



Review

Apoptosis signaling and BCL-2 pathways provide opportunities for novel targeted therapeutic strategies in hematologic malignances

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ABSTRACT

Apoptosis is an essential biological process involved in tissue homeostasis and immunity. Aberrations of the two main apoptotic pathways, extrinsic and intrinsic, have been identified in hematological malignancies; many of these aberrations are associated with pathogenesis, prognosis and resistance to standard chemotherapeutic agents. Targeting components of the apoptotic pathways, especially the chief regulatory BCL-2 family in the intrinsic pathway, has proved to be a promising therapeutic approach for patients with hematological malignances, with the expectation of enhanced efficacy and reduced adverse events. Continuous investigations regarding the biological importance of each of the BCL-2 family components and the clinical rationale to achieve optimal therapeutic outcomes, using either monotherapy or in combination with other targeted agents, have generated inspiring progress in the field. Genomic, epigenomic and biological analyses including BH3 profiling facilitate effective evaluation of treatment response, cancer recurrence and drug resistance. In this review, we summarize the biological features of each of the components in the BCL-2 apoptotic pathways, analyze the regulatory mechanisms and the pivotal roles of BCL-2 family members in the pathogenesis of major types of hematologic malignances, and evaluate the potential of apoptosis- and BCL-2-targeted strategies as effective approaches in anti-cancer therapies.

1. Introduction

Apoptosis is an important process involved in organism development, tissue homeostasis, metabolic regulation immunity, and elimination of damaged, infected and unwanted cells, contributing to the overall health of cells. Apoptosis is mediated by the extrinsic and intrinsic pathways. The extrinsic pathway, also designated as the death-receptor pathway, is mediated by the binding of cell-surface death receptors and their natural ligands. The intrinsic pathway, also known as the mitochondrial pathway and a major focus of this review, is strictly regulated by BCL-2 family proteins. Cell killing also can occur via a substitute pathway, a cytotoxic T-cell and natural killer cell-mediated and perforin-granzyme-dependent killing pathway, in which granzyme A and granzyme B are involved, but this pathway is not discussed in this review.

Numerous experiments have proven that deregulation of apoptosis is a common and causative event in hematologic malignances and is associated with tumor development, prognosis and resistance to chemotherapeutic agents [1–5]. Components of the extrinsic pathway and BCL-2 family are targets for anti-cancer therapies currently in

development. These anti-cancer agents have been proved efficient with enhanced efficacy and reduced side-effects in patients with hematological malignances, especially in refractory and relapsed cases. ABT-199, a selective inhibitor of the anti-apoptotic protein BCL-2, was recently awarded a ‘Breakthrough Therapy Designation’ from the United States Food and Administration (FDA) in recognition of its promise as a treatment for patients with relapsed/refractory chronic lymphocytic leukemia (CLL) [6]. This advance in therapy is evidence of the successful investigation of apoptosis and BCL-2 family proteins in recent years.

In this review, we address recent advances in regard to the structure, function and regulatory mechanisms of the BCL-2 family in the apoptotic pathway, and we highlight the biological role of BCL-2 family proteins in hematological malignances. Potential strategies for targeting components of the apoptotic pathways and BCL-2 members for the treatment of patients with hematological malignances in preclinical and clinical trials are discussed. The principles, dose schedules, adverse events, efficacies, limitations and some response-predicted laboratorial parameters associated with these trials are presented.

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2. Apoptotic pathways

2.1. Extrinsic pathway

The extrinsic pathway is triggered when cell-surface death receptors (DRs) are bound by their natural ligands [7]. DRs are members of the tumor-necrosis factor receptor (TNFR) family and are characterized by intracellular death domains. The group of DRs includes Fas (CD95), tumor necrosis factor α receptor 1 (TNFR1), tumor necrosis factor α ligand-receptor 1 (TRAIL-R1, DR4), tumor necrosis factor α ligand-receptor 2 (TRAIL-R2, DR5), DR3, and DR6. Death-inducing ligands can be soluble factors or cell surface molecules on T lymphocytes, including FasL/CD95 ligand (CD95L), tumor necrosis factor α ligand (TNF α) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). Upon ligand binding, DRs attract the adaptor protein FADD (Fas-associated death domain protein, also known as MORT1), which, in turn, recruits inactive forms of certain members of the caspase protease family, forming a “death-inducing signaling complex” (DISC), and resulting in activation of caspases 8 and 10, thus triggering apoptosis [8]. BID, a pro-apoptotic member in the intrinsic apoptotic pathway, is cleaved by active caspase-8 and translocates to mitochondria.

2.2. Intrinsic pathway

The intrinsic pathway is tightly regulated by the balance of pro-apoptotic and anti-apoptotic BCL-2 family proteins within mitochondria. Intrinsic apoptotic signals from the intracellular microenvironment include toxins, drugs, viral infections, free radicals, hypoxia, hyperthermia, calcium flux, loss of growth factors, cytokines or hormones, and elimination of apoptotic suppression [9,10]. These intrinsic stimuli initiate activation and interactions between BCL-2 family proteins (Fig. 2B–C), forming the mitochondrial outer membrane permeabilization (MOMP) complex. These molecular events result in release of cytochrome *c* and other factors from the mitochondrial inter-membrane space, inducing the formation of the apoptosome (caspase activation complex) which in turn activates caspase 9. The interaction between the anti-apoptotic and pro-apoptotic members can inhibit or activate MOMP and determine the fate of a cell. Activation of apoptotic caspase-9 leads to the activation of downstream “executioner” caspases.

The extrinsic and intrinsic apoptotic pathways converge into a common downstream pathway that includes the “executioner” caspases 3, 6 and 7. These caspases cleave each other, activate cytoplasmic endonuclease, degrade cytoskeletal proteins and polymerase ADP ribosyltransferase (PARP), thereby triggering various biochemical and morphological alterations in apoptotic cells. The last component of apoptosis is their phagocytic uptake.

2.3. Inhibitors of apoptosis proteins

Inhibitors of apoptosis proteins (IAPs) are a family of molecules that are pivotally involved in inhibition of the extrinsic and intrinsic pathways. The human IAP superfamily consists of nine members: NAIP, MLIAP, XIAP, cIAP1, cIAP2, ILP2, survivin, livin and BRUCE [11]. Each member of this family has a common domain composed of 70 amino-acid baculovirus repeats (BIR) that can suppress caspase function by facilitating protein-protein interactions, thus inhibit apoptosis. IAPs have been proven to be dysregulated in a variety of hematological malignances and can potentially serve as targets of anti-cancer therapies (Fig. 1) [12].

3. BCL-2 family

3.1. Classification of BCL-2 family members

BCL-2 is highly expressed and its structure is highly conserved in many hematologic cancers, and constitutes an essential component of

the BCL-2 family in cell death regulation. Since its discovery in follicular lymphoma (FL), BCL-2 has been recognized as a new class of oncogene that does not affect cell proliferation but instead promotes tumorigenesis by preventing cells from undergoing apoptosis. BCL-2 can also protect cells from a broad range of cytotoxic stimuli, including anti-cancer drugs [13,14]. Through three decades of research effort, it has been confirmed that BCL-2 plays a crucial role in apoptosis, heralding a new era in our understanding of cell survival pathways. Currently, > 25 BCL-2 family members have been identified, that are categorized into three main subtypes according to their structural and sequence homology: anti-apoptotic members (for which BCL-2 is the prototype), multi-domain pro-apoptotic members, and BH3-only members.

Anti-apoptotic members share four BCL-2 homology (BH1-4) domains. These anti-apoptotic members include: BCL-2, BCL-X_L (also known as BCL2L1), MCL-1, BCL-W (also known as BCL2L2), A1 (known as BCL2A1 or BFL1 in human), and BCL-B (also known as DIVA, BOO and BCL2L10 in mouse) [15]. Multi-domain pro-apoptotic BCL-2 family members include: BAK, BAX and BOK. BH3-only members include BID, BIM, PUMA, BAD, NOXA (also known as PMAIP1), HRK, BIK, BMF, BNIP3 and NIX [16,17].

The multi-domain pro-apoptotic members BAX and BAK are identified as “effectors”. Upon activation, these molecules directly induce conformational changes, leading to the MOMP complex and thus initiate apoptosis. BH3-only members can be divided into two subtypes according to the functions they play in pro-apoptosis: “activators” and “sensitizer/derepressors”. “Activators”, include tBID, BIM, and PUMA, and can directly interact with the “effectors” BAK, BAX and anti-apoptotic proteins. “Sensitizer/derepressor” molecules including BAD, BIK, BMF, HRK and NOXA, and cannot directly combine with “effectors”, but instead interact with anti-apoptotic members and then free “activators” to combine with BAX and BAK (Fig. 2).

Anti-apoptotic members reside on the outer mitochondrial membrane (OMM), the endoplasmic reticulum membrane and the nuclear envelope [16,18]. These proteins preserve OMM integrity by directly inhibiting pro-apoptotic proteins. BAX and BAK are important due to their critical roles in the formation of the MOMP complex (Fig. 2B–C). BID is induced by caspase 8, and thus connects the extrinsic and intrinsic apoptotic pathways [19].

The complex network of interactions between pro- and anti-apoptotic BCL-2 family proteins tightly regulates the mitochondrial apoptotic response, allowing for a swift response to specific stimuli to prevent deleterious cell death during normal cellular homeostasis. Interruption of the stoichiometric balance between anti- and pro-apoptotic BCL-2 family proteins can lead to a variety of human diseases including cancers and inflammatory or autoimmune disorders.

3.2. Structure of BCL-2 family

The BH domain is the homologous sequence shared by BCL-2 family proteins. All anti-apoptotic and some pro-apoptotic family members (BAX, BAK) share multiple BH1-4 domains. BH3-only proteins, as elucidated above, are a special group of pro-apoptotic members. The BH3 domain is constructed of 9 to 15 amino acids that are required to enable a protein to bind to anti-apoptotic BCL-2. The BH4 domain, a conserved structure-sequence motif which can stabilize BH1-3 domains, is essential for the anti-apoptotic activity of BCL-2 proteins. The highly conserved BH4 domain is located on the native N-terminal domain of BCL-2 and is composed of a stretch of 20 amino acids (residues 10–30) organized in a helical structure f1-f2-X-X-f3-f4. In this structure, X represents amino acids, f1, f2, f4 are aliphatic residues, and f3 is an aromatic residue. The loss of the BH4 domain completely eliminates the anti-apoptotic activity of BCL-2 without influencing the ability of BCL-2 to bind BH3-only proteins [20]. Most BCL-2 members have a trans-membrane (TM) domain that anchors the molecule to the membrane of organelles, most notably the mitochondrial membrane.

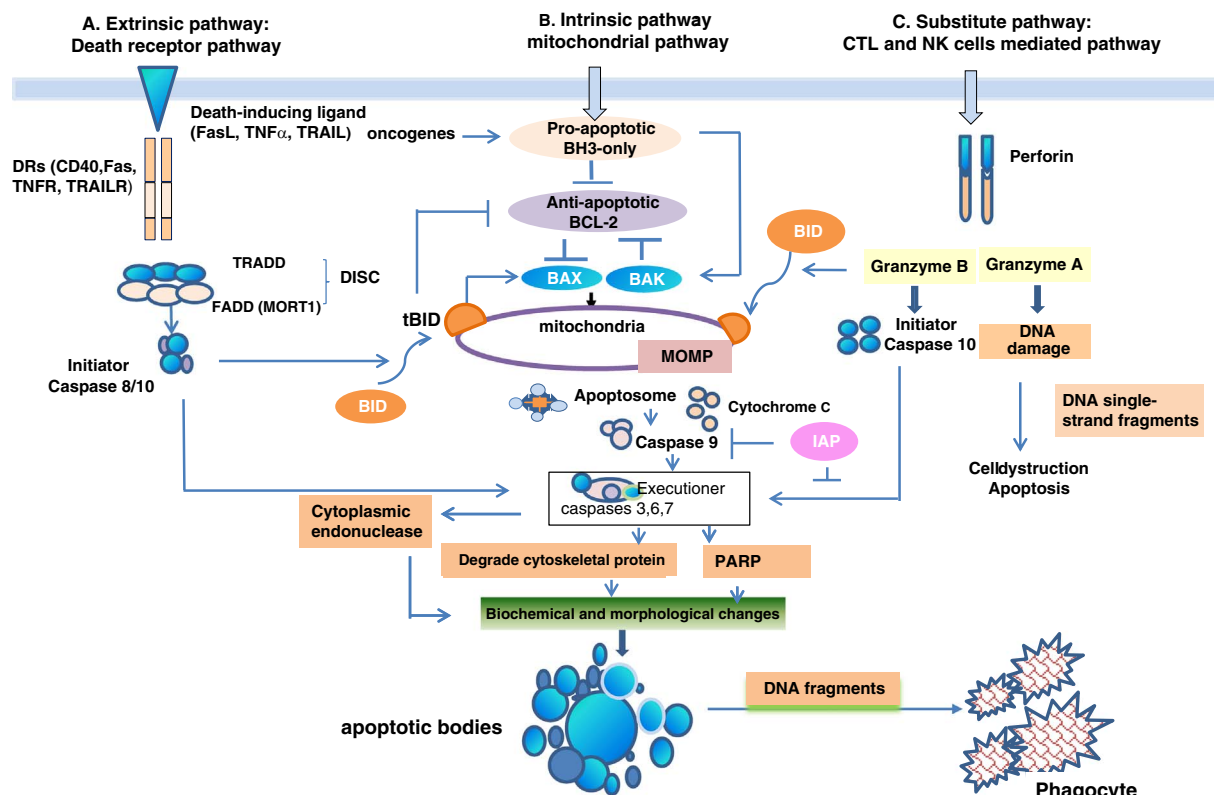


Fig. 1. Illustration of major apoptotic signaling pathways. (A) The extrinsic pathway is initiated by the interaction of death receptors (DRs) and death-inducing ligands via their FADD/TRADD domains. A death-inducing signaling complex (DISC) is formed, which then recruits inactivated initiator caspase-8 and caspase-10. After that, BID is cleaved into tBID and translocates to mitochondria. (B) In intrinsic apoptotic pathway, the stimuli first activate BH3-only proteins, and then pass the apoptotic signals via activating interactions between BCL-2 members (Fig. 2B and C), forming a mitochondrial outer membrane permeabilization (MOMP) complex and eventually releasing cytochrome c. Cytochrome c induces the assembly of the apoptosome, and caspase-9 is activated. The extrinsic and intrinsic apoptotic pathways converge in which caspase-3, caspase-6 and caspase-7 function as executioner caspases that trigger various biochemical and morphological alterations in apoptotic cells that are eventually taken up by phagocytes. Inhibitors of apoptosis (IAP) proteins inhibit both initiator and executioner caspases in apoptotic pathways. (C) The substitute pathway is mediated by cytotoxic T lymphocytes or NK cells which involve perforin and granzyme.

