Thirty years of BCL2 – translating cell death discoveries into novel cancer therapies

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Abstract (100 words max)

The “hallmarks of cancer” are generally accepted as a set of genetic and epigenetic alterations a normal cell must sustain to transform into a fully malignant cancer. It follows that therapies designed to counter these alterations might be effective as anti-cancer strategies. Over the past 30 years research on the BCL2 regulated apoptotic pathway led to the development of small molecule compounds, known as ‘BH3 mimetics’ that bind to pro-survival BCL2 proteins to directly activate apoptosis of malignant cells. This Timeline focuses on the discovery and study of BCL2, the wider BCL2 protein family, and specifically their roles in cancer development and cancer therapy.

Introduction:

In the last 30 years cell death has become a major field of investigation building to a crescendo with the recent award of ‘Breakthrough Therapy Designation’ from the FDA to ABT-199/venetoclax, a selective inhibitor of BCL2, in recognition of its promise as a treatment for patients with chemo-resistant chronic lymphocytic leukaemia (CLL) (http://abbvie.mediaroom.com/2015-05-06).

Although research on cell death extends for over 150 years, until the late 1980’s it remained an esoteric subject. Today, however, it is a major research field, with over 20,000 new publications on apoptosis or programmed cell death appearing each year. The explosion in interest was sparked by research on one protein, BCL2, as experiments on BCL2 showed that mechanisms for cell death are highly conserved throughout the evolution of animals, and because chromosomal translocations that activate the *BCL2* gene are associated with malignant disease in humans. Thus, the identification of BCL2 as an inhibitor of cell death marked recognition of the first component of a cell death mechanism in any organism, and established a new hallmark of cancer – evasion of cell death (apoptosis).

Over the past three decades, research in hundreds of laboratories has identified and characterised at least 16 members of the BCL2 protein family, and categorised them into three functional groups that each bear one or more BCL2 homology (BH) domains (Figure 3). These are the pro-survival BCL2 family members (including BCL2 itself), the multi-BH domain pro-apoptotic members (such as BAX and BAK), and the pro-apoptotic “BH3-only” proteins (such as BIM and PUMA). Many of the upstream pathways that control these

proteins have been elucidated, as well as the effector processes triggered by their activation that are the ultimate cause of cell demolition. Reviews that provide detailed in-depth discussion of BCL2 regulated apoptosis signalling at a molecular level are available [1-7]. While non-apoptotic roles for BCL2 family members have been proposed, their importance in normal physiology and cancer remain unclear, and are beyond the scope of this article (for a review on these topics see [8]). This Timeline will focus on key advances in our understanding of the function of the BCL2 protein family in cell death, the development of cancer, and as targets in cancer therapy.

Early studies on apoptosis and its detection in cancerous cells

In their 1972 paper that adopted the word "apoptosis" to describe a physiological process of cellular suicide, John Kerr and colleagues recognised the presence of apoptotic cells in tissue sections of certain human cancers [9]. Accordingly, they proposed that increasing the rate of apoptosis of neoplastic cells relative to their rate of production could be therapeutic. However, interest in cell death, and its role in cancer, languished until the late 1980's, when genetic abnormalities that prevent cell death were directly linked to malignancy in humans.

Until the early 1980's most oncogenes were discovered as genes carried by transforming retroviruses (e.g. *v-Myc, v-Src*, *v-Abl*), genes located at recurrent chromosomal translocation breakpoints (e.g. *BCR-ABL, c-MYC*), or genes that could transfer oncogenic properties from malignant cells to non-malignant ones (e.g. mutant *RAS*) [10]. While the normal counterparts of these oncogenes promoted cell proliferation in a controlled manner, when they were dysregulated in cancers, they caused uncontrolled cell growth and proliferation.

***BCL2*: a novel class of oncogene**

The discovery of BCL2 started with the association of t[14;18] chromosomal translocations with human follicular lymphoma by Janet Rowley and colleagues [11]. This led to the cloning of the chromosomal breakpoint and subsequently the cDNA, which was termed *BCL2* for B cell leukaemia/lymphoma gene number 2 [12-16].

The strong association of translocations involving *BCL2* with follicular lymphoma suggested it was an oncogene, but did not provide proof, and the amino acid sequence did not provide clues to its function. Because expression of several then known oncogenes, including SV40 large T, *v-abl* and *v-fms*, allowed IL-3 dependent mouse myeloid FDC-P1 cells to grow in the absence of cytokine, and to form tumours in mice (for example [17, 18]), a *BCL2* expression construct was transduced into these cells and they were cultured without cytokine. Although the BCL2 expressing cells did not grow or proliferate in the absence of IL-3, unlike the parental cells, they failed to die when growth factor was removed, and when it was restored (even after weeks), they began to divide once more [19].

These experiments revealed that BCL2 did not affect cell proliferation, but promoted cell survival by preventing the death of cells cultured without growth factor. Stan Korsmeyer and colleagues confirmed these findings by showing that a *BCL2* transgene conferred a 'death-sparing capacity' to primary B lymphocytes in culture [20]. Subsequent studies from numerous groups confirmed that overexpression of BCL2 was able to block apoptosis in cell lines and primary cells in transgenic mice [21-27].

Although it was originally reported to reside on the cytosolic face of intracellular membranes [28, 29], subsequent papers suggested BCL2 is localized to the inner mitochondrial membrane [24], or the plasma membrane [30]. This was resolved by Monaghan et al [31], Jacobson et al [32] and Lithgow et al [33] who showed that the first reports were correct, and that BCL2 resides on the outer mitochondrial membrane, the endoplasmic reticular membrane, and the nuclear envelope.

