hours under the same conditions as described previously. Co-cultured CLL cells were then collected by gentle pipetting and incubated at a density of 4×10^6 cells/mL in the presence of A-1331852 under standard culture conditions for 24 hours. The A-1331852-treated cells were the further incubated with 100 nM ABT-199 for 4 h before cell death analysis by flow cytometry. As shown in Figure 19A, as expected, A-1331852 alone induced concentration-dependent cell death in unstimulated CLL cells, but did not do so in CD40-stimulated CLL cells.

Next, I combined A-1331852 with 100 nM ABT-199 to see if the combination treatment can kill CD40-stimulated CLL cells. As shown in Figure 19B (blue line), 100 nM ABT-199 alone killed almost all unstimulated CLL cells. Again, A-1331852 only modestly increased ABT-199-induced cell death in CD40-stimulated CLL cells, causing 14.45 % of cell death at 10 nM, 19.94 % at 100 nM, 20.38 % at 1,000 nM and 40.12 % at 10,000 nM (Figure 19B, orange line). Comparing to the level of cell death induced by 10 nM ABT-199 of the same CLL sample described earlier (Figure 19B), A-1331852 increased the cell death induced by 100 nM ABT-199, although not to the level of cell death seen in similarly treated unstimulated cells.

(A) Single agent (B) Combination treatment

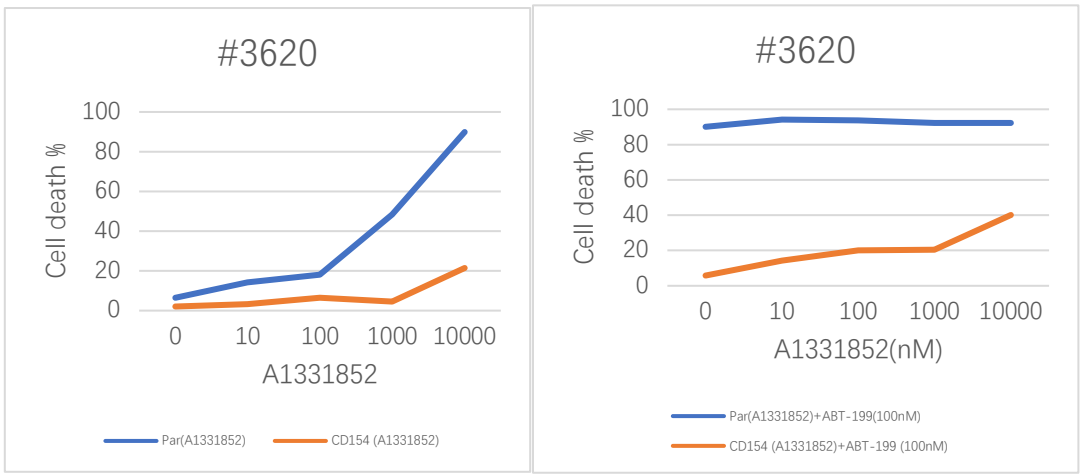


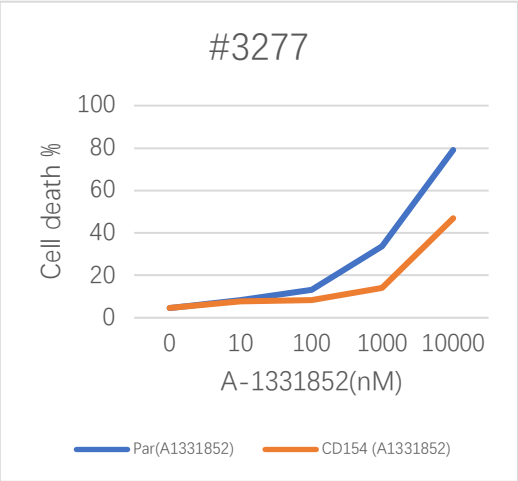
Figure 19. Induction of cell death by A-1331852 with or without 100 nM of ABT-199 (CLL sample 3620). (A) Primary CLL cells were co-cultured with CD154-expressing or parental (PAR) fibroblasts for 24 h. Co-cultured CLL cells were then collected and incubated with A-1331852 at the indicated concentrations for 24 h. Cell death was measured by flow cytometry as previously described. (B) Following incubation of co-cultured CLL cells with A-1331852 for 24 h, as in (A), cells were further incubated with ABT-199 (100 nM) for 4 h before cell death analysis by flow cytometry.

4.3.2.2.2 Result from CLL sample #3277

I then repeated the above experiments using primary CLL cells from another patient sample (#3277). As shown in Figure 20A, A-1331852 alone induced a concentration-dependent cell death in unstimulated CLL cells and, to a lesser extent, in CD40-stimulated CLL cells as well.

Next, I examined the effect of combination treatment of A-1331852 with ABT-199 in these cells. 100 nM ABT-199 alone caused almost 100 % cell death in unstimulated cells and ~40 % in CD40-stimulated cells (Figure 20B, blue line). When used in combination with 100 nM ABT-199, A-1331852 clearly increased ABT-199-induced cell death in a concentration-dependent manner in CD40-stimulated CLL cells, causing 44.32 % of cell death at 10 nM, 61.66 % at 100 nM, 71.69 % at 1,000 nM and 81.83 % at 10,000 nM, respectively (Figure 20B, orange line). Therefore, the above results showed that A-1331852 can overcome the protective effect of CD40-stimulation and restore sensitivity to ABT-199-induced cell death in CD40-stimulated CLL cells.

(A) Single agent



(B) Combination treatment

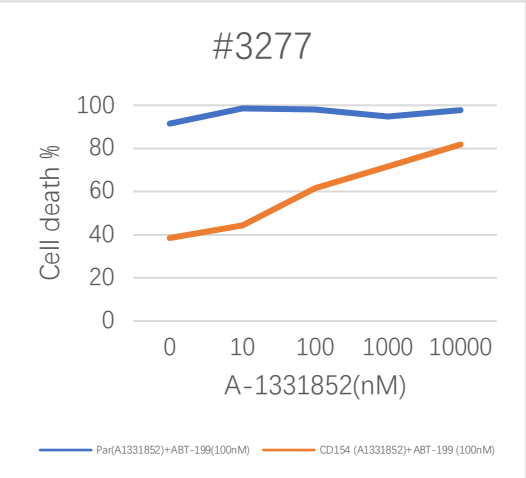
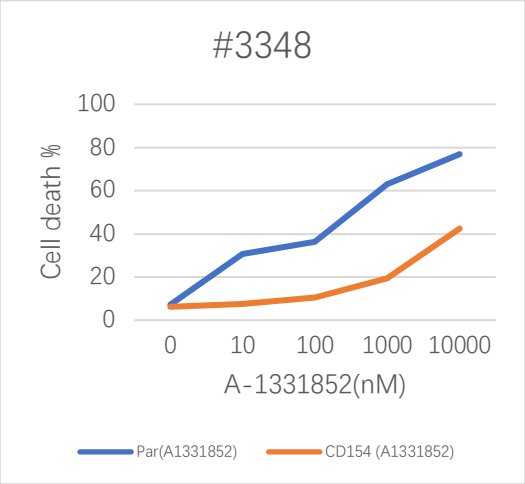


Figure 20. Induction of cell death by A-1331852 with or without 100 nM ABT-199 (CLL sample 3277). (A) Primary CLL cells were co-cultured with CD154-expressing or parental (PAR) fibroblasts for 24 h. Co-cultured CLL cells were then collected and incubated with A-1331852 at the indicated concentrations for 24 h. Cell death was measured by flow cytometry as previously described. (B) Following incubation of co-cultured CLL cells with A-1331852 for 24 h, as in (A), cells were further incubated with ABT-199 (100 nM) for 4 h before cell death analysis by flow cytometry.