BCL-2 family proteins, based on their structure, can be categorized into folded globular proteins and intrinsically unstructured proteins. The globular proteins include all multi-domain anti-apoptotic proteins (BCL-2, MCL1, BCL-W, BCL-B and A1) and multi-domain pro-apoptotic proteins (BAX, BAK and BOK). The intrinsically unstructured proteins include BH3-only proteins and their BH3 domains bind to globular BCL-2 proteins by their amphipathic helices (Fig. 2A). BID is the only BH3-only protein with its structure solved, consisting of a hydrophobic core similar to the BCL-2 groove. BID can be activated by proteolytic caspase-8 and granzyme B cleavage in the $\alpha 1$ - $\alpha 2$ loop, leading to the formation of a stable folded truncated BID (tBID).

Each globular BCL-2 family protein shares a conserved “BCL-2 core”. The BCL-2 core is a ~20 kDa globular domain composed of 7 or 9 amphipathic α helical bundles with a large hydrophobic pocket composed of helices 2, 5, 7 and 8. These α -helix bundles coalesce together and generate a folded hydrophobic groove known as the BH3 and C-terminus-binding groove (BC groove). The BCL-2 protein binds to the BC groove via its α helix at the C-terminal end. The BH4 domain is composed of conserved $\alpha 1$ helix alongside the $\alpha 6$ helix, stabilizing the BH1-BH3 domains. Together, the BCL-2 core is composed of the BC groove and $\alpha 1/\alpha 6$ structural components. The BC groove serves as an important platform for interactions of the BH3 domain with various BCL-2 members, and enables homo and heterodimerization by family proteins [21].

Structural investigation is pivotal in drug discovery and the designation of therapeutic agents that target BCL-2 family proteins. Nowadays, various BH3-mimetics are designed based on disrupting protein-protein interactions (PPIs) between the BCL-2 core and BH3

domains, and many of these agents have shown promising activity as targeted therapies for patients with cancers [22].

4. Functions of BCL-2 family

Aberrant expression of BCL-2 family proteins has been found in many types of cancer. However, expression is not always reflective of the physiological functions of these proteins [23]. Since from the early 1990s, knockout mice models have shown individual and overlapping functions and consequences of disrupted regulation of many components in the intrinsic apoptotic pathway. mRNA levels of BCL-2 proteins that are expressed differentially in diverse tissues and tumor cell lines [24] as well as functional studies of BCL-2 have proven that each individual BCL-2 family member can exert a highly selective role in apoptosis and even in a cell type-dependent manner [23]. Over-expressed anti-apoptotic proteins render various cell types resistant to diverse apoptotic stimuli and promote cancer and drug resistance. Constitutive or conditional gene deletions of anti-apoptotic BCL-2 family members have also showed their crucial roles in maintenance of cell biology and function. BCL-2 mutations, in the coding or non-coding region, may impact its regulatory functions via apoptotic and/or non-apoptotic pathways. Several studies have revealed that the BCL-2 family also exhibits essential functions beyond apoptosis.

4.1. Anti-apoptotic BCL-2

4.1.1. BCL-2

BCL-2 transgenic mice that overexpress BCL-2 developed lymphoma

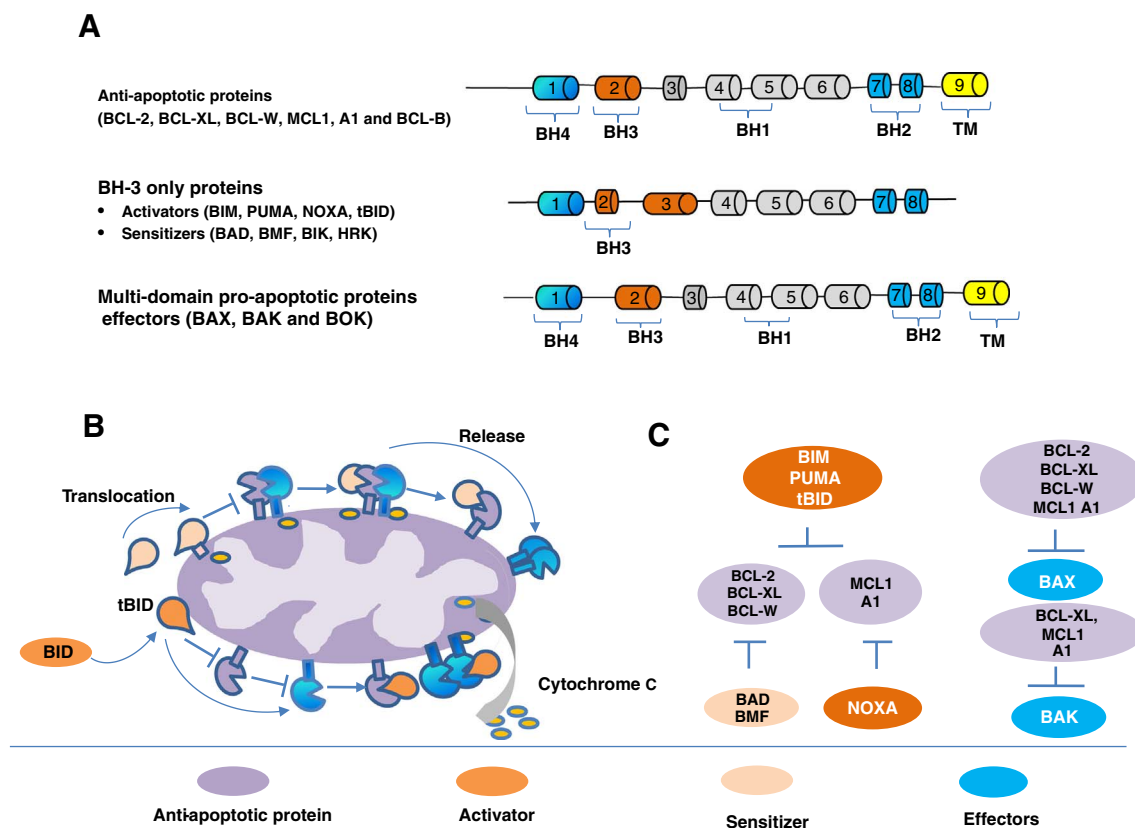


Fig. 2. Classification of BCL-2 family proteins and interactions among BCL-2 family proteins. (A) There are three main subtypes of BCL-2 family proteins: anti-apoptotic members, BH3-only members and multi-domain pro-apoptotic members. Pro-apoptotic proteins can be divided into activators, sensitizers and effectors according to their functions in the apoptotic pathway. Anti-apoptotic BCL-2 family proteins and multi-domain pro-apoptotic members share BH1-4 domains. BH3-only proteins only contain BH3 domain. Each globular protein comprised of seven or nine amphipathic alpha (α) helices. The BH1 domain is composed of α 4- α 5 helices; the BH2 domain is composed of α 7- α 8 helices and BH3 is composed of α 2 helices. The BH4 domain stabilizes the BH1-BH3 domains by the conserved α 1 positioned alongside the α 6. Most BCL-2 members have a trans-membrane (TM) domain for anchoring to organelles. (B) Anti-apoptotic BCL-2 family proteins inhibit apoptosis by three model, neutralizing pro-apoptotic proteins directly, interacting with “effector” proteins, BAX and BAK, and sequestering “activator” proteins from engaging BAX and BAK. (C) Interactions between the subtypes of BCL-2 family proteins. All of the anti-apoptotic proteins can interact with BAX, BIM, PUMA and tBID. Only MCL1 and A1 interact with NOXA; BCL-2, BCL-X_L and BCL-W antagonize BAD and BMF; BCL-X_L, MCL1 and A1 can target BAK.

and drug resistance [13,14,25–27]. *BCL-2* knock-out mice failed to maintain (most) mature T and B lymphocytes [28–30], with inadequately generate activated T and regulatory T cells [31], but showed no defect in myeloid cell development [32,33]. All these phenotypes can be restored by loss of one or two *BIM* alleles.

4.1.2. *BCL-X_L*

BCL-X_L, a product of *BCL-X*, is expressed in pre-B cells and down-regulated in immature and mature B cells. *BCL-X_L* protects mature B cells against antigen-induced activation and proliferation signals [34]. In thymocytes, *BCL-X_L* is expressed at a low level in CD4⁻/CD8⁻ cells, is expressed maximally in CD4⁺/CD8⁺ cells, and down-regulated again in CD4⁺/CD8⁻ or CD8⁺/CD4⁻ thymocytes [35]. *BCL-X_L* expression provides a survival signal for the maintenance of immature CD4⁺/CD8⁺ thymocytes before positive selection.

B cells that overexpress *BCL-X_L* accumulate markedly in lymphoid organs [34], and thymocytes overexpressing *BCL-X_L* are resistant to apoptosis and unable to block clonal deletion of thymocytes reactive to self-super antigens or H-Y antigen. *BCL-X_L* deficient lymphocytes have shortened lifespan and significantly reduced in number. Loss of *BCL-X_L* negatively impacts on the formation of germinal center (GC) B cells, memory B cells and natural killer (NK) cells [36,37]. *BCL-X_L* deficiency models have shown apoptotic accumulation in hematopoiesis. Conditional-deletion of *BCL-X_L* revealed that it acts in conjunction with *MCL1* as a “timer” for platelet and erythrocyte lifespan, and determines megakaryocyte function and immature thymocyte survival [38–40].

BCL-X_L transgenic mice show similar phenomena as in *BCL-2*

transgenic mice, but with more variable phenotypes. Concomitant overexpression of *BCL-X_L* and *BCL-2* significantly inhibits apoptosis and leads to functionally aberrant accumulation of lymphocyte.

4.1.3. *MCL1*

MCL1 is the most crucial pro-survival protein in the BCL-2 family. It is expressed steadily with a propensity to higher levels during cell maturation from pro-B to pre-B cells, and from immature B cells to follicular and marginal zone B cells [41]. *MCL1* is also expressed outside the hematopoietic system. It has a short half-life and has been identified as a molecular “timer” in cell death induction [42].

Along with *BCL-X_L*, *MCL1* is expressed more highly in GC B cells than in non-GC B cells, and its expression is dose-dependent in the formation of pre-formed GC, memory B cells [43], granulocytes, monocytes and mast cells [44]. *MCL1* plays an important role in the survival of NK cells [37], plasma cells [45] and macrophages upon microbial challenge [46]. *MCL1* transgenic mice develop splenomegaly with extended extra-medullary hematopoiesis and a predisposition to different types of B cell lymphoma. Moreover, these models are more sensitive to drug treatment than models that overexpress *BCL-2* [43,47].

4.1.4. *A1*

During B cell maturation, *A1* mRNA levels increase gradually and are highest in activated B cells [48], then decrease in plasma cells due to the transcription factor Blimp-1. In T cells, *A1* increases upon rearrangement of the T cell receptor β (TCR- β) chain, pre-TCR expression,

and TCR mediated activation [49]. *A1* mRNA is expressed either constitutively or in response to inflammatory cytokine stimulation of myeloid cells, or in response to TLR ligation (LPS) in macrophages or FcεRI-mediated activation in mast cells [50]. *A1* has limited expression outside the hematopoietic system. It has a very short half-life and plays a critical role in adaption and selection processes upon challenge, inflammation or drug treatment [51].

A1 transgenic mice have shown that *A1* over-expression is insufficient for tumorigenesis. *A1* gene deletion delays thymic development, shows impaired B cell homeostasis with decreased mature follicular B cells [52], and impairs B-cell activation in response to mitogen and mast cell homeostasis [53].