Collectively, these findings revealed that BCL2 was unlike other oncogenes known at the time, as it did not stimulate cell growth or proliferation, but promoted tumorigenesis by allowing cells that would normally undergo programmed cell death to survive. This abnormal cell survival facilitated acquisition of additional oncogenic lesions to drive neoplastic transformation [34-36]. BCL2 thus became the first component of the cell death machinery to be cloned and recognised, and it became the archetype of a new class of oncogenes, the inhibitors of cell death.

Although over-expression of BCL2 allowed growth factor dependent cell lines (e.g. FDC-P1) to survive in the absence of cytokine, when they were injected into mice, they did not

form tumours, suggesting that inhibition of cell death alone was not sufficient to render a cell fully transformed. In 1983, Land *et al*. had shown that tumorigenic transformation of fibroblasts required not only expression of a *RAS* oncogene, but also *c-MYC* [37]. Furthermore, Croce and Nowell's group had observed the transformation of a follicular lymphoma bearing a *BCL2* translocation into acute pre-B cell leukaemia after acquiring a second chromosomal translocation involving *c-MYC* [38]. To test whether BCL2 could synergise with c-MYC in neoplastic transformation, a retroviral vector bearing *BCL2* was introduced into bone marrow cells from pre-leukaemic *Eμ-Myc* transgenic mice. Cells expressing both BCL2 and c-MYC, but not those expressing either oncogene alone, gave rise to immortalised cell lines *in vitro* that caused lymphoma when transplanted into irradiated mice [19]. This synergy between inhibition of cell death and dysregulated cell proliferation in tumorigenesis was confirmed *in vivo* by generating *Eμ-Bcl2/Eμ-Myc* bi-transgenic mice, which rapidly succumbed to highly aggressive lymphoma [34]. This synergy was explained when it was shown that cells respond to over-expression of c-MYC by undergoing apoptosis by a mechanism that BCL2 can block [39-41].

Although some early studies using over-expression in cell lines suggested that BCL2 might promote cell growth and proliferation [21, 42], investigations of transgenic mice over-expressing BCL2 in B cells, T cells or both demonstrated beyond doubt that BCL2 specifically inhibits cell death and does not promote proliferation [20, 26, 27, 43]. Furthermore, studies with these transgenic mice confirmed *in vitro* studies [23] showing that BCL2 not only inhibited apoptosis due to deprivation of growth factors**,** but protected cells from a broad range of cytotoxic stimuli, including diverse anti-cancer drugs [20, 25-27]. Studies such as these showed that BCL2 acts at the convergence of several upstream apoptosis inducing signalling pathways. Nevertheless, it soon became apparent that BCL2 did not control all types of cell death. For example, BCL2 did not prevent the killing of cells targeted by cytotoxic T cells (mediated by perforin and granzymes) [44], and it did not inhibit apoptosis of primary lymphocytes triggered by ligation of the 'death receptor' FAS [45], even though in other cell types, including certain tumours, BCL2 is able to block FAS induced apoptosis [46, 47] (see below for further discussion).

Evolutionary conservation of cell death mechanisms.

Until BCL2 was recognised to be an inhibitor of cell death, little was known of the mechanism of apoptosis in mammalian cells, but research on programmed cell death in

invertebrates was progressing rapidly, largely through the power of genetics in the nematode *C. elegans*. Sulston, Horvitz and co-workers had shown that the fate of 131 of the 1090 somatic cells formed during development is to undergo programmed cell death [48]. Moreover, by classical forward genetic approaches they had shown that around a dozen genes were needed for operation of this process [49] and, by performing crosses, they could demonstrate that these genes acted in a hierarchy. Many appeared to be specific for cell death and had no other role, but their full characterisation awaited cloning of the genes.

In 1992, Vaux, Weissman, and Kim described the effects of expressing human *BCL2* in *C. elegans* [50]. In worms that expressed *BCL2*, the number of developmentally programmed cell deaths was markedly reduced. This meant that the human BCL2 protein was able to engage with the worm's cell death machinery, implying that the processes of apoptosis (of mammalian cells) and programmed cell death (in *C. elegans*) were implemented by the same molecular mechanism, one that had been conserved for over 500 million years of evolution. The effect of human BCL2 expression most closely resembled that of a gain-of-function mutation to a *C. elegans* gene termed *ced-9*, and suggested they were functionally homologous. The subsequent cloning and sequencing of the *ced-9* gene proved that this was indeed the case [51]. Furthermore, as *ced-9* was known to act upstream of *ced-3* and *ced-4,* it seemed likely that BCL2 would somehow act to negatively control the products of the mammalian homologues of these genes. Cloning of *ced-3* showed that it encoded a latent cysteine protease, that when activated, caused programmed cell death [52], which implied that BCL2 would act like CED-9 to prevent activation of caspases. While this is indeed the case, it is important to note that there are major differences in the regulation of cell death between mammals and nematodes. In the worm, the BCL2 homologue CED-9 directly inhibits the APAF-1 homologue CED-4 (the activator of the caspase, CED3), and mitochondrial factors are not required for cell killing, whereas in mammals BCL2 acts by inhibiting BAX and BAK, for which there are no homologues in *C. elegans*. BAX and BAK promote cell death by forming pores in the outer mitochondrial membrane (see below) [53].

In the mammalian context the high degree of conservation of protein sequence and function between orthologues allowed for rapid progress due the reproducibility of findings in experiments using human and mouse cells. In most cases, especially for the most

important BCL2 family members, mouse and human orthologues can be used interchangeably.