4.3.2.2.3 Result from CLL sample #3348

As the results obtained from the previous two CLL cases indicate that A-1331852 may be a promising agent to be used in combination with ABT-199 to overcome CD40-stimulation mediated resistance, I repeated the experiments using a third CLL sample (#3348). As shown in Figure 21A, A-1331852 again induced a concentration-dependent cell death in unstimulated cells and, to a lesser degree, in CD40-stimulated cells as well. I then examined the effect of combination treatment of A-1331852 with ABT-199 in these cells. Again, 100 nM ABT-199 alone caused almost 85 % cell death in unstimulated cells and ~30 % in CD40-stimulated cells (Figure 21B). When used in combination with 100 nM ABT-199, A-1331852 clearly increased ABT-199-induced cell death in a concentration-dependent manner in CD40-stimulated cells, causing 53.48 % of cell death at 10 nM, 68.27 % at 100 nM, 70.11 % at 1,000 nM and 73.77% at 10,000 nM, respectively (Figure 21B, orange line). Therefore, the above results were consistent with what were observed from the previous two cases, which was encouraging.

(A) Single agent



(B) Combination treatment

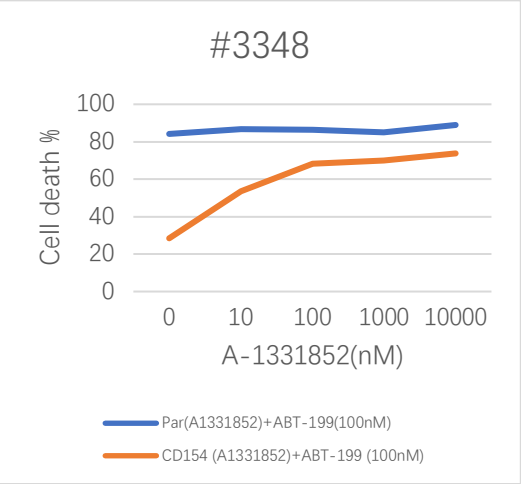
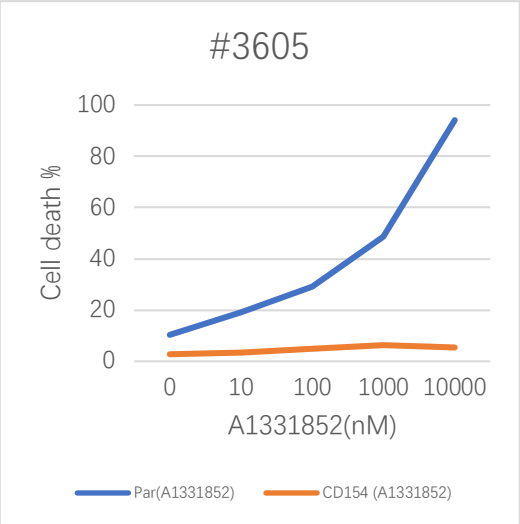


Figure 21. Induction of cell death by A-1331852 with or without 100 nM ABT-199 (CLL sample 3348). (A) Primary CLL cells were co-cultured with CD154-expressing or parental (PAR) fibroblasts for 24 h. Co-cultured CLL cells were then collected and incubated with A-1331852 at the indicated concentrations for 24 h. Cell death was measured by flow cytometry as previously described. (B) Following incubation of co-cultured CLL cells with A-1331852 for 24 h, as in (A), cells were further incubated with ABT-199 (100 nM) for 4 h before cell death analysis by flow cytometry.

4.3.2.2.4 Result from CLL sample #3605

As CLL is a heterogeneous disease, I wished to expand the above combination experiments using primary CLL cells from a fourth CLL sample (#3605). As shown in Figure 22A, A-1331852 again induced a concentration-dependent cell death in unstimulated cells. However, this time it did not induce a significant amount of cell death in CD40-stimulated cells. Next, I examined the effect of combination treatment of A-1331852 with ABT-199 in these cells. Again, 100 nM ABT-199 alone caused almost 100 % cell death in unstimulated cells, but only ~10 % of cell death in CD40-stimulated CLL cells (Figure 22B). When used in combination with 100 nM ABT-199, A-1331852 again increased ABT-199-induced cell death in a concentration-dependent manner in CD40-stimulated cells, causing 45.95 % of cell death at 10 nM, 58.95 % at 100 nM, 60.46 % at 1,000 nM and 69.57 % at 10,000 nM, respectively (Figure 22B, orange line). Therefore, the above results confirmed that A-1331852 could overcome the protective effect of CD40-stimulation and restore sensitivity to ABT-199-induced cell death in CD40-stimulated CLL cells.

(A) Single agent



(B) Combination treatment

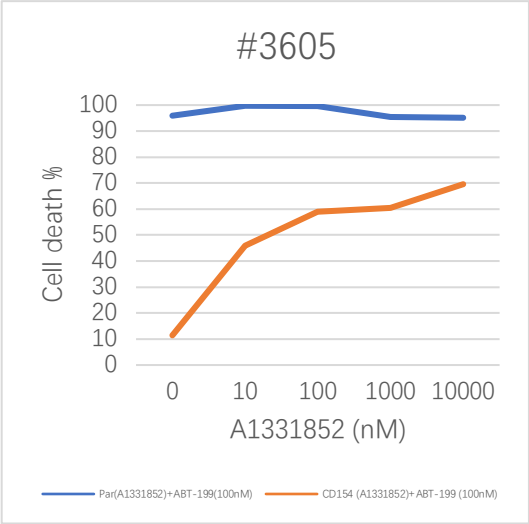


Figure 22. Induction of cell death by A-1331852 with or without 100 nM ABT-199 (CLL sample 3605). (A) Primary CLL cells were co-cultured with CD154-expressing or parental (PAR) fibroblasts for 24 h. Co-cultured CLL cells were then collected and incubated with A-1331852 at the indicated concentrations for 24 h. Cell death was measured by flow cytometry as previously described. (B) Following incubation of co-cultured CLL cells with A-1331852 for 24 h, as in (A), cells were further incubated with ABT-199 (100 nM) for 4 h before cell death analysis by flow cytometry.

4.3.2.2.5 Pooled date analysis of induction of apoptosis by A-1331852 in combination with 100 nM ABT-199 (n=4)

Next, I pooled the cell death data obtained from the above four CLL samples and performed the statistical analysis to determine if the increase in ABT-199-induced cell death by A-1331852 were statistically significant. As shown in Figure 23A, A-1331852, as expected, induced concentration-dependent cell death in unstimulated CLL cells and, to a lesser extent, in CD40-stimulated CLL cells. However, the effect of combination treatment of A-1331852 with ABT-199 was very encouraging. Again, 100 nM ABT-199 alone caused an average of ~90 % of cell death in unstimulated cells and addition of A-1331852 had little effect (Figure 23B, blue line). 100 nM ABT-199 alone also caused an average of about 23 % of cell death in CD40-stimulated CLL cells (Figure 23B, orange line). However, when used in combination, A-1331852 increased ABT-199-induced cell death in a concentration-dependent manner in CD40-stimulated cells, causing an average of 39.55 % of cell death at 10 nM, 52.21 %at 100 nM, 55.66 % at 1,000 nM and 66.32 % at 10,000 nM, respectively (Figure 23B, orange line). Statistical analysis using two-tailed, paired Student's t test showed that increases in ABT-199-induced cell death by A-1331852 were statistically significant (Figure 23B). At 100 nM, A-1331852 significantly increased ABT-199-induced cell death in CD40-stimulated CLL

cells, when compared with cell death of CD40-stimulated cells treated with A-1331852 alone (p=0.026). At 1000 nM, it also significantly increased ABT-199-induced cell death in CD40-stimulated cells comparing to CD40-stimulated cells treated with A-1331852 alone (p=0.019). Finally, at 10,000 nM, A-1331852 again significantly increased ABT-199-induced cell death in CD40-stimulated CLL cells as compared to their counterparts treated with A-1331852 alone (p=0.002).

Therefore, the above results confirmed that A-1331852 could overcome the protective effect of CD40-stimulation and restore the sensitivity of CD40-stimulated CLL cells to ABT-199, although not to the level of cell death seen in unstimulated cells. However, caution is again needed in interpreting the results due to the small number of CLL samples used in the study (n=4).

