Bodyguards and assassins: Bcl-2 family proteins and apoptosis control in chronic lymphocytic leukaemia

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doi:10.1111/j.1365-2567.2005.02117.x
Received 6 December 2004; revised 14 December 2004; accepted 14 December 2004.
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Summary
Chronic lymphocytic leukaemia (CLL) is the most common B-cell malignancy in the Western world and exists as subtypes with very different clinical courses. CLL is generally described as a disease of failed apoptosis. Apoptosis resistance may stem from a combination of microenvironmental survival signals as well as from intrinsic alterations in the apoptotic machinery within the CLL cell. The molecular mechanism involved in controlling apoptosis in CLL is complex and is influenced by many factors, including Bcl-2 family proteins, which are critical regulators of cell death. Here we review the significance of apoptosis dysregulation in CLL, focusing on the role of Bcl-2 and related Bcl-2 family proteins, such as Bax and Mcl-1. The differential properties of the newly described subsets of CLL are also highlighted.

Keywords: apoptosis; Bax; Bcl-2; chronic lymphocytic leukaemia; Mcl-1

Introduction
Chronic lymphocytic leukaemia (CLL) is the most common B-cell malignancy in the Western world and is characterized by the accumulation of CD5-positive monoclonal B cells in the blood, bone marrow and peripheral lymphoid organs. Although long considered as a single disease, the clinical course of CLL is heterogeneous. Some patients have aggressive disease with a rapidly increasing number of malignant cells in the blood, and require treatment most commonly with cytotoxic therapy. Relapses are common and CLL remains incurable. By contrast, others have stable, non-progressive disease that often requires no treatment other than careful monitoring.

Studies of the B-cell receptor (BCR) have provided important clues to the mechanisms that underlie the diverse clinical behaviour of CLL. Most notably, the presence of somatic mutations in the variable region of heavy (VH) and light (VL) immunoglobulin chains is a powerful prognostic marker for CLL. Patients with mutated immunoglobulin genes (M-CLL) have a greatly improved prognosis compared to patients with unmutated germline sequences (U-CLL).

The accumulation of variable-region somatic mutations is a hallmark of antigen-driven affinity maturation within germinal centres, suggesting that these clinically important subsets of disease differ with respect to the stage of maturation reached by the B cell of origin. The usage of selected VH genes in CLL provides evidence for antigenic pressure during the development of both U-CLL and M-CLL. However, the nature and outcome of this antigenic stimulation appears to vary between the subsets. Although gene expression profiling has provided evidence to suggest that all CLLs may arise from (or most closely resemble) memory B cells, the relatively small differences in the gene-expression profiles of naive and memory B cells, and evidence for continued activation, limits the conclusions that can be drawn from these analyses. An alternative proposal is that U-CLL could arise from B cells undergoing a T-independent stimulation by low-avidity (super)antigen, outside germinal centres. By contrast, high-avidity antigen is more likely to contribute to the development of M-CLL via normal T-cell-dependent events within germinal centres (Fig. 1).

Expression of activation markers in both subsets of CLL suggests that environmental antigen continues to play a role in the maintenance of the CLL clones. Expression of the cell-surface activation marker CD38 has also been linked to disease heterogeneity in CLL. Relatively high levels of CD38 are associated with poor prognosis and, although controversial, CD38 expression...
Figure 1. A model for the development of chronic lymphocytic leukaemia (CLL) subtypes. CLL with unmutated, germline immunoglobulin sequences (U-CLL) may develop in response to low-avidity antigens (potentially superantigens) via T-cell-independent events outside the germinal centre. CLL with mutated, germline immunoglobulin sequences (M-CLL) may develop in response to high-avidity antigens that drive T-cell-dependent germinal centre formation and are therefore associated with the accumulation of immunoglobulin mutations. There is evidence for continued antigen interaction in both subsets of CLL. Whereas M-CLL cells appear to be relatively anergic, U-CLL cells are generally more responsive to B-cell receptor signalling. This may be a result of the nature of the antigen (i.e. low avidity) and differences between B cells of differing maturation in the propensity to become anergic. Resistance to apoptosis will probably contribute to both types of CLL. Intercellular heterogeneity in the susceptibility to apoptosis/expression of apoptosis regulators may impact most significantly on the response to therapy, rather than disease progression. Adapted from Stevenson and Caligaris-Cappio.3

appears to be largely independent of VH mutational status.