4.2. Multi-domain pro-apoptotic BCL-2

Most transgenic mice lacking both BAX and BAK die in the perinatal period or develop lymphadenopathy [54–56]. BAX acts in a redundant manner in regulating granulocyte survival and death [33], BAX mutations in primary cells of hematological neoplasms lead to resistance to apoptosis and may be a key feature of lymphoma and leukemia [57]. BAK enhances apoptosis; however, it inhibits cell death in an Epstein-Barr-virus-transformed cell line, probably due to inducing viral anti-apoptotic factors, such as the EBV-encoded BCL-2 homologue BHRF-1 [58,59]. BAK also acts as a continuous molecular timer of platelet lifespan [39]. *BOK*-deleted mice do not develop lymphomas, but develop splenomegaly and an enlarged thymus, partially due to hormonal regulation [60].

4.3. BH3-only proteins

Overexpression of BH3-only proteins, such as the activators BIM, PUMA, and BID, and sensitizers NOXA, BIK, BAD, HRK, and BMF, will trigger apoptosis. In contrast, deletions of these proteins lead to various levels of abnormality, depending on their essential individual roles in apoptosis.

BID plays an essential role in maintenance of myeloid homeostasis and tumor suppression. *BID*-deleted transgenic mice progress to myeloid leukemia including chronic myelomonocytic leukemia, often with chromosomal trisomy or t (6; 11), and thus BID was identified as an upstream factor for CMML progression [61]. PUMA is critical for inducing p53-mediated apoptosis [62] and the recovery of cells after γ -radiation-induced DNA damage, partially via the p53/Arf pathway [63]. Loss of *PUMA* ablates radiation-induced thymic lymphomagenesis [64]. *NOXA* specifically binds to BCL-2 and MCL1, and deletion of *NOXA* accelerates γ -radiation-induced thymic lymphomagenesis. BIM is essential in the regulation of diverse subtypes of T cell and granulocyte apoptosis [65]. Loss of *BIM* and/or *PUMA*, combined with *BID* deletion, leads to lymphoid neoplasms in aged mouse models and accelerates Myc-driven lymphoma [56]. Cells cannot undergo apoptosis without adequate levels of BID, BIM and PUMA, even with sufficient BAX and BAK levels.

Bad-deletion delays thymocyte death after γ -radiation and increases the frequency of hematological malignancies, particularly diffuse large B cell lymphoma (DLBCL) [66]. *Bad* deletion leads to abnormal IgG production and resistance to FAS-induced apoptosis, and causes a mild increase of platelets [41,67]. BMF is a novel component involved in suppression of Myc-aberrant B-cell lymphomas [41], and plays a restrictive role by supporting BIM in some cell death processes [68]. *Bmf*-deletion leads to B cell accumulation and resistance to glucocorticoids and other pro-apoptotic stimuli [69]. *Bmf* mutations lead its aberrant expression and are involved in metastatic carcinomas [70]. Deletion of *Bik* [71] or *Hrk* have been reported to result in minor apoptotic defects (Table 1) [72,73].

4.4. Functions of the BCL-2 family beyond apoptosis

BCL-2 family proteins display multiple non-canonical and non-apoptotic functions and influence cell bioenergetics and metabolism, including regulation of mitochondrial functions (ANT, VDAC, and glucokinase) [74], Ca^{2+} homeostasis at the ER [75] and ATP synthesis [76]. BCL-2 family proteins are involved in autophagy mediated by autophagy regulator, Beclin1 [77]. Via its BH3 domain, Beclin 1 interacts with the hydrophobic groove of BCL-2, preventing autophagy activation [78]. BCL-2 has been shown to prevent cell death and DNA by decreasing the Ca^{2+} sensitivity of MPTP (mitochondrial permeability transition pore) [79].

BCL2 is found to prevent matrix swelling, ROS damage and the loss of mitochondrial membrane potential, therefore, maintaining mitochondrial integrity. BCL2 family proteins have been critical for neural synapse maturation, cell cycle regulation, vascular proliferation via HIF- α , VEGF, and microenvironment stromal components.

Functional studies have helped to better define the roles of BCL-2 family members in apoptotic signaling pathways and to predict the efficacy or possible side effects of BCL-2-targeted therapy. Each individual BCL-2 family member can exert highly selective roles in the development of lymphocytes and other hematopoietic cells, even in a strictly cell type-dependent manner. This functional characteristic of BCL-2 family proteins makes it possible to target different BCL-2 family member proteins regarding specific tissues and developmental stages to obtain an optimal response. However, the phenotypes present in the transgenic and knock-down mice point to potential toxicities in clinical trials. Some of the BCL-2 functions beyond apoptosis are mediated by the BH4 domain which is now becoming a new BCL-2 therapeutic target [20].

5. Activation and regulation of BCL-2 family

The ratio between anti-apoptotic and pro-apoptotic BCL-2 family proteins on mitochondria determines cell fate. Expression of BCL-2 family proteins can be stimulated by a number of factors, such as cytokines, colony-stimulating factors and mitogens, and expression levels of BCL-2 family proteins are controlled via transcriptional, posttranscriptional, and posttranslational modification mechanisms.

5.1. Signaling pathways in the regulation of BCL-2 family members

The ubiquity of apoptosis requires that it be tightly and finely controlled. Several signaling pathways have been shown to impact the apoptotic potential of the cells. The most notable is the phosphatidylinositol 3'-kinase (*PI3K*) pathway. *PI3K* can be activated by growth factors [e.g. epidermal growth factor; EGF] and platelet-derived growth factor (PDGF), cytokines (IL-2 and IL-3) and some hormones (e.g. insulin) [80,81]. When activated, *PI3K* generates 3'-phosphorylated lipid products, activates PKB/Akt leading to anti-apoptotic gene expression by activating nuclear factor κ B (*NF- κ B*), and influences pro-apoptotic gene expression by inactivating the forkhead superfamily transcription factors AFK and FKHL1. At the same time, Akt phosphorylates and inactivates BAD and blocks its pro-apoptotic function [82]. On the other hand, AKT survival signals are counteracted by the tumor suppressor PTEN.

Another prominent regulator of apoptosis is the extracellular signal regulated kinase 1/2 (*ERK1/2*) signaling pathway which influences the expression and/or activity of BCL-2 family members [83]. *ERK1/2* phosphorylation promotes transcription and expression of MCL1, BCL-2 and BCL- X_L , and ubiquitinates BIM, BAD and BIK for degradation. Additionally, BIM can be transcriptionally regulated by FOXO3, which itself is a target of *ERK1/2*. *ERK1/2*-mediated FOXO3 phosphorylation promotes MDM2-dependent ubiquitination and degradation by the proteasome, thereby repressing BIM transcription.

It has been reported that TNFR can activate the *JNK/SAPK* pathway

Table 1
Characteristics and functions of BCL-2 family members in hematologic malignancies.

	Characteristics	Functions
BCL-2 18q21.3, 238aa	Most founding in BCL-2 family	Fail in T production and B maintenance [28–30]; favor the development of M and regulatory T [31–33]
BCL-XL 20q11.21,232aa	Anti-apoptotic; displaces BCL-2 during cell maturation; a “timer” of Plt, Meg and E [38–40]	Reduce the number and shortened the lifespan of immature lymphocytes; essential in GC B, memory B and NK [36,37]
MCL-1 1q21, 349aa	Anti-apoptotic; displaces BCL-XL during cell maturation, expressed stably; short half-life; a ‘timer’ in cell death induction [42]	Expressed higher in GC B cells than non-GC B cells [43]; dose-dependent in preformed GC B cells, memory B, G, Ms. and Mon [44]; critical in NK, Mc and PC [37,45–46]
A1 Chr15 4.7–7.8 kb	Anti-apoptotic; reaches highest in active B cell; short half-life	Pivotal in M, Mc and Ms. [50]; reduce G progenitors and mature follicular B [52]; impair the reaction to mitogen [53]
BAX 19q13.3-q13.4, 191aa	Pro-apoptotic effector; induces MOMP	Lead to lymphadenopathy, hematological malignancies [54]
BAK 6p21.3, 210aa	Pro-apoptotic effector; induces MOMP; a ‘timer’ of platelet [39]	Play a similar role as BAX; inhibit cell death in EBV-transformed cell lines [58,59]
BOK 2q37.3, 211aa	Pro-apoptotic effector; functions in a BAX/BAK-like pro-apoptotic manner	Do not accelerate lymphoma development [60]
BID 22q11.1, 240aa	Pro-apoptotic activator; tBID initiates intrinsic apoptosis; regulates M hematopoiesis	Develop myeloid leukemia, including CMML [61]
BIM 2q13, 198aa	Pro-apoptotic activator; prevents BAX and BAK homo-oligomerization; has spliced isoforms	Essential in diverse subsets of lymphocyte; essential to G apoptosis [33,65]
PUMA 19q13.3, 260aa	Pro-apoptotic activator; prevents BAX and BAK homo-oligomerization	Essential to cell recovery after γ -radiation-induced DNA damage [62,64]
NOXA 18q21.3, 102aa	Specifically binds to BCL-2 and MCL-1	Lead to lymphomas, accelerate γ -radiation-induced thymic lymphomagenesis
BAD 11q13.1,166aa	Pro-apoptotic sensitizer	Develop DLBCL, lead abnormal IgG production [41]; increase Plt numbers [41]; resistant to FAS-induced apoptosis [67]
BMF 15q14, 183aa	Pro-apoptotic sensitizer; weak inducer of apoptosis	Accumulate B; resistant to glucocorticoid stimuli [69]. BMF mutations are responsible for metastatic carcinomas [70]

Abbreviations: Plt: platelet; Meg: megakaryocyte; E: erythrocyte; T: T lymphocyte; B: B lymphocyte; M: myeloid cell; NK: Natural Killer cell; G: granulocytes; Ms.: mast cell; Mon: monocyte; Mc: macrophages; PC: plasma cells; CMML: chronic myelomonocytic leukemia.

(c-Jun N-terminal protein kinase 1/stress activated protein kinase pathway) also inducing BCL-2 phosphorylation, even under IL-3 withdrawal conditions [84]. IL-7-activation of the *JAK/STAT5* pathway also has been reported to control leukemic B lymphocyte survival by activating MCL1 in B acute lymphoblastic leukemia (B-ALL) [85].

The BCR signaling pathway is involved in up-regulating A1 mRNA and protein levels [48,53], and plays a key role in the survival and/or proliferation of peripheral B cells. In naïve, activated and post-effector T cells, A1 expression is dependent on TCR, but not on cytokine receptor engagement, indicating that A1 is differently regulated from BCL-X_L and BCL-2 [86]. Under homeostatic conditions, BH3-only proteins are regulated by growth factor promoting signaling pathways in which several oncogene kinases are involved. Inhibitors of these signaling pathways lead to apoptosis and tumor regression in vivo. Some mutations of oncogenic drivers such as *EGRF*, *HER2* and *K-RAS* can also induce BCL-2 protein alterations (Fig. 3).

5.2. Methylation and mutation of promoters of BCL-2

Hypomethylation occurs at the 5′-end of *BCL-2* within a region corresponding to its promoter and the first major exon promoter. Hypomethylation has been reported to cause a cis-regulatory defect in lymphomas; however, hypomethylation does not appear related to BCL-2 overexpression that may be driven through other mechanisms [87]. Nearly 30% mutations occur in the *BCL-2* promoter region, and could jeopardize the functions of transcriptional factors. *BCL-2* promoter region contains binding motifs for Myc, Miz-1 and p53, and its interaction modulates cell cycle regulation. Mutations in the *BCL-2* promoter region could damage BCL-2 binding specificity.

5.3. Transcriptional factors and oncogenes in BCL-2 regulation

In physiologic conditions, BCL-2 proteins are expressed constitutively. When stimulated by apoptotic signals, the cell initiates the transcription of BCL-2 family proteins. Various studies have reported

that HRK, BAX, BCL-X_L, MCL1, A1 and BCL-2 are transcriptionally responsive.

5.3.1. FOXO3a/FKHR, FKHL

The forkhead transcription factor FKHR-L1/FOXO3a is a downstream target of the PI3K/Akt pathway. When cells are under the conditions of cytokine withdrawal and various chemotherapeutic agents, the cells trigger FOXO3a activation, inducing BIM expression in T lymphocytes, and then triggering apoptosis [88].

5.3.2. Nuclear factor kappa B (NF- κ B)

NF- κ B is a well-known transcriptional factor that is sequestered inactively in the cytoplasm in resting cells, binding to inhibitor of kappa B (I κ B) proteins. Upon stimulation, I κ B is phosphorylated thus releasing NF- κ B which can translocate to the nucleus and bind to target genes [89]. *BCL-2* has two promoter regions, P1 and P2. *BCL-2* expression is transcriptionally regulated by NF- κ B through functional binding with NF- κ B locus at the *BCL2* P2 promoter [90]. A1, TRAF1/2 and cIAP1/2 are also transcriptional targets of NF- κ B [91–93].