（A） Single agent　　(B) Combination treatment

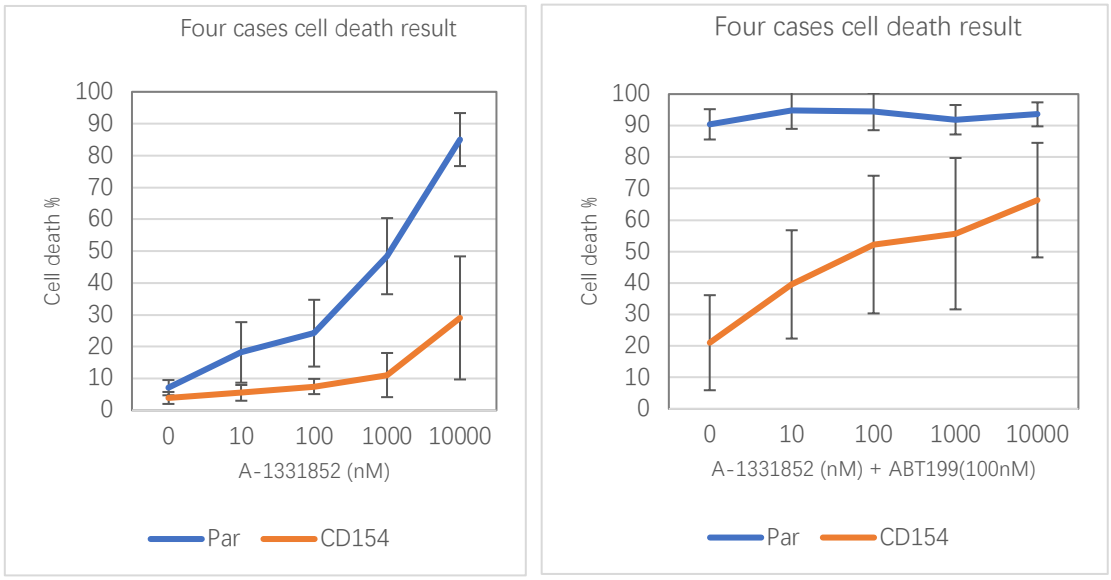


Figure 23. Induction of cell death by A-1331852 in combination with 100 nM ABT-199 (pooled data analysis, n=4). (A) Primary CLL cells were co-cultured with CD154-expressing or parental (PAR) fibroblasts for 24 h. Co-cultured CLL cells were then collected and incubated with A-1331852 at the indicated concentrations for 24 h. Cell death was measured by flow cytometry as previously described. (B) Following incubation of co-cultured CLL cells with A-1331852 for 24 h, as in (A), cells were further incubated with ABT-199 (100 nM) for 4 h before cell death analysis by flow cytometry. In both (A) and (B), data represent the mean ± SD from four independent experiments using four different CLL samples.

4.3.3 A-1331852 sensitises CD40-stimulated CLL cells to ABT-199 by disrupting the BIM-BCL-XL complex

My results have so far shown that BCL-XL inhibitor A-1331852 can restore the sensitivity of CD40-stimulated CLL cells to ABT-199. I thus wished to investigate the underlying mechanisms responsible for the sensitization effect. It has been shown that A-1331852 is able to bind BCL-XL with high affinity and selectively disrupts the BCL-XL-BIM complex (Leverson et al., 2015). I thus hypothesized that A-1331852 sensitises CD40-stimulated cells to ABT-199 by interrupting the binding BCL-XL with BIM, enabling released BIM to activate apoptosis. To test this hypothesis, I used Co-immunoprecipitation (Co-IP) method to investigate the changes in binding of BIM and BCL-XL in CD40 stimulated CLL cells treated with ABT-199 in the presence or absence of A-1331852. Therefore, CLL cells were harvested after co-culture with CD154-expressing fibroblasts for 24 h and then treated with or without 100 nM A-1331852 for 24 h. All co-cultured CLL cells were then further incubated with 100 nM ABT-199 for 4 h before cell death analysis by flow cytometry and Co-IP experiments. I chose 100 nM A-1331852 as it, in combination with 100 nM ABT-199, induced an average of over 50 % of cell death in CD40-stimulated CLL cells, whereas ABT-199 alone caused an average of ~20 % of cell death in these cells (Figure 23B, orange line).

4.3.3.1 Result from CLL sample #3684

After I optimised the Co-IP conditions using a rabbit polyclonal antibody against BCL-XL (from Cell Signaling Technologies), I used the primary CLL cells from sample #3684 and co-cultured the cells with CD154-expressing fibroblasts for 24 h. I then collected co-cultured cells as previously described and incubated them at 4 x 10^6 cells/ml under standard culture conditions with or without 100 nM A-1331852 for 24 h. I then further incubated all co-cultured CLL cells with 100 nM ABT-199 for 4h before harvest. Cell death analysis by flow cytometry showed that co-cultured cells treated with A-1331852 plus ABT-199 had 40 % more cell death than the co-cultured cells treated with ABT-199 alone, confirming that A-1331852 sensitised CD40-stimulated cells to ABT-199. I then lysed remaining cells according to the established procedure as described in Methods. After protein lysates were quantified using a Bradford assay kit (Bio-Rad laboratories), 100 µg of protein from the corresponding lysate samples was used for immunoprecipitation with either 1 µg of polyclonal rabbit IgG control antibody or anti-BCL-XL antibody. The identity of immunoprecipitated proteins was then revealed by Western blotting analysis.

As shown in Figure 24A, BIM (all three isoforms) was detected in the pre-IP lysates from the cells treated with A-1331852 plus ABT-199 or ABT-199 alone. Western blot analysis also showed that BIM was present in the BCL-XL antibody pull-down fraction in the lysate from CD40-stimulated CLL cells treated with ABT-199 alone, indicating that BCL-XL interacted with BIM. However, BIM was absent in the similarly

immunoprecipitated fraction in the lysate from the co-cultured cells pre-treated with A-1331852, suggesting that the BCL-XL inhibitor may have interfered the interaction between BCL-XL and BIM. I next quantified the signals of BIM (BIM-EL and BIM-L) in both immunoprecipitated (IP) and pre-immunioprecipitated (Pre-IP) fractions by densitometry analysis. As shown in Figure 24B, the ratio of BIM in immunoprecipitated (IP) fraction/Pre-IP fraction was lower in samples treated with ABT-199 plus A-1331852 than that treated with ABT-199 alone. This was the case for both BIM-EL and BIM-L.

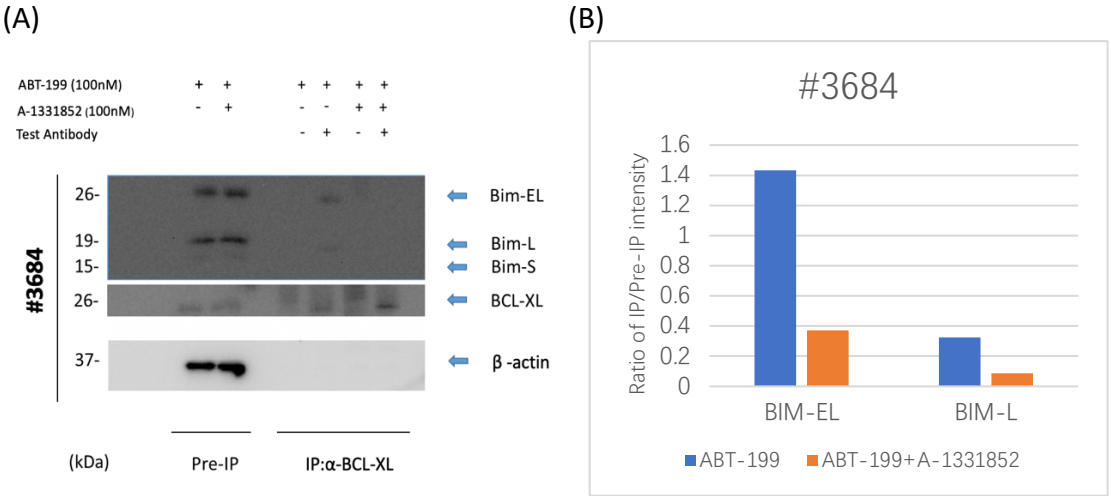


Figure 24. A-1331852 interrupted binding of BCL-XL to BIM in CD40-stimulated CLL cells (sample 3684). Primary CLL cells from sample #3684 were co-cultured with CD154-expressing fibroblasts for 24 h. Co-cultured cells were then collected and incubated at 4 x 10^6 cells/ml under standard culture conditions with or without A-1331852 for 24 h. All co-cultured CLL cells were then further incubated with ABT-199 for 4 h before harvest and used for co-immunoprecipitation experiments. (A) Co-immunoprecipitation using BCL-XL antibody and Western blotting for BIM were performed as described in Methods. 10 µg proteins of un-manipulated cell lysates (Pre-IP) were used as a positive control for BIM. Same blot was also probed for BCL-XL and β-actin. (B) Densitometry analysis of BIM (BIM-EL and BIM-L) in pull-down (IP) and pre-IP factions.