The ability of CLL cells from different patients to trans- mit signals via the BCR varies between the subsets.10–12 Whereas U-CLL cells are generally able to transmit signals following cross-linking of the BCR with antibodies to immunoglobulin M (anti-IgM), M-CLL cells tend to be unresponsive. This might relate to the presence of zeta-associated protein 70 (ZAP-70), a critical T-cell receptor signalling molecule that has been shown to be over-expressed in U-CLL/signal-responsive CLL.12–14 We have hypothesized that the differential response to BCR ligation reflects variable levels of anergy in CLL, with the extent of anergy being influenced by the stage of B-cell maturation and possibly by the nature of the antigen (Fig. 1).3 Anergy may be associated with improved clinical outcome because anergic cells are less responsive to proliferative/survival signals driven by antigen stimulation of the BCR in specific microenvironments.

Alteration in susceptibility to apoptosis is an important feature of many human cancers. Decreased apoptosis contributes not only to tumour development, but also to resistance to conventional anti-cancer therapies, such as radiation and cytotoxic agents. CLL is often considered to be a disease of failed apoptosis.1,15,16 Genetic alterations and changes in the expression of many apoptosis regula-
expression of the intact Bcl-2 protein. Many cell-based studies have demonstrated that overexpression of Bcl-2 enhances resistance to apoptosis, and the oncogenic potential of Bcl-2 has been confirmed in mouse models. Transgenic mice overexpressing Bcl-2 in the B-cell compartment develop B-lymphoid tumours.\(^\text{32}\)

Bcl-2-related proteins can act as cellular bodyguards or assassins to positively or negatively control apoptosis.\(^\text{29–31}\) Bcl-2 family proteins are characterized by the presence of up to four relatively short sequence motifs (less than 20 amino acid residues in length) termed Bcl-2 homology (BH) domains. More than 20 family members have been identified and these can be divided into three subfamilies, based on structural and functional features (Fig. 3). The anti-apoptotic subfamily contains the Bcl-2 and Bcl-X\(_L\) proteins, which suppress apoptosis and contain all four BH domains, designated BH1–4. Mcl-1 is another Bcl-2-related survival protein, but is somewhat structurally distinct and probably lacks a ‘classical’ BH4 domain. Pro-apoptotic proteins, such as Bax, Bak and Bok, contain BH1–3 domains and are termed ‘multidomains’, whereas other pro-apoptotic proteins, such as Bim, Bad and Bid, contain only the BH3 domain and are termed ‘BH3-only’. Some Bcl-2 family proteins also contain a carboxy-terminal transmembrane domain. The targeting of Bcl-2 family proteins to mitochondrial membranes is thought to play an important role in controlling the function of mitochondria, key participants in apoptotic cell death. Bcl-2 proteins also target other organelles: this is probably important for apoptosis control but is less well understood.

The relative expression (or activity) of various anti-apoptotic and pro-apoptotic Bcl-2 family proteins is a critical determinant of apoptosis sensitivity. Precisely how the functions of the separate subfamilies and individual Bcl-2 proteins are coordinated to control apoptosis is far from clear, but one key target appears to be the release of cytochrome \(c\) from mitochondria, and, thereby, downstream caspase activation. Bcl-2 prevents mitochondrial cytochrome \(c\) release, whereas the addition of Bax or BH3 peptides to isolated mitochondria is sufficient to promote cytochrome \(c\) release. Bcl-2 family proteins act in a coordinated manner to control apoptosis, and various homotypic and heterotypic interactions, mediated via the BH domains of these proteins, have been described. This is considered key for their biological function, as mutation of BH domains prevents dimerization and inactivates Bcl-2 family proteins. The basic mode of interaction of Bcl-2 family proteins is the insertion of an alpha-helical BH3 domain into a shallow binding groove formed by the BH1, 2 and 3 domains of a second Bcl-2 family protein (Fig. 4).\(^\text{33}\) Through the control of cytochrome release, Bcl-2 proteins modulate the ‘intrinsic’ cell-death pathway, but direct regulation of caspases, for example, by physical sequestration, may also play a role.\(^\text{34}\) Under some conditions, death receptors which can activate the ‘extrinsic’ pathway can also cross-talk to the ‘intrinsic’