5.3.3. TP53

The tumor suppressor gene TP53 has been shown to be involved in the intrinsic and extrinsic apoptosis pathways. p53 can induce transcription of PUMA, BID, BAX, TRAIL2 and FASL in the nucleus [94], as well as translocate to the mitochondrial membrane to form an inhibitory complex with BCL-X_L and BCL-2 in response to apoptotic signals thus inducing cell apoptosis. Tumor-derived p53 mutations concomitantly lose the ability to interact with BCL-X_L and promote cytochrome *c* release. On the other hand, cytosolic p53 might induce the activation of pro-apoptotic BAX via direct protein-protein interactions and further induce apoptosis [95]. p53 can interact with the TATA-binding motif in the *BCL2* promoter and prevents the pre-initiation complex formation and function. Lymphoma patients with both TP53 mutation and BCL-2 aberrations (mutations and translocation or abnormal expression) showed worse survival than those with TP53

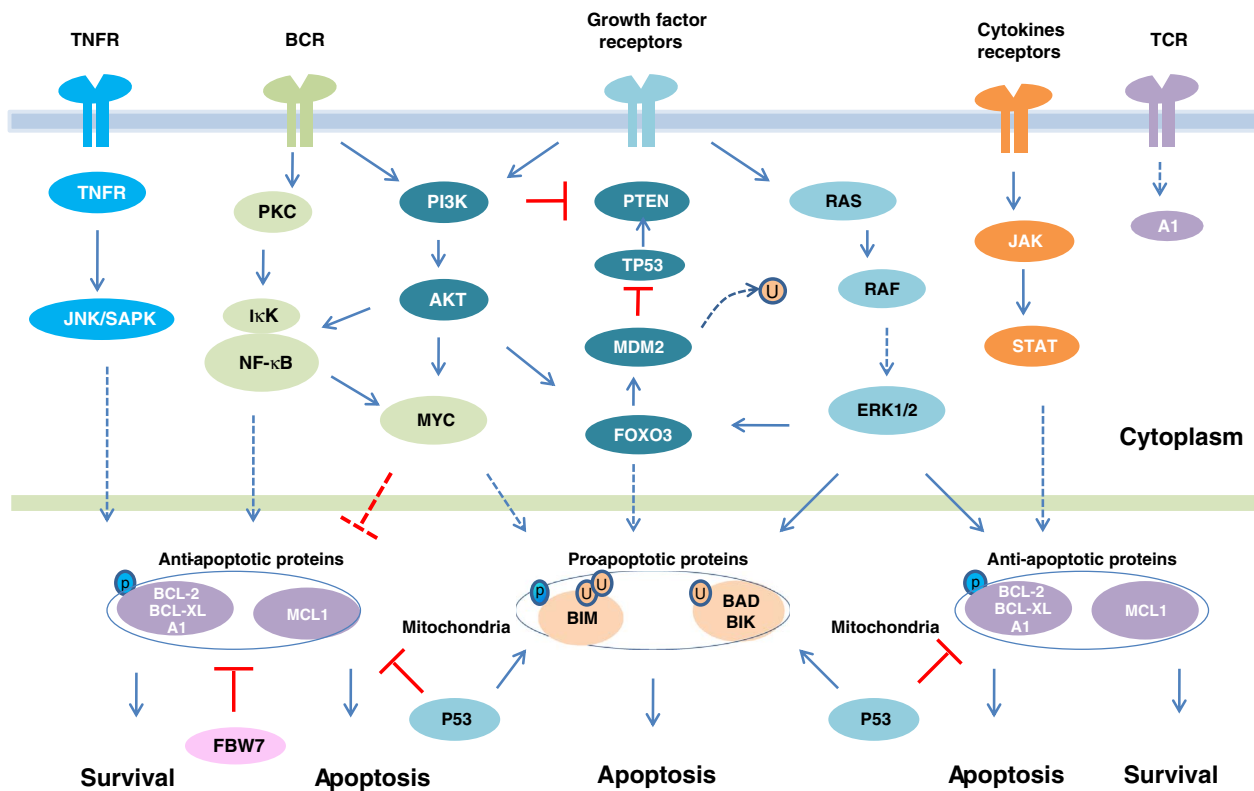


Fig. 3. Signal pathway and transcriptional regulation of BCL-2 family proteins. Phosphatidylinositol 3' kinase (*PI3K*) pathway and grow-factor-activating extracellular-signal-regulated kinase 1/2 (*ERK1/2*) signaling pathway are two essential regulators of apoptosis and influence the expression and/or activity of BCL-2 family proteins. c-Jun N-terminal protein kinase 1/ stress activated protein kinase (*JNK/SAPK*) can be activated by TNFR, then induce BCL-2 phosphorylation even in the condition of IL-3 withdrawal. IL-7-activated *JAK/STAT5* pathway is essential in control of the survival of pro-B lymphocytes by activating MCL1. *BCR* signal pathway is involved in up-regulating *A1* mRNA and protein, and expression of *A1* is dependent on TCR but not on cytokine receptor engagement. *FOXO3a/FKHR*, *FKHRL*, nuclear factor kappa B (*NF-κB*), *TP53* and *Myc* are involved in the posttranscriptional regulation of BCL-2 family proteins.

mutation or *BCL-2* aberrations alone. Biologically, combined *TP53/BCL-2* genetic alterations may resemble “double-hit lymphoma” (*MYC* and *BCL-2* translocation, DHL). Follicular lymphoma patients with few specific *BCL-2* genetic mutations (BH4 lymphoma-specific mutations or gain-of-functional mutation) showed poor response to standard treatment R-CHOP regimen and a shortened duration of transformation to DLBCL. The FLD domain of *BCL2* protein contains negative regulatory regions (AA 32–68). p53 could interact with FLD domain and prevented BAX binding to the *BCL2* hydrophobic groove, reducing its apoptotic activity.

5.3.4. *MYC* gene

MYC encodes a multifunctional nuclear phosphoprotein that regulates the expression of numerous pro-apoptotic genes, such as *BAX* and *BIM*, and negatively regulates *BCL-2* and *BCL-X_L* thereby promoting apoptosis. Aberrant *Myc* expression decreases the expression of *BIM* and *BAX* and increases *BCL-2* and *BCL-X_L* expression, thereby resulting in prolongation of tumor cell survival (Fig. 3) [96].

5.4. Posttranscriptional and epigenetic modifications

5.4.1. microRNA (miRNA) and epigenetic regulation

miRNAs are small noncoding RNA molecules (about 22 nucleotides) that play important regulatory roles by targeting mRNAs for cleavage or translational repression. Many miRNAs have been proven to be anti-apoptotic factors, and they mediate this effect by targeting pro-apoptotic mRNAs or their positive regulatory mRNAs. Conversely, pro-apoptotic miRNAs target anti-apoptotic mRNAs or their positive regulators [97].

miR-15, miR-16 and miR-34 target *BCL-2* mRNA, promoting

apoptosis, and are decreased in CLL patients [98]. miR-491 and miR-153 antagonize *BCL-X_L* and *MCL1*, respectively [97]. miR-29, miR-101, miR-133b and miR-193b are negative regulators of *MCL1* [99–102]. miR-32, miR-25 and miR-17-92 target *BIM*, acting as oncogenes [103]; miR-221, miR-222, miR-483-3p and miR-BRAT5 down-regulate *PUMA* [97], and miR-125b inhibits *BIK*.

miR-15a-16-1 at chromosomal 13q14 is most commonly altered in CLL, which contributes to the pathologic clinical course of CLL due to its abnormal expression and deletion [104]. Low expression of miR-34a, miR-29c and miR-17 is associated with an unfavorable subtype of CLL with *TP53* aberrations [105]. miR-181b decreases during CLL progression. miR-150, miR-155 and miR-34 regulate the *BCR* signaling pathway in CLL cells [106–108]. miR-650 regulates V2 immunoglobulin light chain and is associated with a better prognosis in CLL [109]. miR-17-92 overexpression induces lymphomagenesis [110], and miR-155 is aberrantly upregulated in B-cell malignancies including DLBCL, FL, CLL and mantle cell lymphoma (MCL). miR-155 levels are significantly higher in the activated B cell (ABC)-like than GC-like DLBCL [111]. miR-21 is upregulated in B-cell non-Hodgkin lymphomas (NHLs) and is associated with the ABC-like DLBCL and AIDS-related NHLs [103,111].

5.4.2. Alternative mRNA splicing

After *BCL-2* DNA is transcribed into mRNA, it can be spliced into multiple isoforms which can be translated into multiple proteins with different amino acid sequence and biological functions. For instance, *BIM* can be spliced into 19 isoforms that can be grouped into six subtypes: *BIM_S*, *BIM_L*, *BIM_{EL}*, *BIM_D*, *BIM_{Dd}* and *BIM_{EdD}* [109]. Similar splicing alterations have been reported in the *BCL-X*, *BAK* and *PUMA*, resulting in isoforms that differ in size and apoptotic activities [112].

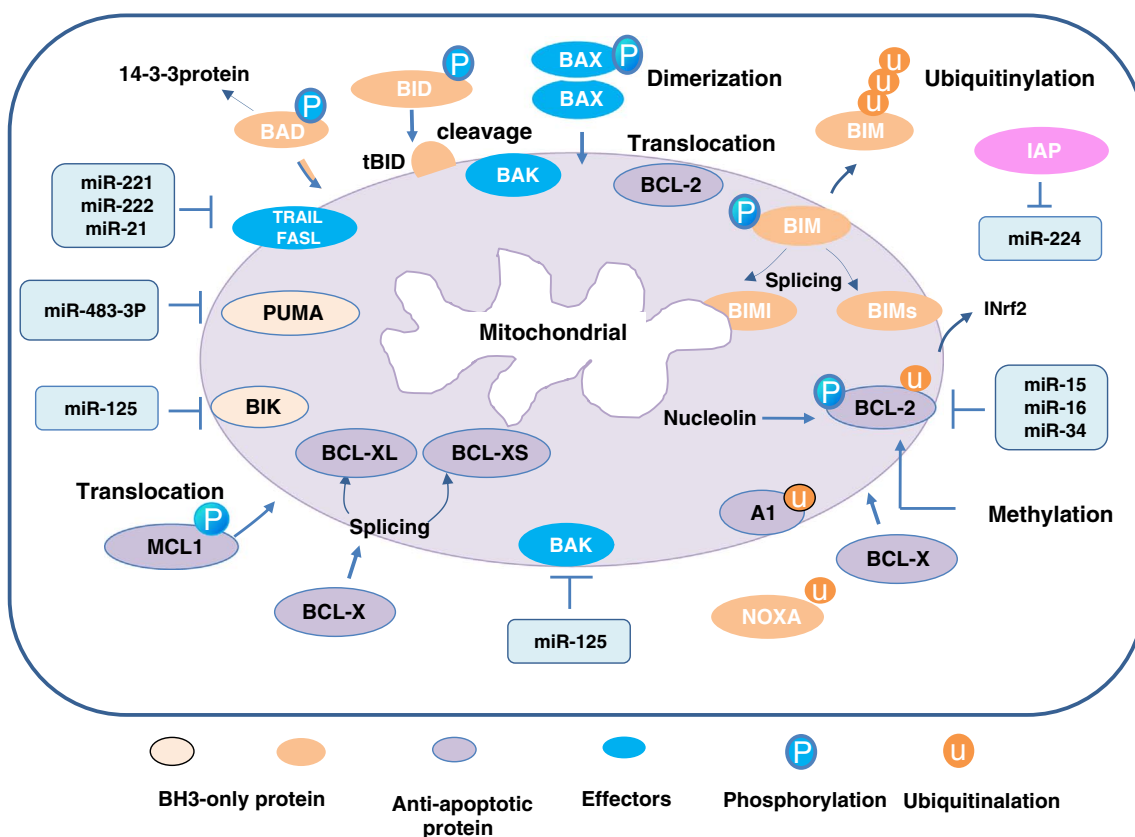


Fig. 4. Diagram of post-transcriptional and post-translational modifications of BCL-2 family proteins. The anti-apoptotic protein BCL-2 can undergo phosphorylation either enhancing or diminishing cell survival according to their differential subcellular locations. miRNAs regulate the expression of BCL-2 proteins by antagonizing targeted mRNA. BID is cleaved into t-BID by caspase 8 and then translocates to mitochondrial membrane. The BAX translocates to the mitochondrial membrane and becomes dimerized. BIM can be spliced into 19 isoforms which can be grouped into six subtypes. Activated nucleolin promotes the stabilization and expression of *BCL-2* mRNA from nuclease degradation, while in its inactivated form, nucleolin decreases *BCL-2* mRNA. BIM and BAX translocate to the mitochondrial outer membrane when they are activated. DNA hypomethylation of CpG islands at the 5'-end of gene sequence of the *BCL-2* promoter is associated with high BCL-2 expression. BIM phosphorylation and BCL-2 dephosphorylation lead to their ubiquitination. A1 can be ubiquitinated, thereby losing its anti-apoptotic function.

For instance, BCL-X_L acts an anti-apoptotic protein, whereas its short isoform BCL-X_S plays a pro-apoptotic role; and BIM_S, the shortest molecule of BIM, kills cells more efficiently than BIM_L and BIM_{EL}.

5.4.3. Nucleolin

Nucleolin is a multifunctional protein of the RNP (ribonucleoprotein)-containing family member of RNA-binding protein (RNA-BP). It binds to an AU-rich element (ARE) in a 3'-UTR of *BCL-2*, and is identified as *BCL-2* mRNA-stabilizing protein [113]. When in its activated form, nucleolin promotes the stabilization and expression of *BCL-2* mRNA; whereas when in inactive form, nucleolin decreases *BCL-2* mRNA and protein expression levels [114,115]. Nucleolin, together with miR-17-92, miR-15 and miR-16, has been found playing important role in CLL pathogenesis (Fig. 4) [116].