4.3.3.2 Result from CLL sample #3702

I next repeated Co-IP experiments using primary CLL cells from another CLL sample (#3702) and treated them exactly the same way as described for the previous case. Cell death analysis by flow cytometry also confirmed that ABT-199 alone induced 16.79% cell death and pre-treatment of A-1331852 increased ABT-199-induced cell death to 64.44% in CD40-stimulated cells. The results of Co-IP were shown in Figure 25. Again, BIM was present in the BCL-XL antibody pull-down fraction in the lysate from CD40-stimulated CLL cells treated with ABT-199 alone, confirming that BCL-XL interacted with BIM. However, BIM was absent in the immunoprecipitated fraction in the lysate from the co-cultured cells pre-treated with A-1331852, again confirming that the BCL-

XL inhibitor may have interfered the interaction between BCL-XL and BIM. Again, the above results were in part in agreement with that from the previous CLL sample.

(A) (B)

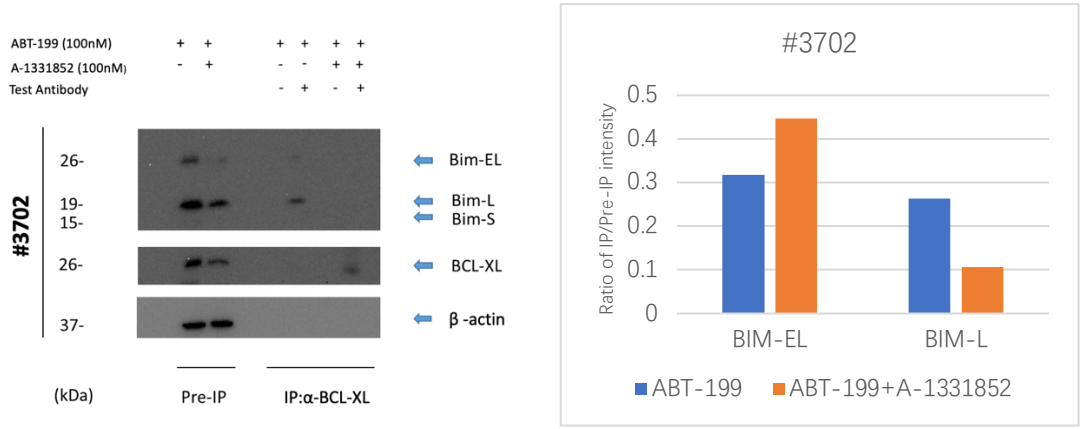


Figure 25. A-1331852 interrupted binding of BCL-XL to BIM in CD40-stimulated CLL cells (sample 3702). Primary CLL cells from sample #3702 were co-cultured with CD154-expressing fibroblasts for 24 h. Co-cultured cells were then collected and incubated at 4 x 10^6 cells/ml under standard culture conditions with or without A-1331852 for 24 h. All co-cultured CLL cells were then further incubated with ABT-199 for 4 h before harvest and used for co-immunoprecipitation experiments. Co-immunoprecipitation using BCL-XL antibody and Western blotting for BIM were performed as described in Methods. 10 µg proteins of un-manipulated cell lysates (Pre-IP) were used as a positive control for BIM. Same blot was also probed for BCL-XL and β-actin.

4.3.3.3 Result from CLL sample #3694

To ensure that the results of Co-IP experiments from previous two CLL samples were reproducible, I repeated the Co-IP experiment using primary CLL cells from a third sample (#3694) and treated them the same way as before. After confirming by flow cytometry that ABT-199 alone induced ~55 % of cell death and A-1331852 increased ABT-177-induced cell death to ~75 % of cell death in CD40-stimulated CLL cells, I proceeded with Co-IP using BCL-XL antibody as described earlier. As shown in Figure 26A, BIM was detected in the BCL-XL antibody pulled-down fraction from lysate of the CD40-stimulated CLL cells treated with ABT-199 alone. In contrast, BIM was not detected in the immunoprecipitated fraction from lysate of the CD40-stimulated CLL cells treated with ABT-199 plus A-1331852. Densitometry analysis also showed that the proportion of BIM (both BIM-EL and BIM-L) in the IP portion that was incubated with BCL-XL antibody was significantly reduced in samples treated with ABT-199 and A-1331852, as compared to that treated with ABT-199 alone.

(A) (B)

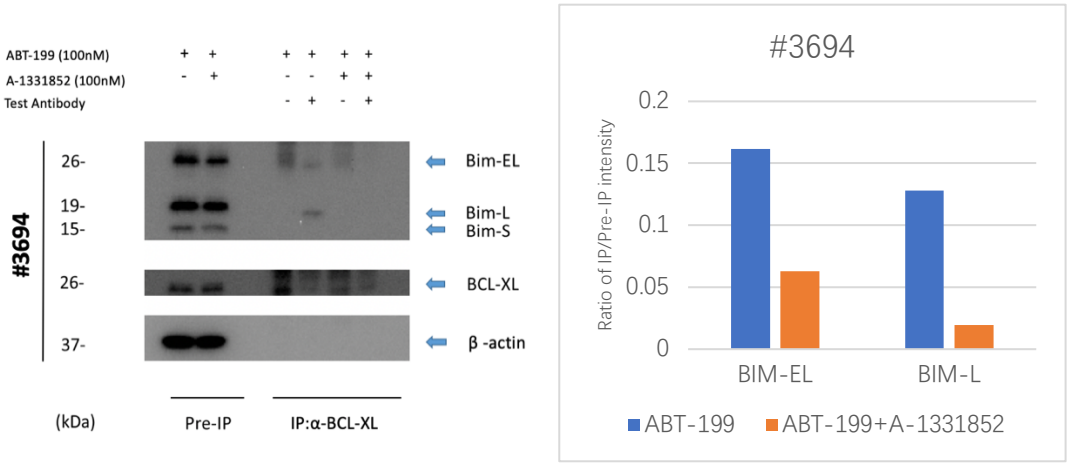


Figure 26. A-1331852 interrupted binding of BCL-XL to BIM in CD40-stimulated CLL cells (sample 3694). Primary CLL cells from sample #3694 were co-cultured with CD154-expressing fibroblasts for 24 h. Co-cultured cells were then collected and incubated at 4 x 106 cells/ml under standard culture conditions with or without A-1331852 for 24 h. All co-cultured CLL cells were then further incubated with ABT-199 for 4 h before harvest and used for co-immunoprecipitation experiments. (A) Co-immunoprecipitation using BCL-XL antibody and Western blotting for BIM were performed as described in Methods. 10 µg proteins of un-manipulated cell lysates (Pre-IP) were used as a positive control for BIM. Same blot was also probed for BCL-XL and β-actin. (B) Densitometry analysis of BIM (BIM-EL and BIM-L) in pull-down (IP) and pre-IP factions.