The BCL2 family expands: three classes of interacting proteins

When they cloned BCL2, Cleary *et al.* noticed that it resembled BHRF1, a product of Epstein Barr virus [15], and since then, many further viruses have been found to carry *BCL2*-like genes [54]. Importantly, since some of these viruses are implicated in cancer, it is possible that their BCL2-related proteins contribute to tumorigenesis and may thus constitute therapeutic targets (see below). The first non-viral pro-survival *BCL2*-like genes to be identified were *MCL1, Bclx* and *A1. MCL1* was identified in 1993 in a screen for genes induced by phorbol acetate in myeloid leukaemia cells [55]. In the same year, the *Bclx* gene, which encodes the pro-survival BCLXL protein, as well as the rarely detected shorter splice variant, BCLXS, was discovered by low stringency hybridisation, initially in chickens, and then mammals [56]. A1/BFL1 was identified as a gene induced by GMCSF in myeloid cells [57], BCLW joined the pro-survival BCL2 family members when it was cloned in 1996 [58], and DIVA/BOO (the human homologue is called BCLB) was cloned in 1998 [59].

Stan Korsmeyer and colleagues identified BAX as a protein that co-immunoprecipitated with BCL2, and found that the two proteins shared similar amino acid sequences within the so-called BCL2 Homology (BH) domains [60]. Surprisingly, BAX was found to promote, rather than inhibit apoptosis when over-expressed [60]. This was the first discovery that the BCL2 family contains both pro-survival and pro-apoptotic members, and that they regulate cell death by physically interacting with each other. The other multi BH-domain pro-apoptotic BCL2 family member, BAK, was cloned in 1995 [61, 62] and the highly related BOK (the function of which is still unclear [63]) was identified in 1997 [64].

BAD was discovered as a BCL2 binding protein in 1995 using the yeast two-hybrid system and lambda phage expression screening [65]. Despite initial controversy about the number and type of BH domains shared by BAD with BCL2, subsequent analysis showed that it has just a BH3 domain. BAD and BIK (also described in 1995 [66]) thus became the prototypic members of a novel subclass of the BCL2 family that are now called the BH3-only proteins. Korsmeyer's lab also described BID, which binds to both BCL2 and BAX, providing the first example of direct activation of pro-apoptotic BAX by a BH3-only protein [67] (Figure 2). Mutations to the BH3 domain of BID abrogated its pro-apoptotic function as

well as its ability to interact with BCL2 and BAX. Later biochemical and genetic studies revealed that all BH3-only proteins can bind to pro-survival BCL2 family members, thereby freeing BAX or BAK activating them indirectly, and some (e.g. tBID, BIM, PUMA) can also directly bind to and activate BAX and BAK [68-71].

Other BH3-only proteins include HRK [72], BIM [73], BMF [74], and the p53 target genes *NOXA* [75] and *PUMA/BBC3* [76, 77]. Several other proteins (e.g. BNIP3, NIX) have been found to have sequences that resemble BH3 domains, but their ability to bind and regulate pro-survival BCL2 proteins or pro-apoptotic BAX/BAK has not been established, and some clearly function in non-apoptotic processes [78, 79]. These and other proteins containing certain BH domains (e.g. BCLG, BFK) are not implicated in cancer and are therefore not further discussed here.

Regulation of apoptosis by the BCL2 family

The generation of knockout mouse strains, starting in the early 1990's, revealed the individual and overlapping functions of BCL2 family members and the consequences of disrupted regulation of apoptosis. *Bcl2*$^{-/-}$ mice completed embryonic development but succumbed to polycystic kidney disease early in life [80-82]. Elevated rates of apoptosis were evident in the lymphoid organs, which were reduced in size, and the mice became prematurely grey due to loss of melanocytes. These defects could all be rescued by concomitant loss of pro-apoptotic BIM [83].

Bclx-deficient mice died around embryonic day 14/15 due to increased apoptosis of erythroid and neuronal cells [84].

Complete loss of *Mcl1* caused embryonic lethality prior to blastocyst implantation [85] and studies with conditional knockout strains revealed that MCL1 is critical for the survival of many cell types, including cardiomyocytes [86, 87], neurons [88], haematopietic stem/progenitor cells [89, 90] and immature as well as mature lymphoid cell subsets [91-93].

Mice lacking the BH3-only protein BIM had increased numbers of lymphocytes, which were resistant to diverse apoptotic stimuli, including cytokine deprivation, abnormal calcium flux and ER stress [94, 95]. Many ageing *Bim*$^{-/-}$ mice developed a fatal SLE-like autoimmune disease with severe glomerulonephritis and auto-antibodies against a range

of self-antigens [94], reminiscent of the pathologies seen in *BCL2* transgenic mice several years earlier [43]. Moreover, some aged mice lacking both BIM and PUMA presented with lymphoid neoplasms [96], demonstrating the overlapping tumour suppressive function of these BH3-only proteins. Loss of BIM in *Eμ-MYC* transgenic mice accelerated lymphoma development beyond all other deficiencies for single-BH3 only proteins in this model [97]. Notably, loss [98] or silencing [99] of the *BIM* gene is frequently found in human cancers, such as mantle cell lymphoma or renal carcinoma.

BID-deficient mice were normal in the absence of stress, but their hepatocytes were resistant to FAS-induced apoptosis [100]. This, together with previous biochemical investigations [101, 102] and later genetic studies [103], revealed that in so-called type 2 cells (e.g. hepatocytes, pancreatic β cells), but not in type 1 cells (e.g. lymphocytes), 'death receptor' (e.g. FAS)-induced apoptosis signalling requires activation of the BCL2-regulated pathway for efficient apoptotic cell death. This cross talk between the BCL2-regulated (intrinsic, mitochondrial) and death receptor apoptotic pathways is achieved by caspase-8 mediated cleavage and activation of BID, which can then both activate BAX and BAK directly and also neutralise BCL2-like pro-survival proteins [101, 102].