5.5. Post-translational modification

Posttranslational regulatory processes, such as phosphorylation, dimerization, translocation, cleavage and ubiquitination, are also important in the regulation and activation of BCL-2 family members.

5.5.1. Phosphorylation of BCL-2

Phosphorylation is one of the most important means of post-translational modification of BCL-2 family proteins. Both protein kinases and phosphatases, including PI3K/Akt, protein kinase C (PKC), MEK/mitogen-activated protein kinase (MAPK), ERK1/2 and c-Jun N-terminal protein kinase 1 (JNK1) [84,117,118], can phosphorylate and

regulate the activities of BCL-2 family proteins.

The anti-apoptotic protein BCL-2 can undergo phosphorylation at multiple amino acid domains within the flexible loop, either enhancing or diminishing cell survival (T56, T69, S70, T74, and S87) [119]. For example, phosphorylation at serine 70 (Ser70) of BCL-2 via PKC is essential for its fully anti-apoptotic function [117] and resistance to chemotherapeutic agents [119]. Phosphorylation contributes to the stabilization of interactions between BCL-2 and BAX, inhibiting BCL-2 degradation and blocks apoptosis due to the loss of p53 binding [117,120]. Similar implications can be found in MCL1 phosphorylation at Thr163 via the ERK1/2 pathway [83].

Phosphorylation of pro-apoptotic proteins seems to inactivate their functions in apoptosis. BAD is phosphorylated by cAMP/PKA or RSK1 at Ser112, Ser136 or Ser155 sites and then sequestered by 14-3-3 protein in the cytosol, thus inactivating its pro-apoptotic function. Conversely, upon growth factor withdrawal, BAD is activated by dephosphorylation at membranous sites to promote cell death [82]. BIM phosphorylation has been reported to be essential for lymphocyte maturation. BID is phosphorylated via the Akt/PI3K and cAMP-PKA pathways and is inactivated by binding to *BCL-2* due to the exposure of the hydrophobic face of the BH3 domain. BIM phosphorylation has been reported to be essential for lymphocyte maturation.

5.5.2. Dimerization

BAX is primarily cytosolic and cycled regularly between the cytosol and outer mitochondrial membrane, whereas BAK is anchored in the outer mitochondrial membrane [121]. Upon apoptotic stimulus, BAK

and BAX translocate from cytosol to the mitochondrial membrane, and undergo major conformational changes. These proteins form symmetric and labile homodimers that coalesce as disordered clusters to perforate the mitochondrial outer membrane for releasing cytochrome *c*. Linkage in the mobile BAK N-terminus (V61C), involved in dimer arrangement, is an important component for association between dimers. This knowledge could enable pharmacologic control of apoptosis in cells, potentially as an interventional treatment strategy.

5.5.3. Translocation

BIM translocates to the mitochondrial outer membrane through its association with the LC8 dynein light chain after stimulation and executes its pro-apoptotic activity via binding BCL-2 [122]. Similarly, BAX translocates from the cytosol to the outer mitochondrial membrane when it is activated [123].

5.5.4. Cleavage

It has been suggested that amino-terminal domains of BCL-2 family proteins can act as inhibitory domains. When stimulated by survival signals, BID can be cleaved into t-BID by caspase 8 and translocates to the mitochondrial membrane, resulting in cytochrome *c* being released from mitochondria [119].

5.5.5. Ubiquitination

Both BIM phosphorylation and BCL-2 dephosphorylation lead to ubiquitination and eventually degradation by the proteasome [124,125]. A1 can be ubiquitinated as its C-terminal portion degraded, losing its anti-apoptotic function [51]. When the inhibitor of NF-E2-related factor 2 (INrf2) interacts with the BH2 domain of BCL-2, BCL-2 initiates Cul3-Rbx1-mediated ubiquitination. Inactivating mutations of INrf2 which can stabilize BCL-2 and confer resistance to apoptotic stimuli have been found in various cancers. MCL1 is unique among anti-apoptotic BCL-2 family proteins for its rapid-turn-over through the ubiquitination. Deubiquitinase USP9X can stabilize MCL1, promotes cell survival, and has been proven as a prognostic and therapeutic target [126]. NOXA which sequester/inactivate MCL1 is degraded by the proteasome in an ubiquitylation-independent manner. NOXA mutations at its C-terminal tail attenuates its rapid degradation and stabilize endogenous MCL1 [127]. Instead, BOK, a multi-domain pro-apoptotic protein, can be degraded via the ER-associated degradation and ubiquitin-proteasome pathways, and it can be stabilized by proteasome inhibition (Fig. 4) [128].

6. BCL-2 family dysregulation in hematological malignances

Cancer is a heterogeneous group of diseases characterized by abnormal cell proliferation and/or decreased cell removal. Evasion of apoptosis by dysregulation of BCL-2 family members is a hallmark of hematological malignances (Fig. 5).

6.1. Dysregulation of anti-apoptotic BCL-2 in hematological malignancies

6.1.1. BCL-2

BCL-2 protein functions as an oncogene. In addition to BCL-2 gene activation by the chromosomal translocation t(14;18)(q32;q21), juxtaposing *IgH* with BCL-2 in FL [4], amplification of BCL-2 is detected in some patients with diffuse large B-cell lymphoma (DLBCL), multiple myeloma (MM) and acute myeloid leukemia (AML), also causes BCL-2 activation [129]. In DLBCL patients, BCL-2 expression levels may be a useful prognostic factor, especially in those with a low International Prognostic Index (IPI) [130]. The t(14;18)/*IgH*-BCL-2 is the most frequent translocation in GCB-DLBCL, 30–40% of the cases and is infrequent in ABC-DLBCL [5]. Concurrent overexpression of BCL-2 (anti-apoptosis) and Myc (pro-proliferation) correlates with a highly aggressive clinical course in DLBCL, associated with high-risk, early relapsed, short overall survival, frequent extranodal disease, central

nervous system involvement and resistance to conventional chemotherapy [3]. BCL-2 mutations are most frequent in GCB-DLBCL and FL, but they are not independently associated with survival [131]. In transformed FL, BCL-2 mutations predict for shortened survival and decreased duration of transformation to DLBCL. The exact role of BCL-2 mutations is not clear in each types of blood cancer. Systemic studies are essential but have been delayed due to the complexity of the mutational events and pleiotropic nature of the gene, resulting in difficulty in analysis of its synthesis and function. Precise sequencing and functional validation will shed new lights for diagnosis and treatment.

In mantle cell lymphoma (MCL) cells, BCL-2 inhibition has been correlated with activation of caspase-3 and caspase 9 and loss of cyclin D1a [132]. A high level copy number gain of the BCL-2 locus has been shown in MCL [133]. Activation of the AKT/mTOR [134], NF- κ B [135] and BCR pathways has been reported to contribute to upregulation of BCL-2, MCL1 and BCL-X_L in MCL, DLBCL and MM patients or cell lines [136].

A group of deleted or silenced miRNAs leading to BCL-2 over-expression were found in most CLL patients. These included miR-15a, miR-16.1, miR-34a, miR-29c, miR-17, miR-181b, miR-150, miR-155, miR-34 and miR-650 [104–106]. Otherwise, dysregulated miR-155 and miR-21 were detected in ABC-like DLBCL [103] and AIDS-related non-Hodgkin lymphomas (NHLs).

6.1.2. BCL-X_L

Elevated BCL-X_L levels have been reported in advanced and relapsed MM [137] and various types of NHL. Antagonism between BCL-X_L and BIM controls the apoptosis rate of Myc-induced lymphomas [138]. Somatic copy number alterations (CNAs) of BCL-X_L and MCL1 (gains) have been detected on a substantial proportion of human cancers [139], and the let-7 family of miRNA has been reported to inhibit BCL-X_L expression in some solid tumors [140].

6.1.3. MCL1

A unique anti-apoptotic member of the BCL-2 gene family with a short half-life, is amplified in diverse tumor cell lines and primary cells in patients with chronic myeloid leukemia (CML) [141], CLL [142,143], MM [144], AML and acute lymphocytic leukemia (ALL), particularly at time of relapse [2]. MCL1, similar to BCL-2, contributes to chemoresistance, disease relapse, poor progression-free survival and failure to achieve complete remission. A polymorphism of MCL1 (G486T) has been reported to be associated with poor overall survival in ALL patients [145]. Silencing MCL1 with interfering RNA (siRNA) can induce malignant cell apoptosis. A deubiquitinase USP9X can stabilize MCL1 and its level is associated with MCL1 expression in MCL, DLBCL and MM cell lines [126].

6.1.4. A1

A1 has been reported to be highly expressed in therapy-resistant CLL, large B cell lymphomas and AML and is associated with as poor prognosis [136,146,147]. However, A1 mRNA levels do not correlate with CLL progression, IGHV mutation or other status in CLL cells [148]. Inhibition of BCL-2 and BCL-X_L are not adequate to achieve complete apoptosis when the cells have a high level of A1 [148,149].

6.2. Dysregulation of pro-apoptotic BCL-2 in hematological malignances

6.2.1. BH3-only proteins

BIM allele has been reported to be deleted in 17% cases of MCL, and has been identified as a novel candidate tumor suppressor gene in MCL [150]. BIM deletion or polymorphism of BIM deletion contributed to the promotion of Myc-driven lymphomas and the development of MCL in cyclin D1 transgenic mice [151]. Loss of a single BIM allele is sufficient to promote Myc-driven lymphomas; however, loss of both alleles of BCL-X_L can attenuate this effect [138]. Polymorphism of BIM deletion results in its aberrant expression characterized by lack of a BH3