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**Figure 2.** Pathways of apoptosis. The ‘intrinsic’ and ‘extrinsic’ apoptosis pathways converge on the effector caspases, such as caspase 3, which promote the biochemical and morphological characteristics of apoptosis. The ‘extrinsic’ pathway is activated by cell-surface death receptors, such as Fas, and is mediated by activation of the initiator caspase, caspase 8, within a death-inducing signalling complex (DISC). The ‘intrinsic’ pathway is activated by a wide range of stimuli which trigger the release of cytochrome \(c\) from mitochondria, leading to the activation of a distinct initiator caspase, caspase 9, within the apoptosome. In some situations, the activation of caspase 8 can lead to the cleavage of Bid, which is also able to promote cytochrome \(c\) release. Bcl-2 family proteins play a key role in controlling mitochondrial function associated with the ‘intrinsic’ cell-death pathway, either preventing or promoting the release of cytochrome \(c\).

**Figure 3.** Schematic diagram of representative Bcl-2 family proteins. Examples of anti-apoptotic (Bcl-2, Bcl-X\(_L\) and Mcl-1), pro-apoptotic multidomain (Bax, Bak and Bok) and pro-apoptotic BH3-only (Bid, Bim, Bad) proteins are shown. Bcl-2 homology (BH) and transmembrane (TM) domains are indicated. Note that Mcl-1 may not contain a classical BH4 domain.
pathway, by caspase-mediated cleavage of a BH3-only Bcl-2 family molecule, Bid, which targets the mitochondria (Fig. 2). Therefore, in some cell settings, Bcl-2 proteins can also control susceptibility to death receptor-induced apoptosis.

**Apoptosis resistance in CLL: bad influences or the devil within?**

CLL is commonly considered as a paradigm for a malignancy of failed apoptosis, as CLL cells circulating in the blood are largely non-proliferating and arrested in the G0/G1 phase of the cell cycle. Cell division must occur, presumably in ‘proliferation centres’ in tissue microenvironments, accounting for the inexorable rise in white blood cell counts in some patients and evidenced by the shortening of telomeres. However, lack of apoptosis is considered a major component of the dysregulation of normal B-cell homeostasis in all subsets of this malignancy.

A critical question is to what extent the apoptotic resistance mechanism of CLL cells are autonomous, for example, owing to genetic or epigenetic alterations in the expression of apoptosis regulators within the CLL cell, or are driven by environmental signals that are received by the CLL in vivo. The role of the microenvironment is clear from the well-recognized propensity of CLL cells to undergo apoptosis when placed in ex vivo culture, but intrinsic cellular alterations could also contribute.

CLL cells generally undergo rapid ‘spontaneous’ apoptosis following ex vivo culture. Clearly the CLL cell does not have autonomous survival capability and must depend, to some extent, on environmental signals. However, does this preclude a role for autonomous genetic/epigenetic alterations that confer further apoptosis resistance? Put another way, without these changes perhaps a CLL cell could have died even more rapidly following removal from the body. Follicular lymphoma cells also undergo apoptosis when placed in culture even though they carry the t(14:18) translocation, the hallmark genetic alteration conferring cellular resistance to apoptosis. Thus, even cells which have genetically deranged apoptosis control mechanisms can readily undergo cell death ex vivo. There is also considerable patient-to-patient heterogeneity in the rate of CLL cell apoptosis ex vivo, suggesting that intrinsic susceptibility to apoptosis may differ. It will be difficult to establish the relative contribution of cell autonomous and environment-derived survival signals for apoptosis dysregulation in the CLL cell, without identifying the elusive normal B-cell counterpart. Even then, comparisons would be difficult, as the CLL cell may be nudged towards apoptosis by oncogene activation/genomic damage. Although environment-derived signals are important, it may be a combination of cell autonomous and exogenous survival signals that underlie defective apoptosis in CLL.

**Bcl-2 and Bax in CLL**

Essentially all CLL cells express high levels of Bcl-2. Expression generally appears to be equivalent to or to exceed that in normal peripheral blood lymphocytes, or even that in cells containing the t(14:18) translocation. The mechanisms that mediate Bcl-2 expression in CLL remain unclear. It has been suggested that a small proportion of patients contain the t(14:18) translocation that is commonly found in FL, although it remains controversial as to whether these cases are truly CLL. In the majority of individuals, the promoter region for Bcl-2 is hypomethylated, which may contribute to increased transcription and Bcl-2 protein expression in CLL. In FL, the t(14:18) translocation provides strong evidence that activation of Bcl-2 is a tumour-associated event. However, the absence of an analogous genetic alteration in the majority of CLL means that it is unclear whether the abundant expression of Bcl-2 in CLL is acquired during leukaemogenesis or reflects the origin of CLL from B cells that normally express Bcl-2.