In 2000, mice were generated that were deficient for both BAX and BAK [104]. Remarkably, although mice lacking either BAX and BAK alone have only minor abnormalities (most notably a defect in spermatogenesis in *Bax*$^{-/-}$ males [105]), the BAX/BAK doubly deficient animals exhibited developmental abnormalities, including persistence of inter-digital webs, imperforate vagina and excess neuronal cells in certain areas of the brain [104]. Although most BAX/BAK double deficient mice died soon after birth, the few surviving animals developed lymphadenopathy, SLE-like autoimmune disease and lymphoid neoplasms when aged [106, 107].

Cells from the BAX/BAK double knockout mice proved to be resistant to many apoptotic stimuli, including enforced expression of BH3-only proteins [104, 108, 109], demonstrating that BAX and BAK have essential (and largely overlapping) roles in unleashing the effector phase of mitochondrial apoptosis. Perhaps even more surprisingly, because some BAX/BAK double mutants survived into adulthood, these experiments show there is no absolute requirement for BAX/BAK-dependent apoptosis during embryonic development of the mouse.

In 1994, Newmeyer et al. used a cell free system to show a mitochondrial component was required for the induction of apoptosis [110]. Xiaodong Wang and colleagues showed that during apoptosis, cytochrome c was released from the mitochondria, and could promote caspase activation *in vitro* [111]. Furthermore, BCL2 could prevent release of cytochrome c [112, 113]. How BCL2 achieved this was revealed by the discoveries that BAX moves from the cytosol to the mitochondria during apoptosis [114], and once there can oligomerise to form channels that allow release of cytochrome c [115].

While the release of cytochrome c was necessary for APAF-1-mediated activation of caspase-9 and downstream effector caspases, it is important to note that activation of BAX and BAK is usually sufficient to cause the death of the cell, even those that lack APAF-1 or caspase-9 [116, 117].

Structural analysis of single proteins [118, 119] and complexes, such as BIM bound to BCLXL [120], revealed how the different members of the BCL2 family interact at the molecular level and how BAX and BAK must change shape to cause mitochondrial outer membrane permeabilisation (MOMP) to cause apoptosis [121, 122]. These interactions mostly take place on or within intra-cellular (e.g. mitochondrial) membranes, and innovative experiments using fluorescence resonance energy transfer (FRET) helped clarify the topology of BCL2 protein family members on membranes, leading to the 'embedded together' model [123].

Genetic and epigenetic deregulation of the BCL2 family in cancer

In addition to its activation by the t[14;18] chromosomal translocation in follicular lymphoma, amplification of the *BCL2* gene has been identified in some cases of diffuse large B cell lymphoma [124]. Furthermore, most cases of chronic lymphocytic leukaemia (CLL) overexpress BCL2 because they have deleted or silenced the mir-15a and/or mir-16.1 micro-RNAs that normally suppress BCL2 expression [125].

Somatically acquired copy number amplifications (SCNA) of *BCLX* and *MCL1* and loss of *BOK* have been detected in a substantial fraction of human cancers [126]. Moreover, whole genome mRNA expression analyses and Western blotting have revealed that a multitude of human cancers present with elevated levels of BCLXL. This is thought to enhance chemo-resistance, for example in subsets of breast cancer [127], neuroblastoma [128], colorectal cancer [129], gastric adenoma/carcinoma [130], hepatocellular carcinoma [131] and

prostate cancer [132]. In a broad range of cancers high levels of BCLXL or MCL1 have been ascribed to the loss or silencing of microRNAs that normally attenuate their expression, such as let7 to target *BCLX* [133] or miR-29, miR-125 and miR-193 to target *MCL1* [134, 135].

Mutations predicted to compromise the pro-apoptotic members of the family have also been observed in human cancer. Homozygous deletion of the *BIM* gene has been found in ~20% of cases of human mantle cell lymphoma [98]. In addition, epigenetic silencing of the *BIM* or *PUMA* genes was reported in several cancers, including renal cell carcinoma and Burkitt Lymphoma [99, 136]. Moreover, frameshift mutations in the *BAX* gene were found in colon cancers with a hyper-mutation phenotype [137] and loss of function mutations in *BAX* were detected in haematopoietic cancers [138].

Role of BH3-only proteins in the response of cancer cells to conventional anti-cancer therapies

The role of the BCL2-regulated apoptotic pathway in the response to anti-cancer therapeutics was first recognised when *Bcl2* vector transfected cell lines or primary lymphoid cells from *BCL2* transgenic mice were found to be profoundly resistant to a broad range of cytotoxic insults, including γ-radiation and several chemotherapeutic drugs (e.g. etoposide, dexamethasone) [23, 26, 27, 139]. Studies with gene targeted mice revealed that combined loss of BAX and BAK rendered cells profoundly resistant to a broad range anti-cancer therapeutics [104], and identified the BH3-only proteins that were necessary for initiation of apoptosis. PUMA (and to a lesser extent NOXA) are required for the killing of normal as well as cancerous cells by therapeutic concentrations of DNA damage inducing drugs (e.g. etoposide, cyclophosphamide) that act at least in part through p53 [140-144]. BIM also plays a role in DNA damage induced cell killing (indirectly activated by p53 and possibly also through a p53-independent pathway), as well as apoptosis induced by taxol, histone deacetylase inhibitors and glucocorticoids (the latter in a manner overlapping with PUMA) [94, 142].

BIM is necessary for the killing of diverse cancer cells by inhibitors of oncogenic kinases, such as treatment of chronic myeloid leukaemia (CML) cells with the BCR-ABL inhibitor imatinib [145, 146], treatment of lung cancer cells with EGF receptor inhibitors gefitinib or erlotinib [147-149] and treatment of BRAF mutant melanoma or colon carcinoma cells with inhibitors of MEK or BRAF [150, 151]. A polymorphism in the *BIM* gene that affects splicing

and is found in certain Asian populations was reported to diminish the therapeutic responses of CML and lung cancers to imatinib or gefitinib/erlotinib, respectively [152].