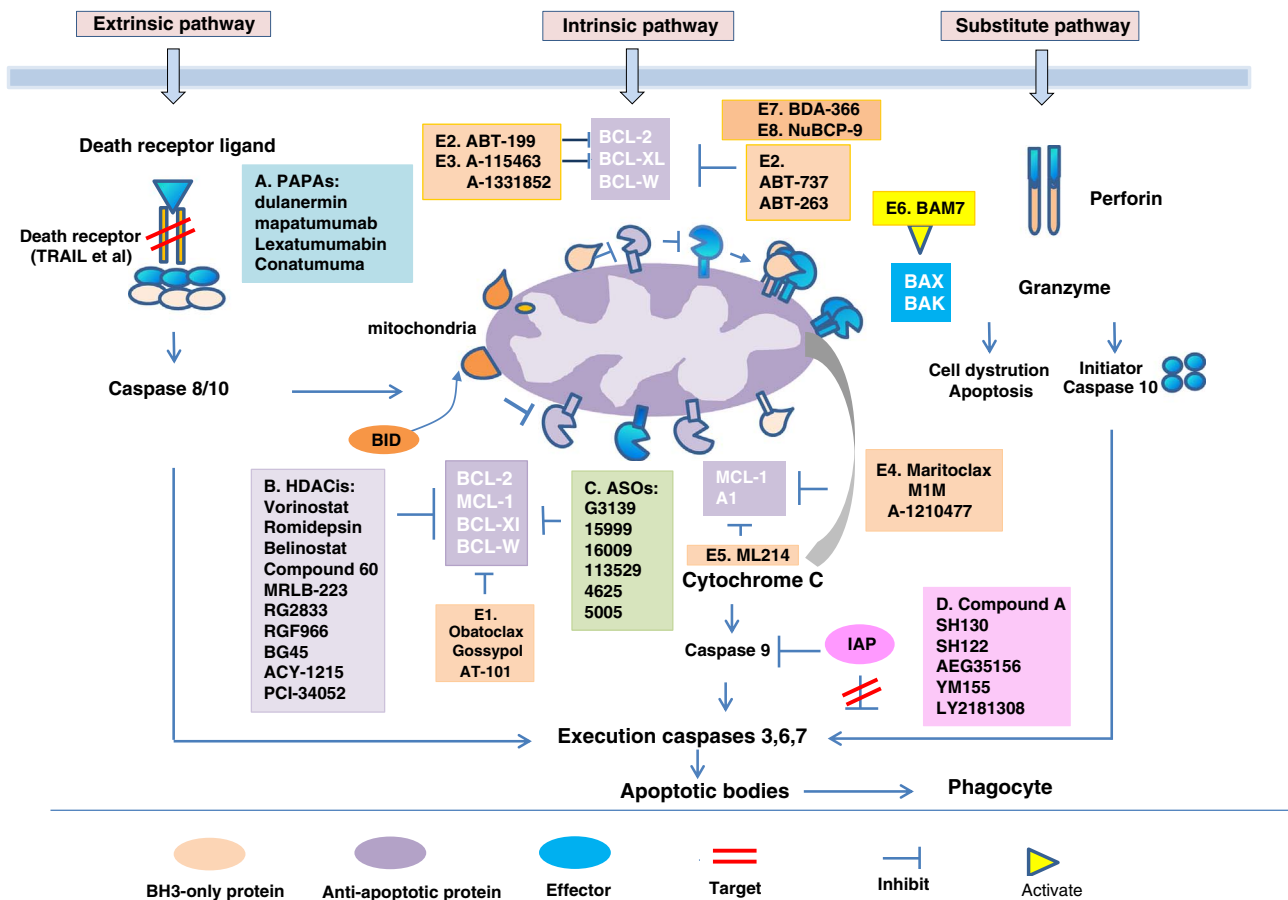


Fig. 5. Diagram of apoptosis- and BCL-2-targeted therapeutics. (A) Pro-apoptotic receptor agonists (PARAs) are antagonists of TRAIL-R in targeting the extrinsic apoptotic pathway. The representative regimens of PAPRs for treatment in hematological malignancies are dulanermin, mapatumumab, drozitumab, lexatumumab and conatumumab. (B) Vorinostat, romidepsin and belinostat are HDAC inhibitors approved by the FDA for the treatment of refractory cutaneous T-cell lymphoma and peripheral T-cell lymphoma. There are relatively specific HDAC inhibitor isoforms such as the HDAC1- and HDAC2-specific inhibitors compound 60 and MRLB-223; HDAC3-specific inhibitor RG2833, RGF966 and BG45; HDAC6-specific inhibitor ACY-121, and HDAC8-specific inhibitors PCI-34052 and C149, are being used in preclinical or clinical trials for the treatment of patients with hematologic malignancies. (C) An antisense oligonucleotide (ASO) is the first approach developed to target the intrinsic pathway. Oblimersom sodium (G3139 or OBL) is an 18-antisense oligonucleotide targeting BCL-2 mRNA. BCL-X_L ASOs 15999, 16,009 and 113,529 and the BCL-2/BCL-X_L-bispecific ASOs 4625 and 5005 have shown more sensitive responses than conventional chemotherapeutics in the treatments of multiple cancers. (D) Antagonists of IAPs are good additions to the list of apoptosis-inducing regimens. The most broadly used antagonists of IAPs include SH130, SH122, compound A, other strategies including AEG35156, YM155, and LY2181308. (E) BH3-mimetics: E1) Gossypol, AT-101 and Abatoclax counteract anti-apoptotic BCL-2 proteins in a nonspecific manner, and their activity seems to be off-targeted; E2) ABT-737, ABT-263 and ABT-199 are designed to specifically displace BH3-domains from the hydrophobic groove of anti-apoptotic BCL-2 proteins. E3) BCL-X_L-selective BH3 mimetics, A-1155463 and A-1331852, enhance the sensitivity of drugs that are resistant to ABT-737, ABT-263 and ABT-199. E4) Maritoclax is a novel MCL1 inhibitor and has shown potent and promising response in the treatment of MCL1-dependent hematological cancers. MIM1 and A-1210477 are two kinds of MCL1 specific inhibitors that have been identified recently. E5) The probe ML214 is a valuable research tool for study of targets to overcome A1. E6) BAM7 can engage in the BAX trigger site and promote the functional oligomerization of BAX. E7) BDA-366 is a potent and effective BH4 domain inhibitor that induces apoptosis and has potential to overcome therapeutic resistance in vitro and in vivo. E8) NuBCP-9 acts as a molecular switch to dislodge the BCL-2 BH4 domain, exposing its BH3 domain is being developed as an anticancer regimen.

domain, and patients experience inferior responses to tyrosine kinases inhibitors (TKIs) compared to patients without the polymorphism [152–154]. In mice, *BIM* gene mutations at the phosphorylation site Thr112 result in decreased binding activity of BIM to BCL-2 and thus increased cell survival, whereas mutation of the phosphorylation sites Ser-55, Ser-65, and Ser-73 increase apoptosis due to reduced proteasomal BIM degradation. PUMA along with NOXA are pivotal for the survival and recovery of hematopoietic cells after radiation damage [64]. NOXA is now being tested as a promising therapeutic target in cancer patients. PUMA and BIM expression are decreased in diverse hematologic malignancies, including Burkitt lymphoma, owing partially to hypermethylation of their promoter regions [155,156]. BID, a BH3 only pro-apoptotic protein connecting the extrinsic to intrinsic pathways, is involved in myeloid malignancies and associated with CMML progression in mouse models [61].

BIK, even in combination with NOXA, is neither a suppressor nor a factor that promotes chemotherapeutic killing in Myc-driven tumors [157]. The functions of *BIK* in apoptosis must overlap with other BH3-

only proteins [71]. BAD itself [66], or together with BMF [41], is involved in long-term suppression of Myc-aberrant B-cell lymphomagenesis. BAD deficiency is associated with the development of DLBCL, partly via inactivation of phosphorylation in the AKT/PKB pathway [82]. BMF is a novel component in the *TP53*-independent tumor suppressor pathway triggered by Myc [158]. Loss of BMF has been reported in CLL, and *BMF* mutations have been implicated in metastatic carcinomas [70].

6.2.2. Multiple-domain pro-apoptotic BCL-2

BAX functions as a tumor suppressor in human hematopoietic cells. BAX mutations occur in about 20% of hematologic malignancies, and result in premature termination of translocation; these mutations are thought to be key genetic alterations in lymphomas and leukemias [159,160]. In vitro experiments have shown that BAX alterations at its promoter and coding sequences abolish its apoptotic initiation function [57]. However, in a study that enrolled 112 nodal and extranodal B-cell lymphoma patients, only two patients had BAX mutations in the open

Table 2
Pathogenesis of BCL-2 family member dysregulation in hematologic malignancies.

CLL	DLBCL	MCL	FL	HL	MM	Myeloid leukemia	ALL	Other subtypes
BCL-2 overexpression [129]; PI3K/AKT and/or NF-KB activation [134,135]; A1 overexpression [148]; BAX mutations [159,160]; B1M loss [70]; Increased miR15/16, miR-17-92 [104–106]; Nucleolin [103]	t(14:18) [4,5]; BCL-2 amplification [129]; BCL-2 mutations [131]; USP9Xupregulation [126]; A1 Overexpression [146]; BAX mutations [154]; BAD deficiency [82]; miR-21 dysregulation [97]	BCL-2 copy number gains [133]; PI3K/AKT and/or NF-KB activation [134,135]; USP9Xupregulation [126]; B1M deletion or polymorphism of B1M [150,151]; Caspase 3, 9 cyclin D1a [132,151]	t(14:18) [4]; BCL-2 mutations [131]	PUMA and B1M promoter hypermethylation [155,156]	BCL-2 and BCL-XL overexpression [136,137]; MCL-1 overexpression [144]; USP9Xupregulation [126]	BCL-2 overexpression [114–116]; MCL-1 overexpression in CML and AML [141,2]; A1 overexpression in AML [146]; B1M deletion polymorphism in CML [152,154]; B1D deletion in CMMML [61]; BAK mutations [159]	BCL-2 and MCL CNAs gains [132]; MCL-1 overexpression [2]; BAX mutations [159]; Polymorphisms of both MCL-1 and B1M [145]	BCL-2 and MCL CNAs gains in human cancers [132]; BOK CNAs loss in human cancers [139]; B1M mutations in metastatic carcinomas [70]

Abbreviations: CLL: chronic lymphocytic leukemia; SLL: small lymphocyte lymphoma; cHL: classical Hodgkin lymphoma; NHL: non-Hodgkin's lymphoma; MCL: mantle cell lymphoma; FL: follicular lymphoma; DLBCL: diffuse large B-cell lymphoma; BL: Burkitt lymphoma; AML: acute myeloid leukemia; CML: Chronic myelogenous leukemia; ALL: acute lymphoblastic leukemia; SCNA: somatic copy number alteration;

reading frame [161]. BAK enhances the apoptotic pathway, however, it inhibits cell death in an EBV-transformed cell line [58,59]. Loss of somatic copy number of BOK has been reported in a substantial proportion of cancers [139]. These genetic and epigenetic defects of the BCL-2 family contribute to the design and development of novel anti-cancer strategies in hematological malignancies (Table 2).

7. Implication of apoptosis- and BCL-2-targeted therapies

Evasion of apoptosis is a hallmark of tumorigenesis and also a crucial acquired capability used by cancer cells to fend off anticancer therapies. Although chemotherapeutic agents and other immunologic therapies have improved the outcome of patients with hematological malignancies, not every patient can benefit from these therapies, especially elderly patients and patients with relapsed or refractory disease. Apoptosis- and BCL-2-targeted therapeutics have been developed to enhance the efficacy and reduce side-effects of treatments, many of which have shown promising efficacy in preclinical and clinical trials for patients with hematologic neoplasms.

7.1. Targeting inhibitors of apoptotic proteins (IAPs)

IAPs are involved in inhibiting the extrinsic and intrinsic apoptotic pathways. Expression and/or functions of IAPs are altered, due to genetic aberrations, increased mRNA or protein levels, or loss of their endogenous inhibitors in various cancers [12,162]. These abnormalities are associated with poor prognosis and chemo-resistance in human adult T-cell leukemia [163], AML [12], T acute lymphoblastic leukemia (T-ALL) [164], CLL [165] and DLBCL [166].

Antagonists of IAPs are good additions to the list of apoptosis-inducing regimens [167]. Among the therapeutic strategies designed to target IAP proteins, the most broadly used was designed based on mimicking the IAP-binding motif of SMAC (second mitochondria derived activator of caspase), such as SH130, SH122 and compound A (CA). Other strategies included XIAP antisense oligonucleotides (such as AEG35156, YM155) and a survivin mRNA anti-sense mediated interference regimen (LY2181308). These agents have shown their pro-apoptotic activities in preclinical and clinical trials for patients with lymphoma and leukemia [168,169]. The efficacies of the treatments, however, need further validation [170].

7.2. Targeting death receptors (DRs)

Extrinsic pathway apoptosis is mediated by death receptors and their ligands, including TRAIL-TRAIL-R interactions. TRAIL-R is highly expressed in a variety of tumor cells (mainly in solid tumors) [171], thereby rendering TRAIL-R a potential target in anti-cancer therapy. Pro-apoptotic receptor agonists (PARAs) include antibodies against TRAIL-Rs, recombinant human TRAIL antibodies, recombinant human proteins and small molecules against TRAIL-R. Representative agents include dulanermin, mapatumumab, drozitumab, lexatumumab and conatumumab. Although dulanermin has displayed activity as a single agent, as well as in combination with chemotherapy and radiotherapy in a variety of pre-clinical models, this agent has not demonstrated efficacy in clinical trials because of its short bioavailability [172–175]. Mapatumumab has shown promising single-agent efficacy in patients with refractory/relapsed NHLs [11,176,177].

7.3. Targeting BCL-2 family members

BCL-2 family proteins determine the commitment of cells to apoptosis. Deregulation of BCL-2 proteins has been detected in hematological malignancies and is associated with the prognosis and resistance. Inducing apoptosis by targeting BCL-2 proteins is considered a potentially promising therapeutic approach, not only by causing cancer cells death but also reversing resistance or inducing sensitivity to the current

Table 3
Clinical trials of BCL-2- targeted therapeutics in hematological malignancies.

Regimen	Subject	Trial	Dose schedule	Adverse effect	Efficacy
ABT-737 — target to BCL-2, BCL-XL, BCL-W Primary cells from CLL (n = 30)	Combine with chemotherapy Preclinical Single or combine with HDAC inhibitors Preclinical		5–200 μM + Dex 0–10 μM, or Eto 0–200 μM, or Dox 0–5 μM, or F-Ara-A 0–10 μM		RR improvement and potential efficacy in CLL [220]
Primary Eμ-Myc lymphoma cells			0.5or 1 μM, + HDACi 0.5–5 μM		Single efficiency in overexpressing BCL-2 lymphomas and synergic efficiency in primary Eμ-Myc lymphoma cells [222]
ABT-263 (Navitoclax) — target to BCL-2, BCL-XL, BCL-W Relapsed/refractory lymphoid malignancies (n = 55)	Single Phase I		Intermittently 10–440 mg/d, × 14 days, 21-d cycle Continuous doses of 200–425 mg/day, × 21 days, 21-d cycle	D 56%, N 38%, A 75%, Neu33%, T 52%	PR 22%; PFS 455 days High efficacy with durable responses in drug-resistant malignancies [223]
Relapsed/refractory CLL (n = 26)	Single Phase I		10–250 mg/d for 14-d cycle or 125–300 mg/d for 21-d cycle	D 76%, N 59%, F 35%, RI 31%, Neu28%, T 28%	PR 35%, SD ≥ 6 m: 27%, PFS 25 m valid therapeutic in CLL [224]
Relapsed/refractory CLL (n = 29)	Combine with R Phase Ib		Lead-in dose150 mg/d × 7d on Week 1 Day-2 (W1 D-2);200–325 mg/d lead-in dose; R: 375 mg/m ² × 4 doses on W1 D-1 before ABT-263 escalation	D 79%, N 72%, RI 8%, Neu28%, T 7%	PR 35%, CR 5%; SD ≥ 6 m = 27% FL: ORR 75%, CLL: PR 100% Combination is more efficient than in monotherapy [225]
ABT-199 (Venetoclax, RG7601, GDC-0199) — target to BCL-2 Relapsed/refractory CLL/SLL (n = 56/60)	Single Phase I		56 pts: Venetoclax ranged from 150 to 1200 mg/d 60 pts: a weekly 20 mg/d stepwise ramp-up to 400 mg/d	D 52%, N 47%, F 27%, RI 48% Neu28%, T 7%	ORR 79%; CR 20%, MRD negative 5% PFS = 15 m: 69% (at dose of 400 mg) 35% occurred disease progression OS = 2 year: 84%
Relapsed/refractory NHL(MCL, FL, DLBCL, WM) (n = 44)	Single Phase I		ABT-199 given on W1 D-7, lead-in 2–3 weeks, final to 200–900 mg	D 25%, N 65.4%, RI 27% F 21%	Safe and substantial responses in relapse CLL or SLL [228] ORR 48% (9/12 MCL, 3/11 FL, 3/9 DLBCL). ABT-199 monotherapy showed efficacy in a range of NHL, especially in MCL and WM [229]
Relapsed/refractory CLL (n = 37)	Combine with R Phase Ib (NCT01682616)		ABT-199 began at 20 mg to 200–600 mg weekly R dose at 375–500 mg/m ² for 6 m	D 30%, N30%, Neu 43%	The combination has potential in hematological malignancies [233]
Relapsed/refractory CLL (n = 49)	Combine with R Phase II		ABT-199400 mg/d; R from 375 to 500 mg/m ² monthly for 6 doses	D 37%, N 41%, Neu47%, P 3%, H 31%, F and RI each 29%	ORR 88%, CR/CRi 32%; PR 56%; 29% MRD negative The combination is safe and effective in CLL/SLL [234]
Relapsed/refractory NHL (n = 26)	Combine with B and R Phase I (NCT01328626)		ABT-199 50–400 mg on 3, 7, 28 d, 28 d/cycle × 6 cycles; Standard B 90 mg/m ² , 2 d/cycle; R: 375 mg/m ² , 1 d/cycle; Combination 4–6 cycles	D 38.5%, N65.4%, A 42.3%, Neu 42.3%, T 34.6%	ORR(CR + PR) 61.5%; 73.3% in FL; 37.5% in DLBCL The combination is tolerable and enhance the efficacy of BR [235]
Relapsed/refractory AML (n = 32)	Single Phase II		20 mg/d on week 1 day 1, final dose to 800 mg/d on day 6 and daily after, or to 1200 mg	D 38%, N 44% RI 9%, Neu 9% T 25%	ORR 15.5%; CR 3%; CRi 12.5% MRD negative 6%; 31% progressed Considerable single-agent activity in R/R AML, particularly to pts with IDH mutations [236]
Untreated older (≥ 65 years) AML	Combine with DEC or AZA (NCT02203773)		ABT-199 escalation to 800 mg DEC 20 mg/m ² [iv] daily or AZA 75 mg/m ² , 28 d cycle	Neu 30% T 8–30%	ORR 76% Combination with DEC or AZA demonstrates a tolerable safety profile [237]