Therefore, co-IP experiments using 3 different CLL samples showed that A-1331852 appeared to sensitize CD40-stimulated cells to ABT-199 by interrupting the binding BCL-XL with BIM in two of the three samples. However, the result from the third sample was inconclusive. Further experiments are still required to confirm the above observation.

4.3.4 Decreased BIM expression reduced ABT-199-induced cell death in CD40-stimulated CLL cells

In order to determine whether BIM was required for ABT-199-induced cell death and the increased sensitivity to ABT-199 by A-1331852 in CD40-stimulated CLL cells, I wished to use BIM siRNA to knock down BIM protein in these CLL cells. I first used unstimulated primary CLL cells from three different CLL patients for BIM siRNA validation and the results showed that BIM siRNA was effective in knocking down BIM expression. Western blotting analysis showed that BIM-EL was significantly reduced in all three CLL samples used (#3679, #3684, and #3702) when incubated with 0.5 µM BIM siRNA, as compared with cells incubated with 0.5 µM control siRNA (Figure 27A).

Densitometry analysis showed that BIM siRNA reduced expression of BIM-EL in CLL sample #3679 by 47 %, sample #3684 by 15% and sample #3702 by 49%, respectively (Figure 27B).

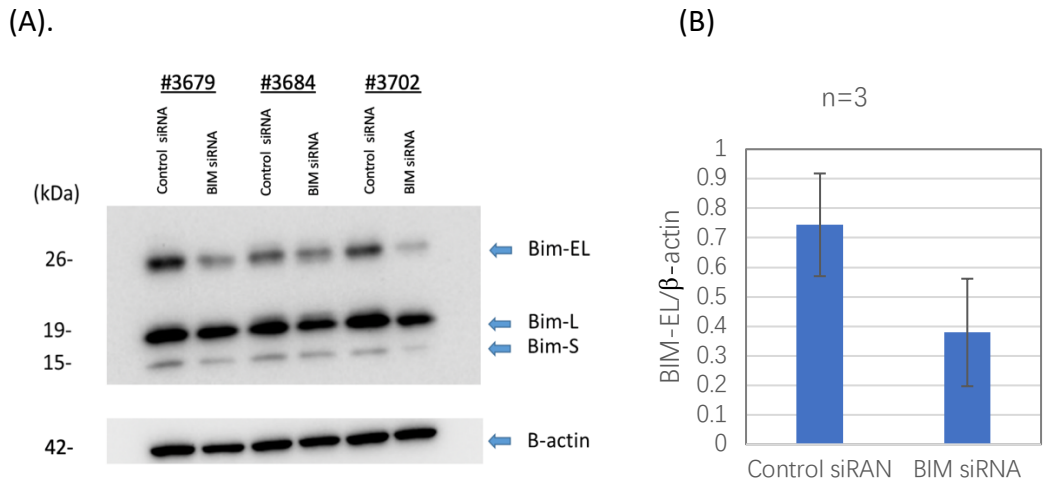


Figure 27. Expression of BIM in CLL cells treated with or without BIM siRNA. (A) Analysis of BIM expression by Western blotting. (B). Densitometry analysis of relative expression of BIM-EL and BIM -L to β-actinin primary CLL cells treated with or without BIM siRNA. Each bar represents ± standard deviation of the results from independent experiments using three CLL samples.

Based on the results from the optimising experiment described above, I used BIM siRNA to knock down BIM proteins in CD40-stimulated CLL cells.

First, I used BIM siRNA to knock down BIM protein from primary CLL cells from patient samples. Non-specific siRNA was used as a control. After incubation with respective siRNA oligos, CLL cells were co-cultured with CD154-exopressing fibroblasts for 24 h. Co-cultured cells were then collected and incubated at 4 x 10^6 cells/ml under standard culture conditions with or without 100 nM A-1331852 for 24 hours. At the end of 24 h incubation, the CLL cells were then further incubated with 100 nM ABT-199 for 4 hours. A portion of the cells were used for cell death analysis by flow cytometry, and the other was used for protein analysis by Western blotting. As shown in Figure 28A, transfection of BIM siRNA reduced expression of BIM, in particular the main isoform BIM-EL, in CD40-stimulated CLL cells. The reduced expression of BIM was associated with the reduction in cell death induced by 100 nM ABT-199 with or without 100 nM A-1331852 (Figure 28B). Statistical analysis showed that the reduction in ABT-199-induced cell death is statistically significant ($p<0.05$). Therefore, decreased expression of BIM resulted in a reduction in cell death induced by ABT-199.

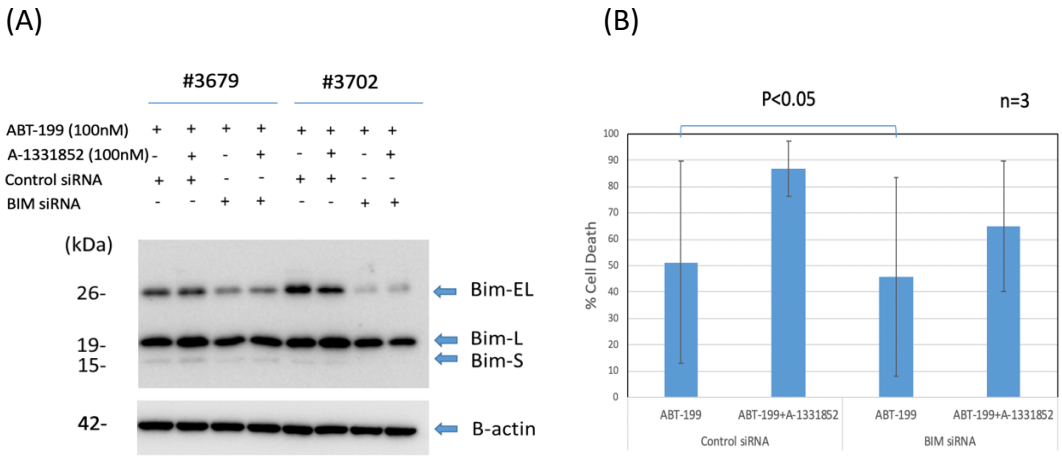


Figure 28. Effect of BIM siRNA in CD40-stimulated CLL cells. (A) Analysis BIM expression in CD40-stimulated CLL cells treated with 0.5 µM BIM siRNA or 0.5 µM control RNA. (B) Primary CLL cells (sample 3679, 3702 and 3711) treated with 0.5 µM BIM siRNA or 0.5 µM control RNA, as in (A) were co-cultured with CD154-expressing fibroblasts for 24 h. Co-cultured CLL cells were then collected and incubated with or without 100 nM A-1331852 for 24 h at the standard culture conditions. Cells were then further incubated with 100 nM ABT-199 for 4 h. Cell death was measured by flow cytometry as previously described. Data represent the mean ± SD from three independent experiments using three different CLL samples.

4.4. Discussion

The purpose of this part of the study was to investigate if the use of CDK-9 inhibitor voruciliib or BCL-XL inhibitor A-1331852 could restore sensitivity of CD40-stimulated CLL cells to ABT-199 and, if so, what the underlying mechanism was. First, I tested the combined effect of CDK-9 inhibitor voruciclb and ABT-199 by performing a concentration-response analysis of cell death using primary CLL cells from 3 independent CLL patient samples. The results showed that up to 1,000 nM, voruciclib alone does not induce apoptosis in CD40-stimulated CLL cells, neither does it sensitise CD40-stimulated CLL cells to ABT-199. Interestingly, in a study using cell lines representative of high-risk diffuse large B-cell lymphoma (DLBCL), Dey et al. found that voriciclib can inhibit MCL-1 expression in cell lines and xenograft models of DLBCL (Dey et al., 2017). It was also shown that the combination of voriciclib and venetoclax (ABT-199) resulted in stronger tumor growth inhibition than either drug used alone. In contrast, under the experimental conditions used in my study, I did not observe the reduction of MCL-1 by voruciclib (up to 1,000 nM) in CD40-stimulated CLL cells, nor the sensitization of CD40-stimulated CLL cells to ABT-199-induced cell death. Further experiments are thus required to confirm the biological activity of voruciclib such as inhibiting MCL-1 expression in CD40-stimulated CLL cells before it can be used for combination with ABT-199.