Despite this unanswered question, the expression of Bcl-2 and Bax and, perhaps more significantly, the relative expression of these functional antagonists, is an important variable in CLL. CLL cells can have increased Bcl-2/Bax ratios (favouring cell survival) compared to normal controls in at least some individuals. Individual variation in the expression of Bcl-2/Bax correlates with apoptosis and clinical outcome. For example, decreased Bcl-2/Bax ratios are associated with increased sensitivity to cytotoxic drugs in vitro and improved responses to chemotherapy in patients. The relevance of these differences for
apoptosis control is demonstrated by experiments using antisense oligodeoxynucleotides (AS-ODN). Those aimed specifically to interfere with the expression of Bcl-2, enhanced spontaneous apoptosis and sensitivity to chlorambucil.\textsuperscript{44,45} By contrast, Bax AS-ODN delayed spontaneous apoptosis.\textsuperscript{46} However, other proteins are also likely to contribute to apoptosis control in CLL. For example, the expression of other Bcl-2 family proteins, such as Mcl-1 (see below) and unrelated survival proteins (Bag-1), may also correlate with response to cytotoxic therapy in patients.\textsuperscript{42,47} Notably, variation in susceptibility to apoptosis and in the expression of the regulatory proteins, Bcl-2, Bax and Mcl-1, do not appear to correlate consistently with stage.\textsuperscript{42,47} Correlations with disease subtypes identified by the presence of VH mutations have not been reported.

Do tumour-associated events also underlie variation in the expression of Bax in CLL? Bax mutations have been described in malignant B cells, leading to a lack of expression or functional inactivation of the protein.\textsuperscript{48–50} In some cases, these are clearly associated with microsatellite instability [where single base insertions or deletions in a G(8) tract within the Bax open-reading frame result in a frameshift mutation and premature termination of translation], but the absence of significant microsatellite instability in CLL and direct sequence analysis does not support the idea that mutations of the coding sequence alter the function/expression of Bax in CLL.\textsuperscript{51}

Sequence alterations in the Bax promoter may contribute to decreased expression in some cases of CLL. In a study of 34 patients, Saxena et al. described a G-to-A polymorphism in the Bax 5′ untranslated region, at position −248 relative to the translation start site and 146 nucleotides downstream from the TATA box.\textsuperscript{51} A heterozygous polymorphism was present in 69% of stage I–IV CLL, but in only 6% of stage 0 CLL and 4% of controls. The polymorphism was homozygous in RL lymphoma cells, which did not express Bax protein. In CLL, the presence of the polymorphism was associated with decreased Bax expression (\(P = 0.05\), progression beyond stage 0 (\(P = 0.0002\)) and failure to achieve complete remission (\(P = 0.038\)).\textsuperscript{51} Although of significant interest, further work is required to determine the frequency of the polymorphism in larger cohorts of CLL patients and normal individuals and to determine directly whether the G-to-A change directly modulates Bax expression, e.g. via modulating RNA stability or promoter activity. Given the familial component to CLL it would be interesting to determine the frequency of the polymorphism in CLL twins and families.

**Mcl-1: new kid on the block**

More recent analyses have revealed an important role for another Bcl-2 family protein, Mcl-1, in CLL. Like Bcl-2, Mcl-1 is a survival protein, but one with distinct features. It was first discovered in differentiating myeloid cells where it is thought to play a transient role in promoting cell survival (reviewed in refs 52,53), but has since been shown to be expressed in various tissues and malignant cells, including CLL, where its expression is significantly associated with a failure to achieve complete remission following cytotoxic therapy.\textsuperscript{42,47} Overexpression of Mcl-1 in transgenic mice predisposes to lymphomagenesis, and antisense ablation experiments have demonstrated that Mcl-1 is required for the survival of multiple myeloma and lymphoma cells.\textsuperscript{52–54} Interestingly, the incidence of tumours is higher in mice expressing an Mcl-1 transgene than in those expressing Bcl-2, and the tumours that develop more closely resemble lymphomas. Mcl-1 is an essential gene in the mouse, and deletion results in very early preimplantation lethality.\textsuperscript{55} Experiments with chimeric mice demonstrate that Mcl-1 is also essential for B- and T-cell development.\textsuperscript{56}