Abbreviations: R: rituximab; Dex: Dexamethasone; Eto: etoposide; Dox: doxorubicin; B: bendamustine; DEC: decitabine; AZA: azacitidine; Bor: bortezomib; I: imatinib; PSI: prednisolone; LEN: lenalidomide; Pts: patients; D: diarrhea, N: nausea; F: fatigue; RI: respiratory infection; Neu: neutropenia; T: thrombocytopenia; P: pyrexia; H: headache; A: anemia; ORR: overall response rate; CR: complete response; PR: partial response; SD: stable disease; MTD: maximum tolerated dose; R/R: refractory/relapsed; PFS: progression-free survival; OS: overall survival; CRi: complete response with incomplete bone marrow; MRD: minimal residual disease. RR: response rate; WM: Waldenström macroglobulinemia.

Table 4
Preclinical trials of BH3 mimetics in hematological malignancies.

Regimens	Preclinical trial	Target disease	Conclusion
ABT-199 - target to BCL-2 Combination with I and Car		Primary cells from MCL and CLL	More efficient compared to each single agent alone; minimal influence on normal cells; a clinical trial with ABT-199 and I is planned [238].
Single and in combination with HTT Combined with Bor and JQ1 Combined with TKI		DLBCL primary cells or cell lines DHL cells Primary cells from CML resistant to TKIs; CML mouse model	Significant synergistic efficacy in BCL-2 expressed DLBCL cell lines and mice models [329]. The combination provides a rational trial for relapsed DHL [240]. ABT-199 alone or in combination was better than TKIs alone in eradicating CML stem cells in vivo [241].
WEHI-539, A-1155463 and A-1331852 - target to BCL-X _L , oral available Single and in combination of Bor		Human MM cell line and mouse models	Xenografts that expressed BCL-X _L , MCL1 and BCL-2 were more sensitive to the Bor combination with A-1331852 than with venetoclax and better than in monotherapy [245].
Combination with D		AML cell lines	Synergistic efficacy and should get higher exposures before dose-limiting effect on neutrophils and platelet [246].
MIM1 - target to MCL1 Single or combined with ABT-737		Leukemia cell lines	Has MCL1 dependent antileukemia activity and synergy with ABT-737 [247].
A-1210477 - target to MCL1 Single or combined with ABT-263		Multiple cancer lines including MM cell lines	Induced MCL1 - dependent apoptosis in single and in synergy with ABT-263; potential therapeutics for cancer treatment [246,248].
CID701939Probe ML214 - target to A1 On research			A valuable research tool for further study to overcoming A1 in cancer cells [250]
BAM7 - target to Trigger BAX Report a molecular modulator			Engages the BAX trigger site and promotes the functional oligomerization of BAX [123].

Abbreviations: see abbreviations of Table 2.

treatments. Advancements in our understanding of the structure and functions of BCL-2 family proteins and mechanisms of BCL-2 protein interactions in the apoptotic pathway, together with the developing technologies, provide improved opportunities. To date, many of these therapies have shown high efficacies in preclinical and clinical trials.

7.3.1. Histone deacetylase inhibitors (HDAC) block gene expression of BCL-2 family proteins

Histone deacetylases are a class of epigenetic regulators and play an essential role in controlling *BCL-2* gene expression. Altered expression and/or functions of HDACs have been reported in a variety of blood cancers and are often associated with poorer prognosis [178,179]. HDAC inhibitors have been shown to be potent anticancer agents. Vorinostat and romidepsin are two kinds of HDAC inhibitors that have been approved by FDA for the treatment of patients with refractory cutaneous T-cell lymphoma (CTCL) [180]. In addition, belinostat and romidepsin have been approved by the FDA for the treatment of patients with peripheral T-cell lymphoma (PTCL). Many HDAC inhibitors have been on the list of clinical trials either singly or in combination therapy applications [181–186]. HDAC inhibitors combined with proteasome inhibitors such as bortezomib have shown promising effects in patients with refractory/relapsed MM [187]. Some specific HDAC inhibitor isoforms are being used in preclinical or clinical trials, such as the HDAC1- and HDAC2-specific inhibitors compound 60 and MRLB-223 [188], HDAC3-specific inhibitor RG2833 [189], RGFP966 [190] and BG45 [191], HDAC6-specific inhibitor ACY-1215 [192], and HDAC8-specific inhibitors PCI-34052 and C149 [101,193]. These HDAC inhibitor agents have shown variable efficacy in different types of blood cancer.

7.3.2. Antisense oligonucleotides silence BCL-2 mRNA

An antisense oligonucleotide (ASO) is the first approach developed to target the intrinsic apoptotic pathway, using a single complementary oligonucleotide strand to target the mRNA of anti-apoptotic BCL-2 members, down-regulating the expression of anti-apoptotic proteins, and ultimately decreasing cell survival.

Oblimersom sodium (G3139 or OBL) is an 18-antisense oligonucleotide targeting *BCL-2* mRNA, that has single-agent activity or combination cytotoxicity in preclinical and clinical trial studies.

Combination studies with other agents have been explored including daunorubicin in patients with AML [194,195], rituximab in B-cell NHLs [196], dexamethasone in relapsed MM [197], fludarabine/cyclophosphamide in relapsed/refractory CLL [198], and imatinib in CML [199]. These results showed that all patients achieved a better overall response rate or high partial or complete remission rate when compared to patients treated with these regimens without oblimersom sodium.

BCL-X_L ASOs (15999, 16009 and 113529) and BCL-2/BCL-X_L-bispecific ASOs (2'-O-(2-methoxy) ethyl (2'-MOE)-modified gapmer antisense oligonucleotide 4625 and 5005) have revealed more sensitive responses than conventional chemotherapeutics in patients with multiple cancers [200,201].

However, most clinical trials have failed to show a significant improvement in survival when compared with standard treatment. ASOs are still not approved by FDA due to their low stability, short half-life, non-specific binding and insufficient survival advantage.

7.3.3. BH3-mimetics interact with BCL-2 proteins by mimicking BH3 domains

One major strategy targeting BCL-2 has been the development of small molecules that mimic the BH3 domain. BH3 mimetics are small molecules or peptides that functionally mimic the pro-apoptotic effects of the BH3-only subset counteracting the hydrophobic grooves of anti-apoptotic proteins in cancerous tissues. Gossypol, AT-101 and obatoclax, identified through compound library screening, counteract anti-apoptotic BCL-2 proteins in a nonspecific manner but also show off-target activity. ABT-737, ABT-263 and ABT-199 were designed by 3D structural technology, which specifically displaced BH3-domains from different anti-apoptotic BCL-2 proteins. To date, about 20 compounds and BH3-mimetics have been developed (Table 3 and Table 4).

7.3.3.1. Gossypol. This compound is derived from a natural product of cottonseed and roots, and functions as a kind of BH3 mimetic that acts as pan-BCL-2-superfamily inhibitor. Gossypol disrupts the heterodimerization of BCL-2 with pro-apoptotic proteins, partly by inhibiting IL-6 prosurvival signals [202]. In the preclinical setting, gossypol exhibited single-agent activity in DLBCL [203], CML [204], MM [205], CLL [206,207], NHL cell lines [208] and synthetic efficacy in some solid tumors cells [155]. There is no phase I/II clinical data

reported for gossypol reported in hematological malignancies. AT-101 (R(-)-gossypol or λ -isomer of gossypol) was the first orally active pan-BCL-2 inhibitor, demonstrating promising efficacy when combined with rituximab in CLL patients but only showed limited efficacy in a phase II study [209]. The toxicity profile of gossypol was tolerable, including diarrhea, fatigue, nausea and anorexia. However, the serious off-target effects and thrombocytopenia prevented gossypol for further development in clinical trials.

7.3.3.2. Obatoclox (GX15-070). This agent is pan-BCL-2 inhibitor derived from prodiginine that influences apoptosis partially by inhibiting the AKT/mTOR pathway [210]. Obatoclox displayed single-agent activity in preclinical settings, and it showed limited activity in phase I/II trial for older AML patients [211], untreated myelodysplastic syndrome (MDS) [212], primary infant ALL with MLL translocation [213], CLL, refractory/relapsed MCL [214] and classical Hodgkin lymphoma (HL) [215]. The side-effects of obatoclox were largely neurological, including fatigue, euphoria, somnolence, ataxia, diarrhea, nausea, thrombocytopenia, anemia and pneumonia.

7.3.3.3. ABT-737. This agent is a BH3 domain mimetic of BAD, which induces apoptosis by binding selectively to BCL-2, BCL-X_L and BCL-W. In preclinical assessment, ABT-737 demonstrated encouraging efficacy singly or in combination with various cytotoxic drugs in MM [216], AML [217], Myc-driven lymphomas [218,219], CLL [220], HL [221] and early T acute lymphoblastic leukemia [222]. The BCL-2/BAX ratio could be used as a parameter to predict treatment response and can reflect early response of ABT-737 treatment in CLL and MM cells [216]. However, clinical application has been hampered by its poor oral bioavailability and low aqueous solubility making intravenous drug delivery a challenge.

7.3.3.4. ABT-263 (navitoclax). It is an orally available derivative of ABT-737 that exhibits encouraging efficacy in inhibiting BCL-2, BCL-X_L and BCL-W. In clinical settings, this drug was well-tolerated with moderate efficacy as a single-agent and showed high synergy with traditional chemotherapeutic agents in hematological malignancies, including relapsed/refractory disease.

In an initial study of 55 patients with relapse/refractory lymphomas, ABT-263 demonstrated objective partial remission and tumor size reduction in patients with CLL [223]. A follow-up study of ABT-263 in 29 patients with refractory CLL, including those with resistance to fludarabine, those with bulky adenopathy and those with 17p-CLL, exhibited high percentage of stable disease with a long median progression-free survival [225]. When combined with rituximab, ABT-263 has demonstrated higher activity in patients with relapsed/refractory B-cell NHL [224]. Common toxicities included mild diarrhea, nausea, fatigue, dose-limiting neutropenia and thrombocytopenia. The BIM/MCL1 or BIM/BCL-2 ratio could be used as a biomarker to evaluate the anticancer effects of ABT-263. Low MCL1 level before treatment and high BIM/MCL or BIM/BCL-2 ratio during the therapy were associated with better clinical response to ABT-263 [224]. The key side effect of dose-dependent thrombocytopenia limited the use of ABT-263, particularly in leukemia patients who often present with thrombocytopenia.

7.3.3.5. ABT-199 (venetoclax). ABT-199 is a BCL-2-specific BH3-mimetic developed from ABT-737. It has been identified as having robust pro-apoptotic function in treating hematologic tumor cells without reducing platelet lifespan even at doses 20 times higher than that of ABT-263 [226].