These experiments showed that at clinically achievable doses, many chemotherapeutic agents do not kill cells directly, but cause changes that are detected by the cells which respond by killing themselves as a stress response. Overall, cancer cells are genetically unstable and hence more fragile than normal cells, which has led to the concept of cancer cells being 'primed for death' [153, 154]. On one hand, tumour cells that express high levels of cell death inhibitors, such as BCL2 or MCL1, will be chemo-resistant, but on the other, drugs that target cell survival molecules, such as BCL2, might increase the sensitivity of such tumour cells to chemotherapy.

Development of BH3 mimetic drugs for cancer therapy

As it became clear BCL2-like proteins promoted the survival of tumour cells, it was apparent that drugs that inhibited these proteins might be useful therapeutically. Perhaps due to the historical challenges associated with drugging protein-protein interactions, early drug development programs focussed on inhibiting BCL2 expression through the use of antisense oligonucleotides [155]. Despite initial promise, subsequent studies have revealed that much of the activity of these compounds likely derives from their ability to induce interferon rather than via their ability to repress BCL2 expression [156].

The 3D structure of BCLXL, both on its own [157], and in complex with a BH3 peptide from BAK [118] led to a different approach. Steve Fesik and co-workers developed compounds to mimic the function of BH3-only proteins (BH3 mimetics) with the goal of bypassing the block in apoptosis signalling that exists in many tumour cells, for example due to mutations in *p53*. ABT-737, which inhibits BCL2, BCLXL and BCLW, was the 'first-in-class' of such compounds. *In vitro* and *in vivo*, ABT-737 killed certain cancer cell lines as a single agent, and could kill further cancer lines when it was combined with standard chemotherapeutic drugs [158]. Studies using cells from gene-targeted mice confirmed that ABT-737 and its clinically used successor ABT-263/navitoclax [159] kill through a BAX/BAK-dependent (i.e. on-target) manner [160, 161].

In clinical trials, ABT-263 as single agent caused significant reduction in tumour burden in most CLL patients [162, 163], and in pre-clinical studies in combination with other conventional

treatments it showed efficacy in several additional cancers, including certain types of breast cancer [164-167]. However, since BCLXL is critical for the survival of platelets [168, 169], BAX/BAK-mediated thrombocytopenia limits the dosing of ABT-263 and BCLXL-selective BH3 mimetics [170] in patients.

For the treatment of cancers that depend on BCL2, the BCL2-specific BH3-mimetic ABT-199/venetoclax was developed. Because it does not target BCLXL, ABT-199 does not reduce platelet lifespan [171], and is therefore better tolerated than ABT-263. This compound has rapidly progressed into phase III clinical trials for the treatment of patients with relapsed or refractory lymphoid malignancies (particularly CLL), and is also being investigated in combination with other anti-cancer therapies, such as anti-CD20 monoclonal antibodies (e.g. rituximab, ofatumumab, obinutuzumab), to reduce tumour burden [172]. These trials are supported by pre-clinical studies in tissue culture and with transplanted tumours in mice, which showed that ABT-199 augments the killing of haematological [171, 173, 174] as well as breast cancers [175] elicited by conventional chemotherapeutics or targeted inhibitors of oncogenic kinases [176].

Since MCL1 is abnormally over-expressed (e.g. due to SCNA) in many cancers [126] and has been proven to be necessary for the sustained survival and growth of diverse types of tumours [177-181], high affinity MCL1-specific BH3 mimetic compounds are eagerly awaited, both as research tools and drugs. However, caution will have to be taken with the use of MCL1 inhibitors, since this pro-survival BCL2 family member is essential to many normal cell types, including cardiomyocytes and neuronal cells [86-90, 93].

Combinations of BH3 mimetics with standard chemotherapeutics, particularly those inducing DNA damage, are likely to cause substantial side effects, because both drugs will affect not only the malignant but also the normal cells. Combinations of BH3 mimetics with drugs that only (or at least preferentially) affect the cancer cells, such as CD20 antibodies [172] or inhibitors of oncogenic kinases, such as imatinib, gefitinib/erlotibib or vemurafinib [182], provide a promising strategy for the selective killing of cancer cells while minimising bystander killing of normal cells. While for all combinations, the therapeutic window will have to be determined in clinical trials, *in vitro* tests on cancer cells, such as “BH3 profiling” might allow the effectiveness of combining BH3 mimetics and conventional chemotherapeutics to be predicted [153, 154, 183].

Conclusions/perspectives:

Thirty years have passed since the cloning of the t[14;18] chromosomal breakpoint in human follicular lymphoma, and the naming of BCL2 by Tsujimoto et al. [12]. Since then researchers have shown that the role of BCL2 is to inhibit cell death, have identified a large number of cell death regulators, and have uncovered a complex web that regulates apoptosis. Inhibitors of BCL2 and some of its relatives (the BH3 mimetic drugs) are currently showing great promise in clinical trials.

Nevertheless, we face some challenges in the coming years: (1) to bring the BH3 mimetic drugs into clinical practice and identify which other therapeutics they can best be combined with in different types of cancer; (2) develop BH3 mimetic drugs that specifically inhibit MCL1, A1/BFL1 and pathogen-encoded pro-survival BCL2 family members for use in the treatment of cancer, autoimmune as well as infectious diseases; (3) attempt to develop BCLXL inhibiting BH3 mimetics for cancer therapy by targeting them selectively to tumour cells (thus sparing platelets and other normal cells); (4) identify the upstream signalling mechanisms that control the expression and function of the different pro-apoptotic BH3-only proteins and the pro-survival BCL2 family members. Some of these regulators may well be promising cancer drug targets in their own right.