Next, I used BCL-XL inhibitor A-1331852 in my study. In order to investigate the effect of the combination of A-1331852 and ABT-199, I first performed a concentration-response experiments on cell death induced by A-1331852 alone using primary CLL cells from 3 independent patient samples. A-1331852 alone clearly induced cell death in a concentration-dependent manner in unstimulated CLL cells, but only managed to induce cell death modestly in CD40-stimulated CLL cells. When I combined A-1331852 with 10 nM ABT-199 to see if it could increase the sensitivity to ABT-199 in the CD40-stimulated cells. The results were not very impressive as it only slightly increased ABT-199-induced cell death in these cells, with the increase not statistically significant. Next, I used A-1331852 in combination with 100 nM ABT-199 and again performed concentration-response experiments on induction of cell death using primary CLL cells from 4 independent samples. The results from these experiments showed that A-1331852 significantly increased ABT-199-induced cell death of CD40-stimulated cells in a concentration-dependent manner.

I next investigated how A-1331852 sensitised CD40-stimulated CLL cells to induction of cell death by 100 nM ABT-199. Based on the available information in the literate regarding the mode of action of A-1331852, I hypothesized that A-1331852 interfered the binding of BCL-XL with BIM, enabling released BIM to activate apoptosis. I therefore used the Co-IP method to examine the changes in interaction between BCL-XL and BIM in CD40-stimulated cells treated with ABT-199 alone versus ABT-199 plus A-1331852. Co-IP experiments allowed me to obtain some new interesting findings. First, I showed that BIM was bound to BCL-XL in CD40-stimulated CLL cells, suggesting that BCL-XL was probably involved in mediating resistance to ABT-199. Secondly, I showed that A-1331852 displaced BIM from complex with BCL-XL, which was related to the increased sensitivity of CD40-stimulated CLL cells to ABT-199.

The above results were obtained through the use of anti-BCL-XL antibody and isotype control antibody in pull-down steps of the Co-IP experiments. Attempts have been made to confirm these findings using a rabbit polyclonal anti-BIM antibody (also from Cell Signaling Technologies) in reversal Co-IP experiments. Unfortunately, I could not detect interaction of BCL-XL with BIM using the anti-BIM antibody under the experimental conditions used. Due to the time constraint, I did not continue with the reversal Co-IP experiments and moved on with other experiments. Therefore, future work is still needed to confirm the interaction of BCL-XL and BIM via the reversal Co-IP using an anti-BIM antibody.

Nevertheless, the results of my study demonstrated that the combination of A-1331852 and ABT-199 may have the potential to eliminate therapy resistant populations of CLL cells in lymph nodes and bone marrow. Although the previously developed BH3 mimic ABT-737 can simultaneously antagonize the anti-apoptotic effects of BCL-2 and BCL-XL and show impressive anti-cancer activity, the loss of platelets leading to thrombocytopenia due to BCL-XL inhibition makes it a dose-limiting factor for further clinical development. ABT-199 has been used as a single drug

for treatment of patients with relapsed or refractory CLL with encouraging clinical outcome (Stilgenbauer et al., 2018, Roberts et al., 2016). However, development of venatoclax resistance has recently been reported among CLL patients receiving the treatment (Herling et al., 2018a, Blombery et al., 2019). The development of a new, specific BCL-XL inhibitor such as A-1331852 leads us to believe that the use of specific BCL-XL inhibitors could overcome resistance to ABT-199 in patients with relapsed and refractory CLL.

In summary, the data from my study demonstrated that combination of A-1331852 and ABT-199 may be useful to overcome CD40 stimulation-induced resistance to ABT-199. Co-IP experiments showed that the increased sensitivity by A-1331852 to ABT-199-induced apoptosis in CD40-stimulated CLL cells was related to the disruption of interaction between BIM and BCL-XL by A-1331852. BIM siRNA knockdown experiments demonstrated that reduced BIM expression led to decreased ABT-199-induced apoptosis in CD40-stimulated CLL cells, which highlighted the importance of BIM in mediating ABT-199-induced cell death. These findings highlight the therapeutic potential of A-1331852 in combination with ABT-199 to eliminate therapy resistant populations of CLL cells in the lymph nodes and bone marrow.

Chapter 5. General discussion

The overall purpose of my research was to explore if CD40 stimulation-mediated resistance to ABT-199 in CLL cells can be overcome with a combination treatment involving an inhibitor of BCL-XL A-1331852 and the CDK-9 inhibitor vorucilib. To this end, I first set out to characterise the sensitivity of CD40-stimulated CLL cells to ABT-199 and compared this to that from unstimulated cells. I then examined the expression of MCL-1 and BCL-XL in both CD40-stimulated and unstimulated CLL cells to see if their expression was correlated with the sensitivity to ABT-199. I then used vorucilib or A-1331852, in combination with ABT-199, to determine whether inhibition of CDK-9 or BCL-XL could restore the sensitivity of CD40-stimulated CLL cells to ABT-199. I also examined the molecular mechanisms mediating the sensitisation effect of CD40-stimaulated CLL cells to ABT-199 by the BCL-XL inhibitor. Here I will focus on the main findings from my research project for discussion.

5.1 CD40-stimulated CLL cells were not as sensitive as unstimulated cells to ABT-199-induced cell death.

In the first step, I used primary CLL samples from 4patients with CLL for my study. The results clearly showed that CD40-stimulated CLL cells are significantly less sensitive to ABT-199 than unstimulated cells. Compared to unstimulated CLL cells, ABT-199 was less effective in killing CD40 stimulated cells at all concentrations tested. This was the case in all 4 CLL samples used in my study. The above results were also consistent with the reported findings from previous studies that demonstrated that CD40 stimulation can protect CLL cells from cell death induced by multiple drugs (Kitada et al., 1999, Kater et al., 2004, Hallaert et al., 2008, Vogler et al., 2009b, Tromp et al., 2012, Dietrich et al., 2012, Zhuang et al., 2014). The possible mechanisms include the CD40 stimulation-mediated changes in the balance between pro-apoptotic and anti-apoptotic BCL-2 family of proteins, resulting in a net effect favouring CLL-cell survival(Smit et al., 2007, Willimott et al., 2007). When I used Western blotting to investigate changes in the expression of anti-apoptotic BCL-2 family proteins, I observed that the protein expression of MCL-1 and BCL-XL was significantly increased in CD40-stimulated CLL cells, whereas the level of BCl-2 expression remained unchanged. The up-regulation of MCL-1 and BCL-XL in CD40-stimulated cells was also associated with reduced induction of cell death by ABT-199 in these cells.

In my study, using an established in-vitro model of CD40 stimulation to simulate the *in-vivo* interaction of CLL cells with activated T cells in the lymph node microenvironment, I demonstrated that CD40 stimulation can induce increased expression in MCL-1 and BCL-XL and that this increase also corresponded to a reduction in ABT-199-induced cell death in CD40-stimulated CLL cells. Therefore, my results are also consistent with the results of previous studies in which CD40

stimulation induced upregulation in expression of anti-apoptotic members of BCL-2 family proteins including BCL-XL and MCL-1 (Kitada et al., 1999, Kater et al., 2004, Hallaert et al., 2008, Vogler et al., 2009b, Tromp et al., 2012, Dietrich et al., 2012, Zhuang et al., 2014).

In CLL, the recurrence of relapsed disease following remission after initial treatment is an important challenge for clinical management of the disease. Therefore, understanding how resistance develops has been the focus of extensive scientific research conducted by the CLL research community for many years. One of the causes for relapsed disease is that chemotherapy drugs cannot effectively kill CLL cells residing in bone marrow and lymph nodes, resulting in minimal residual disease (MRD) (Burger and Gandhi, 2009, Shain et al., 2015). The co-culture system used in my study mimics CD40 stimulation of CLL cells by T cells in lymph node and bone marrow microenvironment. (Burger and Gandhi, 2009, Caligaris-Cappio, 2003).