In preclinical trials, ABT-199 demonstrated single-agent efficacy in AML [227], ALL and NHLs (DLBCL, FL and MCL) [170]. In clinical trials, ABT-199 demonstrated high single-agent activity in 116 patients with relapsed/refractory CLL or NHLs. This cohort included CLL with 17p-deletion, unmutated IGHV and those resistant to fludarabine. ABT-199 achieved a 79% overall response rate and 20% complete remission

in those CLL/SLL patients, with a low of or no minimal residual disease. Dose-escalation protocol minimized the key side effect of tumor lysis syndrome by ABT-199 [228]. Another single-agent administration of ABT-199 in 44 patients with relapsed/refractory NHLs (15 MCL, 11 FL, 10 DLBCL, 4 Waldenström Macroglobulinemia) showed a 48% overall response rate, most notably in MCL and WM [229], and responses were observed at high doses in DLBCL and FL.

The high-quality response with MRD-negative complete remission using ABT-199 in Phase I trial pushed it rapidly into Phase II-III trials as a single agent for the treatment of patients with relapsed/refractory NHLs (particularly CLL). This distinguished ABT-199 from the B-cell receptor antagonists (ibrutinib and idelalisib), where complete remission has been rarely reported in the relapsed/refractory patients [230–232]. ABT-199 has been approved by the FDA for use as monotherapy in CLL with 17q- who have received at least one prior therapy.

In the followup studies, combination of ABT-199 with rituximab showed high degree of overall response (80%), complete remission (39%) and partial remission (39%) in relapsed/refractory CLL [233,234]. When combined with bendamustine and rituximab in 26 relapsed/refractory NHL [235], ABT-199 demonstrated much higher activity than single-agent administration (overall response rate 61.5% vs. 48%) [229].

In a phase II study, ABT-199 also exhibited satisfying activity as a single agent in 32 patients with high risk relapsed/refractory AML or patients unfit for intensive therapy, showing a 15.5% overall response rate and 19% of the patients achieving > 50% reduction of blast cell percentage in bone marrow at first assessment; notably 33% of patients with *IDH1/2* mutations achieved complete remission [236]. The combination of ABT-199 with low dose cytarabine or HMAs showed encouraging results in untreated older (≥ 65 years) AML, demonstrating a 44% overall response rate with acceptable tolerability [162]. When combined with decitabine or azacitidine, ABT-199 showed an overall response rate of 76% [237]. These results suggest that combining ABT-199 with other agents in older untreated AML patients was encouraging and further validation may refine better the scheduling.

ABT-199 is currently in phase ½ clinical trials as monotherapy and in combination with bortezomib plus dexamethasone in relapsed MM. Studies that combined ABT-199 with ibrutinib [NCT02471391], anti-CD20 mAbs or other chemotherapeutic agents in patients with NHL are in progress with impressive preliminary results.

Preclinical studies in which ABT-199 was combined with rifampin, ibrutinib and carfuzomib in primary MCL and CLL cells [238], with homoharringtonine [239] or bortezomib and JQ1 in primary DLBCL cells [240], and with tyrosine kinase inhibitors in primary CML cells [241] have shown variable activity.

The main adverse events of ABT-199 included nausea, diarrhea, fatigue, upper respiratory tract infections, anemia, neutropenia and thrombocytopenia. Tumor lysis syndrome (TLS) can be mitigated by a stepwise dose escalation strategy.

For BCL-2 targeted therapy, early predictive biomarkers are important to assess the efficacy of apoptosis-targeted therapies and ensure adequate safety. “BH3 profiling” parameters are considered as a promising predictive biomarker for investigating the response to ABT-199 or ABT-737 in clinical trials for blood cancers [242]. A typical BH3 profiling that measures the MOMP by cytochrome *c* release is quantified by ELISA (heavy membrane cytochrome *c* assays or HM-CC assay). It has been developed into whole cell JC-1-based BH3 profiling (WC-JC-1) which is more convenient to measure the release of cytochrome *c* by adding fluorescent probe JC-1. FACS-based-WC-JC-1 can even distinguish different BH3 profiling in heterogenous populations of cells. All of these methods of BH3 profiling are proved to be effective and uniformly measure the BCL-2 expression and interaction of > 25 pro- and anti-apoptotic BCL2 family members, therefore functioning as a robust predictor of response to targeted therapies. It may prospectively supervise the treatment selection and evaluate mechanisms of drug resistance [243,244].

The current preclinical and clinical studies have shown that ABT-199 has a manageable safety profile, high-quality specific efficacy and a higher overall response rate for patients with hematological malignancies, especially for those with refractory/relapsed disease ABT-199 is likely to be a breakthrough compound worthy of further clinical investigations due to its highly selective inhibitory affinity for BCL-2 and promising therapeutic effectiveness (Table 3).

7.3.3.6. BCL- X_L -selective BH3 mimetics. These agents, including WEHI-539, A-1155463 and A-1331852, enhance the sensitivity of drugs for diseases that are resistant to ABT-737, ABT-263 and ABT-199. Several studies have suggested that BCL- X_L is heterogeneously expressed in MM cells [245] and AML cells [246]. BCL- X_L overexpression may be a potential factor accounting for resistance to ABT-199 therapy. A-1155463 enhanced the sensitivity to these drugs, which efficiently disrupted BCL- X_L /BIM complexes in MM cells [245].

7.3.3.7. MCL1 antagonists. MCL1, an anti-apoptotic protein that is frequently amplified in blood cancers, has been identified as an attractive target for therapy. However, MCL1 was found bind to BCL-2 inhibitor (ABT-263) with low affinity [246]. The natural product marinopyrrole A (maritoclax) is a small molecule that can trigger MCL1 degradation and mitochondrial fragmentation. Combined with its derivatives KS04 and KS18, maritoclax is potent in patients with MCL1-dependent blood cancers, and also shows significant synergetic efficacy with ABT-737 [247]. MIM1, a hydrocarbon-stapled MCL1 inhibitor, exhibited apoptosis-inducing efficacy in leukemia cells. BI97C1, BI112D1 and TW-37, that are developed with improved and selective affinity in binding to MCL1, induced apoptosis in a BAX/BAK- and caspase-9-dependent manner. Combination of ABT-263 with TW-37 induced extensive apoptosis, whereas TW-37 alone induced apoptosis in a BAK-dependent and partly NOXA-dependent manner [248].

A-1210477 and S63845 show more selective and higher affinity in binding to MCL1. Both of them have demonstrated singly and synergetic effect in various lymphoma and leukemia cell lines [248,249]. A-1210477 binds to MCL1 and disrupts MCL1/BIM complex, and S63845 induces apoptosis in a BAX/BAK dependent pattern. Higher expression of MCL1 has been reported in ABC-DLBCL compared to GCB-DLBCL, and conceivably, MCL1 might represent a new drug target in patients with ABC-DLBCL [248]. However, the requirement for high affinity protein-protein interaction in targeting MCL1, short half-life of MCL1 and side effects of MCL1 drugs including cardiac and neurological toxicity make the selection process difficult.

7.3.3.8. A1 (human Bfl-1 protein) antagonist and BAX triggers. The probe ML214 was reported to be a valuable research tool for further study to overcome A1 in the treatment of cancers [250]. BAM7 was identified as a molecular modulator, which can engage the BAX trigger site and promote BAX functional oligomerization without interacting with the BC groove and BAK. This observation may help design a new generation of apoptotic modulators that can directly activate BCL-2 pro-apoptotic proteins as cancer therapeutics (Table 4) [123].

7.4. Targeting BH4 domain

The conserved BH4 domain is crucial for BCL-2 anti-apoptotic activity. Different from the BH3 domain, the BH4 domain is involved in many cellular functions beyond apoptosis including participating in interactions with BAX, forming a heterodimer with BAX and failing to inhibit apoptosis. Furthermore, BCL-2 regulates Ca^{2+} signals from the endoplasmic reticulum through the interaction of the BH4 domain with the regulated and coupling domain of IP3R. Based on the structural and functional properties of the BH4 domain, targeting the BH4 domain of BCL-2 may be a novel and attractive strategy for blood cancer patients [20].

BDA-366, a small molecule, which can induce BCL-2-dependent BAX activation and BCL-2-IP3R binding, was recently discovered as a potent and effective BH4 domain inhibitor that has the potential to overcome therapeutic resistance in vitro and in vivo [20]. The combination of BDA-366 with an inhibitor of mammalian target of rapamycin (mTOR) exhibited excellent synergistic effects in overcoming chemoresistance of lung cancer [251]. However, its efficacy in hematological malignancies has not been evaluated.

7.5. NuBCP-9, a Nur77-derived BCL-2 converting peptide

NuBCP-9, a Nur77-derived BCL-2 converting peptide, is a 20 amino acid peptide that acts as a molecular switch to dislodge the BH4 domain of BCL-2. As a result, the BH3 domain is exposed and converts BCL-2 into a “BH3-like” pro-apoptotic killer molecule that in turn blocks the activity of anti-apoptotic BCL- X_L . Therefore, NuBCP-9 has broad potential as an anticancer therapy [252].

8. Conclusions and perspective

Apoptosis is central to both cell survival and death. BCL-2 family members are sentinel molecules governing apoptosis via a complex network that is regulated at multiple molecular levels. Dysregulation of apoptosis is a hallmark of hematological malignancies and contributes to disease progression and chemoresistance. Studies of BCL-2 superfamily regulation and mechanisms have elucidated the roles of BCL-2 in the development of hematological malignancies and have contributed to the design of novel anti-cancer strategies to target the BCL-2 alterations. Functional studies have shown that each of the individual BCL-2 family members exerts a highly selective role in apoptotic pathways. This characteristic creates a unique opportunity for individual agents or combined regimens in regard to specific cell-of-origin and developmental cell stages, to achieve an optimal therapeutic response.

With an increased understanding of the extrinsic and intrinsic apoptotic pathways in recent years, novel approach to regulate each component of the apoptotic pathways has been tested in pre-clinical and clinical trials. Targeting BCL-2 family members has become one of the highly valuable anti-cancer strategies. Upstream signaling regulators and post-transcriptional and post-translational factors that modulate expression and function of BCL-2 family members might also broaden the anti-cancer landscape specific to this pathway. Many pathologic functions of the BCL-2 proteins are also attributed by mutations, especially those in the BH4 and FLD domains. These mutations may show gain-of-function, and impact cell cycle regulation, autophagy, mitochondrial energetics, Ca^{++} homeostasis, vascular proliferation, environmental stromal reaction via non-canonical and non-apoptotic mechanisms. In addition, structural investigation of BCL-2 family proteins could significantly enhance drug discovery and target designation. Interaction of BCL-2 with p53 and Myc renders a complex picture of mechanism in blood cancers. It is believed that systemic and functional evaluation of BCL-2 genetic alterations will be valuable to unfold molecular events underlying disease progression and therapeutic resistance.

Apoptosis- and BCL-2-targeted therapeutic agents have been developed to improve the efficacy and reduce side-effects of anti-cancer therapies. Many of these drugs have shown promising efficacy in pre-clinical and clinical trials for the patients with hematological tumors. ABT-199, which is designed to mimic the BH3 domain of BCL2 proteins, is approved by the FDA as a single-agent regimen for patients with relapsed/refractory CLL, and may emerge as an attractive agent for treatment of other hematological malignancies. Optimal use and combination with other conventional or targeted agents needs to be further explored. It is believed that novel approaches for promoting apoptosis of tumor cells will be continuously developed as one effective anti-cancer therapy. Such strategy may not only enhance the therapeutic

efficacy, but also potentially reduce adverse events by increasing cancer cell sensitivity to the specific targeted drugs.

Practice points

- *BCL-2* is a new class oncogene that functions as an anti-apoptotic molecule preventing cells from normal apoptosis. Anti-apoptotic *BCL-2* family members protect cells from apoptosis against a broad range of cytotoxic stimuli.
- The balance between anti-apoptotic and pro-apoptotic *BCL-2* family members is finely regulated and controlled at a multitude of molecular levels, from initial signaling to post-translational modification.
- Structural and functional studies are pivotal in drug discovery and will identify more efficient *BCL-2*-targeted drugs stimulating cancer cell apoptosis.
- The exact role of *BCL-2* mutations is not clear. Systemic studies are essential and will shed new lights for diagnosis and treatment in patients with hematologic neoplasms.
- Dysregulation of the *BCL-2* pathway occurs frequently in hematological malignancies and variable strategies have been designed to target anti-apoptotic *BCL-2* and/or activate pro-apoptotic *BCL-2* pathway molecules. ABT-199 is one of these promising compounds and has been approved by the FDA for its superior efficacy in patients with relapsed/recurrent CLL.

Research agenda

- Appropriate diagnostic and biomarker testing platforms for assessing the *BCL-2* signaling pathway for risk stratification in hematological malignancies, such as lymphoma, MM, AML, MDS, MPN or MDS/MPN.
- Clinical value of *BCL-2* pro-apoptotic and pro-apoptotic pathway dysregulation, structural alterations and relevant therapeutic regimen selection.
- Relationship between monotherapy and combination regimen by targeting PI3K/AKT, Myc, PD-1/PD-L1, p53/MDM2, ubiquitination and associated potential side effects.
- Development of a clinical algorithm for patient evaluation using BH3 mimetic profiling and antagonists.
- Molecular mechanism and regulatory processes of *BCL-2* family members in the immune response and tumor microenvironment.

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