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Conflict of interest

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Figure 1:

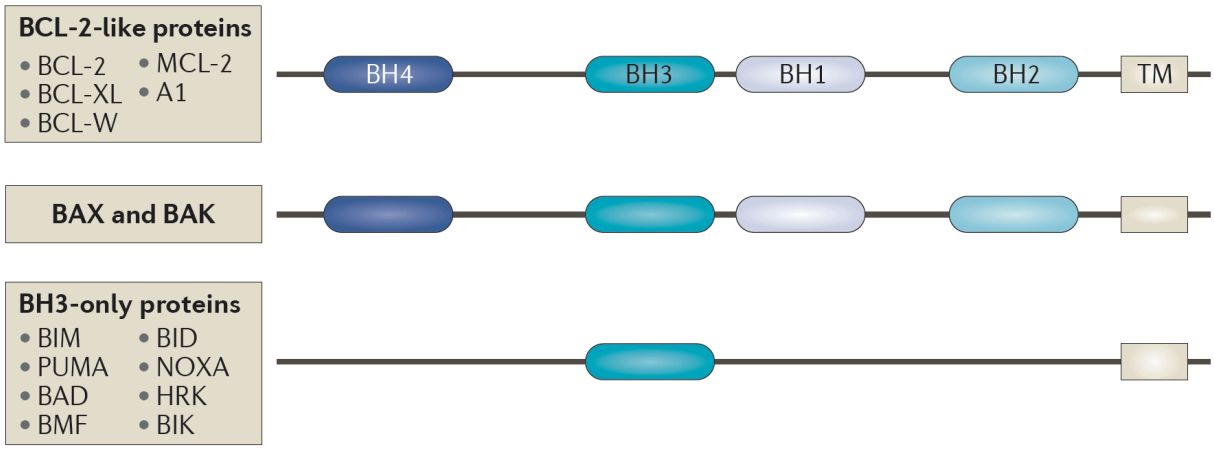
Timeline figure of key discoveries - *ref and year indicated for each*

Year	Event	Reference
Late 1970's	t14;18 translocation identified	[11]
1984	Cloning of *BCL2* gene	[12]
1988	Identification of function, and oncogenic activity of BCL2	[19]
1989	BCL2 transgenic mice	[20]
1989	Bcl2 protects against wide range of cytotoxic stress agents	[23]
1990	Synergy of defects in apoptosis and cell proliferation control in lymphomagenesis demonstrated in *Bcl2/Myc* bi-transgenic mice	[34]
1990	*Bcl2* tg mice develop autoimmune disease	[25]
1991	BCL2 inhibits anti-cancer drug induced cell killing	[26, 27]
1992	Subcellular localisation of Bcl2 resolved	[28, 31]
1992	Not all apoptosis is blocked by BCL2. It does not block CTL mediated target cell killing	[44]
1992	BCL2 is a functional homolog of *C elegans CED9*	[50]
1993	Cloning of *Mcl1*	[55]
1993	Cloning of *A1*	[57]
1993	Cloning of *Bclx*	[56]
1993	Cloning of *Bax*	[60]
1993	BCL2 can block death in cells lacking mitochondrial DNA	[32]
1993	*Bcl2* knockout mice	[80, 82]

1994	BCL2 and CED-9 are sequence homologues	[51]
1994	Requirement for a mitochondrial component in a cell-free system of apoptosis	[110]
1995	Demonstration of two distinct but converging pathways to apoptosis: BCL2-regulated and 'death receptor' pathways	[45]
1995	Cloning of *Bad*	[65]
1996	Cloning of *Bid*, BH3-only protein concept; evidence for direct activation of BAX by a BH3-only protein	[67]
1996	Structure of BCLXL	[157]
1997	Inhibition of BAX channels by BCL2	[115]
1997	Discovery that BCL2 inhibits cytosolic release of cytochrome c with role of cytochrome c in activation of the caspase cascade	[112, 113]
1997	Translocation of BAX to mitochondria	[114]
1998	Discovery of BIM	[73]
1997-8	*BAX* loss of function mutations in haematopoietic cancers and colon cancer with hyper-mutation phenotype	[137, 138]
1999	BIM knockout mice; demonstration that BH3-only proteins are essential for initiation of apoptosis	[94]
2000	MCL1 knockout mice	[85]
2000	Discovery of NOXA	[75]