Compared with peripheral blood CLL cells, CLL cells derived from LNs show changes in expression of BCL-family of proteins, namely increased expression of BCL-XL and MCL-1 and decreased expression of pro-apoptotic BH3-only protein Noxa (Thijssen et al., 2015, Smit et al., 2007, Tromp et al., 2012). CD40 stimulation of CLL cells also results in up-regulation of BCL-XL and MCL-1 and down-regulation of Noxa (Tromp et al., 2012, Kater et al., 2004, Willimott et al., 2007) and leads to resistance to apoptosis induced by multiple drugs (Romano et al., 1998, Hallaert et al., 2008). This shows that the CD40 stimulation through co-culture systems closely resembles the lymph node microenvironment. The up-regulation of MCL-1 and BCL-XL in CD40-stimulated cells is associated with reduced ABT-199-induced apoptosis in these cells. Therefore, targeting the anti-apoptotic proteins BCL-XL and MCL-1 may provide new treatment options for overcoming drug resistance in CLL. If the drug resistance caused by the CD40 stimulation can be overcome, it will be of great significance to eliminate CLL cells hiding in lymph nodes and bone marrow, thus minimising the risk of developing MRD.

Recently, studies have shown that stimulation of CD40 using a co-culture system can also lead to resistance to ABT-199-induced CLL cell apoptosis (Thijssen et al., 2015, Oppermann et al., 2016). This resistance is associated with increased expression of MCL-1, BCL-XL and A1/BFL1. It has also been shown that CLL cells co-cultured with autologous activated T cells are also protected against ABT-199-induced apoptosis and this protective effect has been shown to be associated with upregulation of MCL-1 and BCL-XL in CLL cells (Elías et al., 2018). Since it has been shown that activated T cells are in close contact with CLL cells in the lymph nodes and activate CLL cells through the CD40 signaling pathway (Granziero et al., 2001, Ghia et al., 2002, Pascutti et al., 2013), the above research results from my study and others may to some extent reflect real conditions of CLL cells in the lymph node microenvironment. In fact, CLL cells located in lymph nodes have shown higher levels of expression in MCL-1 and BCL-XL protein compared to their blood counterparts (Smit et al., 2007, Thijssen et al., 2015).

Based on the above observations, I thus hypothesized that use of a specific inhibitor of MCL-1 and BCL-XL will reduce or even overcome resistance of CD40-stimulated CLL cells to ABT-199. This led me to test the idea that BCL-XL or MCL-1 inhibitors might help increase ABT-199-induced apoptosis in CD40-stimulated CLL cells. Therefore, in the next part of the study, I used voruciclib (a CDK9 inhibitor which can decrease the expression of MCL-1) or BCL-XL inhibitor A-1331852 to test if the sensitivity of CD40-stimulated CLL cells to ABT-199-induced apoptosis could be improved when used in combination.

5.2 Inhibition of BCL-XL significantly increase sensitivity of CD40-stimulated CLL cells to ABT-199.

I first tested the combined effect of the MCL-1 inhibitor voruciclib and ABT-199 in inducing cell death using primary CLL cells from 3 CLL patients. The results showed that the combined effect did not achieve the desired effect. At concentrations up to 1000 nM, voruciclib itself did not induce apoptosis in CD40-stimulated CLL cells, nor did it significantly increase the sensitivity of CD40-stimulated CLL cells to ABT-199. Results from Western blotting showed that there was no significant change in MCL-1 protein in CD40-stimulated cells treated with voruciclib at concentrations up to 1000 nM. The expression of MCL-1 was decreased in these cells treated with 10,000 nM voruciclib. At 10,000 nM concentration, voruciclib also induced extensive cell death in both CD40-stimulated and unstimulated CLL cells, suggesting that voruciclib at this concentration could kill cells in a non-specific manner. Reduction in MCL-1 therefore likely results from the extensive cell death caused by 10,000 nM voruciclib. In a study by Dey et al, authors found that voruciclib can inhibit MCL-1 expression in DLBCL cell lines (Dey et al., 2017). It has also been shown that the combination of voriciclib and venetoclax (ABT-199) can inhibit tumor growth more strongly than either drug used alone. In our experiments, the combination of the two did not achieve the desired effect. One reason to explain the negative finding is that in my study the effect of voruciclib on expression of MCL-1 was not established under the experimental conditions used. Time-course experiment was required to determine the time point at which treatment of voruciclib would reduce the expression of MCL-1 in CD40-stimulated cells. Unfortunately, due to time restriction, I did not perform the time course experiments. Therefore, further experiments are needed to confirm the biological activity of voruciclib, such as inhibiting the expression of MCL in CD40-stimulated CLL cells before it can be used in combination with ABT-199.

In the meantime, since BCL-XL was also upregulated in CD40-stimulated CLL cells, I used the BCL-XL inhibitor A-1331852 to see if it can increase their sensitivity to ABT-199. I first performed a concentration-response experiment on cell death induced by A-1331852 alone using primary CLL cells from 3 independent CLL patients. A-1331852 alone induced cell death in a concentration-dependent manner in unstimulated CLL cells, but could only induce a moderate level of cell death in CD40-stimulated CLL cells. .

When used in combination with 10 nM ABT-199, A-1331852 slightly increased the cell death induced by ABT-199 in CD40-stimulated cells. However, when used in combination with 100 nM ABT-199, A-1331852 significantly increased the ABT-199-induced cell death in CD40-stimulated CLL cells in a concentration-dependent manner. Although it did not reach the level of apoptosis seen in unstimulated cells, this result clearly indicates that the combined use of the BCL-XL inhibitor A-1331852 is effective in restore sensitivity of CD40-stimulated CLL cells to ABT-199.

The combined effect of A-1331852 and ABT-199 may thus have the potential to overcome the drug resistance of CLL cells in lymph nodes and bone marrow. The main reason for the withdrawal of the first generation BH3 mimetic ABT-737 is its dual inhibition of BCL-2 and BCL-XL, resulting in severe thrombocytopenia in patients due to suppression of BCL-XL in the platelets (Schoenwaelder et al., 2011, Wilson et al., 2010, Roberts et al., 2012). Now we have two separate inhibitors available which specifically targeting BCL-2 and BCL-XL, respectively, and should be able to determine what the optimal concentration of A-1331852 to use in combination with ABT-199 without causing cytotoxicity to the platelets. The oral bioavailability of A-1331852 and ABT-199 provides an attractive prospect to find an ideal combination therapy for CLL, which would more accurately control the dosage of different inhibitors to achieve the maximum effect of promoting apoptosis whilst avoiding other side effects (Leverson et al., 2015a).

And recently, venatoclax resistance has been reported in CLL patients receiving the treatment (Herling et al., 2018a, Blombery et al., 2019). Mechanistically, the resistance to venetoclax can be caused by the acquired mutation in BCL-2 (Gly101Val mutation) in CLL patients undergoing venetoclax treatment (Blombery et al., 2019). However, out of 15 patients who developed progressive disease, Gly101Val mutation in BCL-2 was detected only in 7 patients (Blombery et al., 2019). This indicates that other, yet unknown, factors can also contribute to the development of resistance to venetoclax. It is known that CLL microenvironment provides a safe haven for CLL cells, shielding the leukemic cells from the cytotoxic effect of therapeutics. Here I have shown that inhibition of BCL-XL can increase the sensitivity of CD40-stimulated CLL cells to ABT-199. The development of specific BCL-XL inhibitors (such as A-1331852) would allow us to test if the use of specific BCL-XL inhibitors can overcome resistance to ABT-199 in CLL patients with relapsed and refractory disease.

5.3 BCL-XL inhibitor A-1331852 sensitised CD40-stimulated CLL cells to ABT-199 by disrupting the binding of BCL-XL to BIM.