One of the remarkable features of Mcl-1 is its rapid regulation, and Mcl-1 is thought to play a critical role in regulating apoptosis in response to rapidly changing environmental cues.\textsuperscript{52,53} For example, Mcl-1 expression is rapidly induced via control of transcription and protein stability by various growth factors and survival signals. Compared to other Bcl-2 family survival proteins, Mcl-1 is structurally distinct, containing PEST sequences in its amino-terminal regions. Mcl-1 protein has a rapid turnover, and is targeted for degradation by the proteasome through ubiquitination. Mcl-1 typically has a half-life of a few hours, a common feature of highly modulated proteins. Although PEST sequences are often important determinants of degradation, the role of the Mcl-1 PEST sequences in determining rapid protein turnover remains unclear.\textsuperscript{57}

Mcl-1 can also be negatively controlled, down-regulated through control of transcription, translation and stability in response to a wide range of pro-apoptotic signals. Early down-regulation of Mcl-1 may be essential for initiation of the apoptotic cascade in response to DNA damage, in at least some systems.\textsuperscript{58,59}

Although the rapid turnover of Mcl-1 in healthy cells is largely caused by proteasome-mediated degradation, other proteolytic fates await Mcl-1. During apoptosis, Mcl-1 is a very efficient substrate for caspses.\textsuperscript{53,60–62} Caspase cleavage of Mcl-1 simultaneously inactivates the survival function of this protein and converts Mcl-1 into a cell death-promoting molecule, activating a positive feedback loop that results in increased caspase activation.\textsuperscript{53} Therefore, Mcl-1 acts as a molecular switch during apoptosis, converted from a molecular bodyguard to assassin, by proteolytic cleavage. This mechanism has also been described for Bcl-2 and Bcl-X\textsubscript{L}, but it appears that Mcl-1 is a particularly efficient substrate for caspses. In CLL cells undergoing apoptosis, both caspase cleavage\textsuperscript{53,60} and...
down-regulation of expression, via mechanisms apparently independent of caspase cleavage, have been described, possibly influenced by the nature of the apoptotic signal.

As discussed above, microenvironment signals are thought to play a critical role in CLL. How are microenvironment-derived survival signals communicated to the apoptosis control machinery? A great many signals can promote the survival of CLL cells in culture, including cytokines and 'nurse' and other stroma-like cells. Although it is difficult to know which of these signals might play a predominant role in promoting CLL survival in specific microenvironments within the body, the up-regulation of Mcl-1 appears to play a key role in at least one of these systems. The HK cell line resembles follicular dendritic cells and is able to promote the survival of CLL cells for extended periods of time. HK-promoted survival is mediated, in part, by CD44, and is associated with an increased expression of Mcl-1. Antisense ablation of Mcl-1 reverses the survival mediated by HK cells, confirming the importance of Mcl-1 in this system. However, in other systems, alternate apoptosis control molecules may play a predominant role. CLL-associated microenvironments are rich in T cells, and T-cell-derived survival signals mediated by engagement of the CD40 receptor on CLL cells could provide another relevant pathway of CLL survival in vivo. In contrast to HK cells, CD40 up-regulates the expression of Bcl-XL and also of survivin, an inhibitor of caspases. In complex tissues, multiple microenvironment-derived signals may act on the CLL cells to co-ordinately modulate cell survival.

Finally, the recent identification of small sequence insertions in the Mcl-1 promoter in a subset of CLL has provided further evidence that Mcl-1 can play an important role in CLL. The study of Saxena et al. demonstrated the presence of 6- or 18-base pair (bp) insertions in the Mcl-1 promoter in 17/58 (29%) patients with CLL, but in none of the controls. The insertion site and the sequences of the 6- or 18-bp inserted regions were the same in all affected individuals. Although the effect of the insertions on promoter activity was not tested, the presence of insertions was correlated with elevated Mcl-1 expression at both RNA and protein levels. The presence of insertions was correlated with rapid disease progression (P = 0.012) and, more strongly, with a poor response to chemotherapy (P = 0.001) and shorter disease-specific survival (P < 0.001). The insertions were more commonly found in CD38-negative patients, suggesting that they may present a poor prognostic marker in what is otherwise a relatively favourable group.