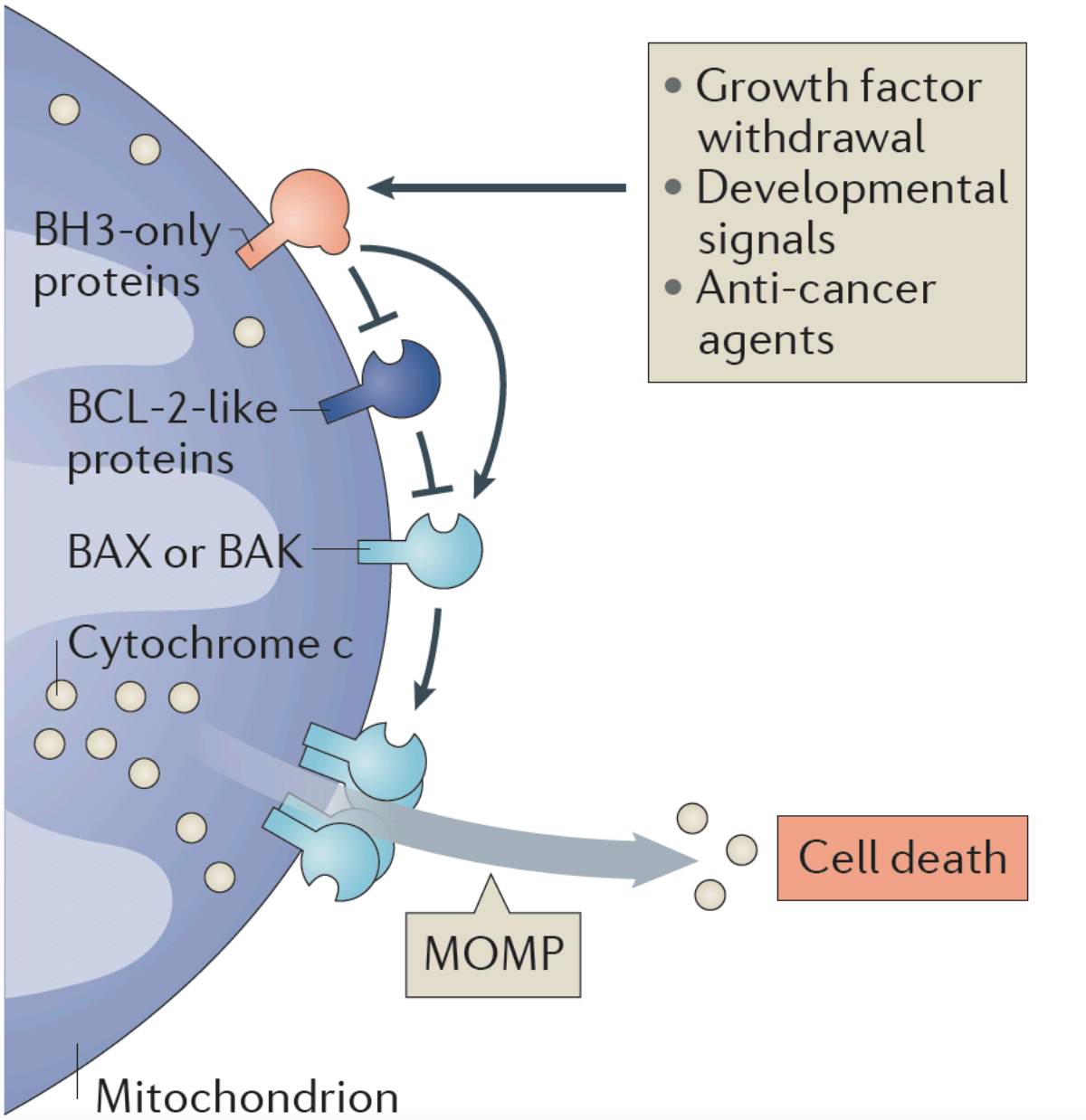
2000	BAX/BAK double knockout mice; demonstration that BAX/BAK have essential overlapping roles in execution of apoptosis	[104]
2001	Loss of BIM rescues developmental defects in BCL2-deficient mice	[83]
2001	Discovery of PUMA/BBC3	[76, 77]
1999-2003	Roles of BH3-only proteins	[94, 140, 141, 184, 185]
2005	different BH3-only proteins have different abilities to bind to different pro-survival BCL2 family members	[69, 71]
2004-2006	inhibitors of oncogenic kinases kill tumour cells by activating BIM and this killing is enhanced by BH3 mimetic compounds	[145, 146]
2005	Development of BH3 mimetics	[158]
2006	Clinical trials with ABT-263	[162]
2007	Discovery that platelets require BCLXL for survival	[168]
2011	Development and clinical trials with ABT-199/venetoclax	[171]
2012	Genetic analysis to determine which pro-survival BCL2 family member is needed for sustained growth of which cancer	[177, 178, 180, 181, 186]
2015	ABT-199 granted breakthrough therapy designation by the US FDA	