Based on the literature on the mode of action of A-1331852 (Leverson et al., 2015b), I speculated that the BCL-XL inhibitor disturbs the binding of BCL-XL to BIM, thereby causing the release of BIM from the complex, with released BIM able to activate apoptosis. Therefore, I used the Co-IP method to examine changes in the interaction

between BCL-XL and BIM in CD40-stimulated cells treated with ABT-199 alone versus ABT-199 plus A-1331852. The Co-IP experiment gave me some interesting new findings. First, I demonstrated that BIM binds to BCL-XL in CD40-stimulated CLL cells, suggesting that BCL-XL may be involved in the mediation of resistance to ABT-199. Second, I showed that A-1331852 displaces BIM from the complex with BCL-XL, which is associated with the increased sensitivity of CD40 stimulated CLL cells to ABT-199.

The above results were obtained by using anti-BCL-XL antibody and isotype control antibody in the pull-down step of the Co-IP experiment., Attempts have been made to confirm these findings in reverse Co-IP experiments using rabbit polyclonal anti-BIM antibodies (also from Cell Signaling Technologies). Unfortunately, under the experimental conditions used, I was unable to detect the interaction of BCL-XL with BIM using anti-BIM antibodies. Due to time constraints, I did not continue the reverse IP Co-IP experiment, but proceeded with other experiments instead. Therefore, further work is needed to confirm the interaction of BCL-XL with BIM by using an anti-BIM antibody in Co-IP experiment.

In the study by Choudhary et al (2015), the authors explored the mechanism of resistance to ABT-199 by establishing an ABT-199 resistant cell line. In their study, the cells resistant to ABT-199 showed an increased activation of AKT signaling as well as upregulated expression of MCL-1 and BCL-XL. Authors used the dual inhibitors of p-AKT and mTOR, NVP-BEZ235and showed that expression of MCL-1 was decreased with increased induction of cell death in these cells (Choudhary et al., 2015). They also showed that BIM was an indispensable part of the induction of apoptosis by ABT-199 as BIM was found to be inhibited by MCL-1 in the resistant cells. Treatment of NVP-BEZ235 resulted in the release of BIM from MCL-1 complex. In my study, I hypothesized that A-1331852 interfered with the binding of BCL-XL with BIM, resulting in release of BIM, which then activates BAX and/or BAK, leading to the release of cytochrome c and eventually cell death. In a study by Vogler et al. (2009), an increase in BCL-XL levels also conferred resistance to ABT-737 in CLL cells co-cultured with transfected fibroblasts expressing human CD154 (Vogler et al., 2009a). Therefore, the above results indicated that BIM displacement from BCL-XL complex by A-1331852 is a likely mechanism responsible for increased sensitivity of CD40-stimulated CLL cells to ABT-199. However, the above interpretation does not exclude the possibility that A-1331852 can increase sensitivity of resistant cancer cells to ABT-199 through other mechanisms. For example, it has recently been shown that apoptosis induced by BCL-XL inhibitor A1331852 was associated with a displacement of both BAX and BAK from BCL-XL in in diffuse large B-cell lymphoma (Smith et al., 2020). Nevertheless, it supports the idea that targeting BCL-XL is a valid approach to restore the sensitivity of resistant cells to ABT-199.

This notion is also consistent with the findings of a study by Thijssen and colleagues who used siRNA to directly inhibit the expression of BCL-XL and showed that CD40-stimulated CLL cells treated with the BCL-XL siRNA were sensitive to apoptosis induced

by ABT-199 (Thijssen et al., 2015).

I next tested the role of BIM in ABT-199-induced cell death in CD40-stimulated CLL cells. According to the results of Co-IP experiments, the increase of sensitivity by A-1331852 to ABT-199 in CD40-stimulated CLL cells is related to the disruption of BIM-BCL-XL complex. I used BIM siRNA to reduce BIM expression to determine if BIM was required for ABT-199-induced cell death. After using siRNA to reduce BIM expression, I found that reduced BIM expression was associated with reduced induction of cell death by ABT-199 in CD40-stimulated CLL cells. This shows the BIM also played an important role in ABT-199-induced cell death. It has previously been reported that BIM is required in induction of apoptosis of CLL cells by several drugs such as fludarabine (Sharma et al., 2013) and glucocorticoids (Melarangi et al., 2012). BIM is required for IL-21-induced apoptosis of CLL cells (Gowda et al., 2008). Here, my observation that decreased expression of BIM by siRNA knockdown reduced cell death induced by ABT-199 in CD40-stimulated CLL cells also suggested that BIM was most likely required in ABT-199-induecd cell death.

5.4 Suggestion for future work

As discussed earlier, due to the small number of CLL samples used throughout my study, caution is clearly required in interpreting the results and making conclusion. For the same reason, I was not able to correlate the sensitivity of CLL cells to ABT-199 to clinical features of the patients. Therefore, significantly increased number of CLL samples from patients with different clinical features (e.g. different disease stage, various cytogenetic defects and treatment history) should be used in future experiments. Also, I have shown that inhibition of BCL-XL can increase the sensitivity of CD40-stimulated CLL to ABT-199, However, it did not fully restore the sensitivity to the level seen in unstimulated cells. This suggests other anti-apoptotic proteins can work together with BCL-XL in mediating resistance. My work as well as work of others have shown that CD40 stimulation upregulated the expression of many anti-apoptotic members of BCL-2 family proteins including BCL-XL and MCL-1. Further studies are thus needed to combine BCL-XL inhibitor and inhibitor of other anti-apoptotic proteins and determine whether the combination can fully restore the sensitivity of CD40-stimulated CLL cells to ABT-199. Furthermore, in my study, due to time constraints, I was not able to determine the biological activity of the CDK-9 inhibitor voruciclib for combination study. Future work can also include other novel MCL-1 inhibitors such as new BH3 mimetic (S63845) to MCL-1 (Kotschy et al., 2016) or Mcl-1-specific, small molecule inhibitor AZD5991 (Tron et al., 2018) or AMG176 (Yi et al., 2020). It is anticipated that, given the important role of MCL-1 in protecting cancer cells against apoptosis in general, inhibition of MCL-1 and BCL-XL will be an attractive strategy to sensitise resistance cells and restore sensitivity of CD40-stimulated CLL cells to ABT-199.

In addition, the co-culture model used in my study only simulated the effect of CD40

stimulation. In the *in-vivo* microenvironment, there are many factors/stimuli that can affect the sensitivity of CLL cells to therapeutic drugs. The drug resistance mediated by the microenvironment is thus likely a result of the multiple mechanisms in operation. Therefore, future work is required to develop an *in-vitro* model to accommodate multiple stimuli relevant to CLL and make it more closely represent the *in-vivo* CLL microenvironment. Recently, a research group tried to combine stimulation of BCR andCD40 to establish an *in vitro* model involving the CD40 ligand, IL-4 and IL-21, together with anti-IgM antibodies and their results showed that this mode can increase the proliferation of CLL cells *in vitro* (Schleiss et al., 2019). Another group used a different approach to create a three-dimensional (3D) culture system by plating primary CLL cells onto a polyurethane scaffold medium mimicking the architecture of the *in vivo* microenvironment. Their results showed that this method can keep the primary CLL cells surviving for a long-term (up to 8 weeks) in the absence of cytokines, serum or feeder layers (Dos Santos et al., 2017). This new culture system could also provide a novel platform for drug testing (Dos Santos et al., 2017).

In conclusion, my study showed that BCL-XL inhibitor A-1331852 can partially overcome resistance to ABT-199 caused by CD40 stimulation. Co-IP experiments showed that the increased sensitivity by A-1331852 to ABT-199 in CD40-stimulated CLL cells was related to its disruption of the interaction between BIM and BCL-XL. BIM siRNA knockdown experiments showed that reduced BIM expression results in reduced ABT-199-induced apoptosis in CD40-stimulated CLL cells, highlighting the importance of BIM in mediating ABT-199-induced cell death. These findings thus emphasize the therapeutic potential of A-1331852 in combination with ABT-199 to eliminate the drug resistant CLL cells in lymph nodes and bone marrow.

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