Conclusions

Although widely considered as a paradigm for a malignancy of failed apoptosis, the mechanisms and significance of resistance to apoptosis in CLL remain poorly defined. In this review, we have focused on the role of Bcl-2 family proteins in apoptosis control in CLL. The presence of high levels of Bcl-2 in the majority of patients, and the genetic alterations of Mcl-1 in a subset, contribute to an emerging picture that these critical control molecules are dysregulated in CLL. However, it is important to consider that other apoptosis regulators, such as p53 and ATM, are also altered in CLL cells.

The first key question is, ‘To what extent does apoptosis resistance in CLL stem from cell autonomous or exogenous survival signals?’. The well-described propensity of CLL cells to undergo rapid apoptosis when removed from the body clearly shows that survival is not ‘hard wired’ in CLL cells and that these cells require specific signals in the body for survival. The impact of a multitude of potential microenvironment-derived signals on CLL cells has been described in detail, but the precise nature of the signals relevant for CLL survival in the body remain unclear. It is probable that multiple environmental factors and target genes contribute to apoptosis control in CLL cells.

In contrast to FL, where the recurrent t(14:18) translocation provides direct evidence for tumour-associated dysregulation of apoptosis, it is unclear whether the high-level expression of Bcl-2 in CLL is acquired during tumorigenesis or whether it reflects abundant Bcl-2 expression in the cell(s) of origin of CLL. However, as there is now evidence for genetic alteration of apoptosis control molecules in at least some CLL (p53, ATM, Bax and Mcl-1 promoter alterations), it seems probable that the intrinsic apoptosis sensitivity is altered in at least some CLL, and this may contribute to patient-to-patient heterogeneity.

Resistance to apoptosis can contribute to the development of cancer and determine the response to therapy. A second key question is therefore, ‘To what extent does variation in apoptosis sensitivity impact on the clinical behaviour of CLL?’. Although CLL exists as at least two distinct subsets of disease (Fig. 1), there is no compelling evidence that consistent differences in apoptosis regulation underlie the variable clinical course associated with these subtypes. By contrast, differences in BCR signalling capacity do appear to correlate. We favour a model where an underlying resistance to apoptosis, potentially driven by both environmental and/or cell autonomous events, contributes to all CLL, providing a foundation on which different (super)antigen-driven events (such as anergy) make a significant contribution to variation in the clinical course. However, interpatient variability in the expression of Bcl-2 family proteins has been demonstrated in CLL. Although identified in some studies, these variables are not consistently associated with disease stage, suggesting that they are not important determinants of clinical progression, and direct correlations with VH status have not
been reported. By contrast, these changes are relatively strongly correlated with differences in response to cytotoxic therapy. For example, differences in expression of the Bcl-2/Bax ratio, Mcl-1 expression and the presence of potentially activating insertions in the Mcl-1 promoter, all correlate with response to therapy.41,42,47,68 Therefore, we suggest that a degree of apoptosis resistance provides a stage on which antigen/BCR-mediated events modulate the clinical course of the disease. However, once the disease progresses and therapy is initiated, individual differences in apoptosis susceptibility, mediated in part by Bcl-2 family proteins but also influenced by deficits in p53 and ATM, may play a significant role in determining response to therapy.

The expression and functional role of Bcl-2 and related survival proteins in CLL cells suggests that targeting these molecules has therapeutic potential. Indeed, results of antisense ablation of Bcl-2 in preclinical experiments have provided some encouragement and trials are ongoing. However, delivery and specificity remain problematic. A new generation of small-molecule inhibitors of Bcl-2-related proteins, the so-called BH3-mimetics, which can neutralize survival proteins by mimicking the binding of proapoptotic BH3-only proteins, may offer a new avenue.69 Fine tuning of the structures of these molecules may permit the inhibition of specific Bcl-2 proteins. Their potential activity in CLL must be weighed against the risk of toxicity; for example, deletion of Mcl-1 is lethal in mice. Regardless, they will be exciting agents to assess in clinical trials and valuable chemical tools for using to further investigate the function of Bcl-2 family proteins in CLL.

Acknowledgements

We wish to thank Kathy Potter, Ian Mockridge, Myra Armstrong, Louise Neville, Christian Ottensmeier, Andrew Duncombe, Debbie Richardson and Becky Pickering for invaluable contributions. Work on CLL in the Stevenson and Packham laboratories is funded by Cancer Research UK, Tenovus and the Leukaemia Research Fund. We apologise for not being able to cite all primary literature; this is because of space constraints.

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