Figure 2



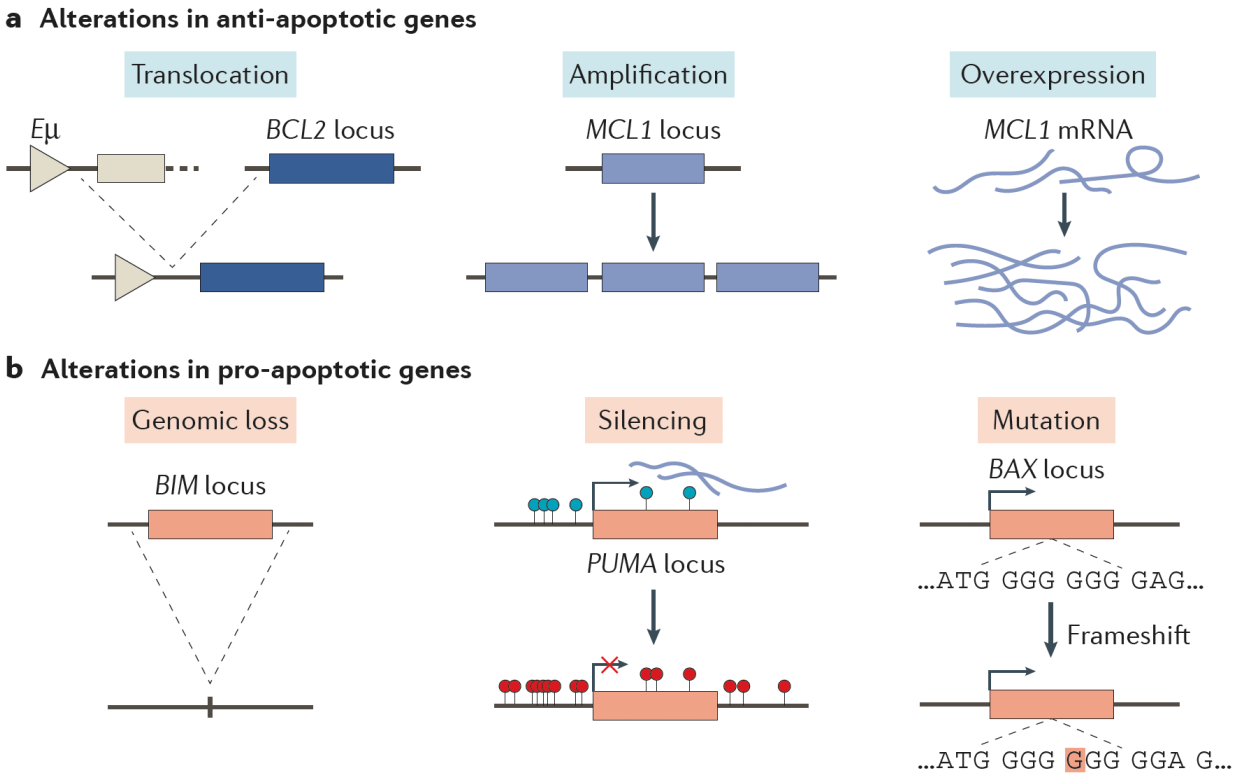
BCL2 regulated apoptosis signalling. Cytotoxic stimuli, such as growth factor deprivation or anti-cancer drugs, activate the expression of the pro-apoptotic BH3-only proteins through transcriptional or post-transcriptional processes. Some BH3-only proteins (e.g. BAD) initiate apoptosis signalling only by binding to pro-survival BCL2 proteins, thereby preventing them form keeping pro-apoptotic BAX and BAK in check. Other BH3-only proteins (e.g. BIM, PUMA) can initiate apoptosis signalling by binding to pro-survival BCL2 proteins (see above for mechanism) and by direct binding and activation of the multi-BH domain pro-apoptotic BAX and BAK proteins. Activated BAX and BAK cause mitochondrial release (MOMP = mitochondrial outer membrane permeabilisation) of apoptogenic factors (e.g. cytochrome c) into the cytoplasm where they promote activation of caspases, the proteases that mediate cell demolition.

Figure 3



BCL2 family: three subsets of interacting family members. Schematic showing the presence of BCL2 Homology (BH) domains, defined solely by sequence similarity to BCL2, that facilitate sub-classification of the BCL2 family into the three major subgroups of proteins. In the case of the BH3-only proteins the BH3 domain acts as a ligand domain to facilitate interaction with the other subgroups. Many pro- as well as anti-apoptotic BCL2 family members also have a trans-membrane (TM) domain to facilitate association with the outer mitochondrial membrane; the exceptions are A1/BFL-1, BAD, BID, PUMA and BMF.

Figure 4



Mechanisms of BCL2 family deregulation in human cancer. Schematic depicting genetic (e.g. chromosomal translocations or somatic gene copy number amplifications) or epigenetic alterations (e.g. gene silencing due to hyper-methylation) that were shown to cause over-expression of pro-survival or loss of pro-apoptotic BCL2 family members in human cancers using illustrative examples, further detail is available in the main text.

Table 1:

BH3-mimetics in clinical development. Listing of BH3 mimetic compounds that are currently undergoing clinical trials. The cancers being treated are indicated and also whether the BH3 mimetic compounds are used as single agents or in combination with other drugs (standard of care regimens).

BH3-mimetic	Alternative name	Targets	Therapy	Indication	Clinical trial stage
ABT-263	Navitoclax	BCL-2, BCL-X_L BCL-W	Single agent	Chronic lymphocytic leukaemia	Phase I/II
				Cutaneous T cell lymphoma	Phase I/II
				Follicular lymphoma	Phase I/II
				Indolent lymphoma	Phase I/II
				Mantle cell lymphoma	Phase I/II
				Non-Hodgkin lymphoma	Phase I/II
				Peripheral T cell lymphomas	Phase I/II
			Combination*	Prostate cancer	Phase II
				Colon cancer	Phase I/II
				Melanoma	Phase I/II
				Non small-cell lung cancer	Phase I/II
				Pancreatic cancer	Phase I/II
				Rectal cancer	Phase I/II
				Skin cancer	Phase I/II
				Small-cell lung cancer	Phase I/II
				Chronic lymphocytic leukaemia	Phase I
				Diffuse large B cell lymphoma	Phase I
				Follicular lymphoma	Phase I
				Hepatocellular carcinoma	Phase I
				Hodgkin lymphoma	Phase I
				Lymphoblastic lymphoma	Phase I
				Lymphoma	Phase I
				Non-Hodgkin lymphoma	Phase I
				Other haematological disorders	Phase I

ABT-199	Venetoclax	BCL-2	Single agent	Chronic lymphocytic leukaemia	Phase III
				Acute Myeloid Leukaemia	Phase I/II
				Diffuse large B cell lymphoma	Phase I
				Follicular lymphoma	Phase I
				Lymphoma	Phase I
				Mantle cell lymphoma	Phase I
				Multiple myeloma	Phase I
				Non-Hodgkin lymphoma	Phase I
			Combination*	Chronic lymphocytic leukaemia	Phase III
				B cell non-Hodgkin lymphoma	Phase I/II
				Diffuse large B cell lymphoma	Phase I/II
				Follicular lymphoma	Phase II
				Non-Hodgkin lymphoma	Phase II
S-055746	None	BCL-2	Single agent	Haematological malignancies including myelodysplasia	Phase I
PNT-2258	None	BCL-2	Single agent	Diffuse large B cell lymphoma	Phase II
				Follicular lymphoma	Phase II
				Non-Hodgkin lymphoma	Phase II