Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Review Emerging understanding of Bcl-2 biology: Implications for neoplastic progression and treatment

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ARTICLE INFO

Article history: Received 28 January 2015 Received in revised form 20 March 2015 Accepted 22 March 2015 Available online 27 March 2015

Keywords: Apoptosis BH3 mimetic Activation-induced cytidine deaminase Mutation Follicular lymphoma

ABSTRACT

Bcl-2, the founding member of a family of apoptotic regulators, was initially identified as the protein product of a gene that is translocated and overexpressed in greater than 85% of follicular lymphomas (FLs). Thirty years later we now understand that anti-apoptotic Bcl-2 family members modulate the intrinsic apoptotic pathway by binding and neutralizing the mitochondrial permeabilizers Bax and Bak as well as a variety of pro-apoptotic proteins, including the cellular stress sensors Bim, Bid, Puma, Bad, Bmf and Noxa. Despite extensive investigation of all of these proteins, important questions remain. For example, how Bax and Bak breach the outer mitochondrial membrane remains poorly understood. Likewise, how the functions of anti-apoptotic Bcl-2 family members such as eponymous Bcl-2 are affected by phosphorylation or cancer-associated mutations has been incompletely defined. Finally, whether Bcl-2 family members can be successfully targeted for therapeutic advantage is only now being investigated in the clinic. Here we review recent advances in understanding Bcl-2 family biology and biochemistry that begin to address these questions.

intermembrane space proteins [13].

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1. Introduction

Apoptosis is a distinct form of cell death initiated by various physiological and pathological stimuli. Morphologically, apoptosis is characterized by cell shrinkage followed by formation of cell fragments, which are rapidly cleared by phagocytes that recognize "eat me" signals on the plasma membrane [1–3]. Biochemically, apoptosis typically involves the conversion of various signals into caspase-mediated intracellular protease activity [1.2.4].

One of the two major pathways leading to apoptosis is the mitochondrial or intrinsic biochemical pathway [5,6]. The signature biochemical change during activation of this pathway (Fig. 1) is the leakage of cytochrome c into the cytoplasm [7], where it facilitates caspase 9 activation to initiate a caspase cascade [1,3,8]. This release of

apoptotic proteins Bcl-2, Bcl-x_L and Mcl-1 are observed in a variety of neoplasms [17,18]; and genes encoding pro-apoptotic proteins such as Bax and Bim are mutated or deleted in selected cancers [19,20]. Accordingly, dysregulation of apoptosis - and particularly the intrinsic apoptotic pathway – is considered a hallmark of cancer [21]. Although the intrinsic pathway of apoptosis was broadly outlined

For example, genomic changes leading to overexpression of the anti-

cytochrome c from mitochondria is regulated by members of the Bcl-2 protein family, which include three functionally and structurally

distinct subfamilies: i) pro-apoptotic effector proteins Bax and Bak,

which mediate mitochondrial outer membrane permeabilization

(MOMP); ii) anti-apoptotic family members, including Bcl-2, Bcl-x₁,

Mcl-1, Bcl-w and A1, which antagonize MOMP; and iii) pro-apoptotic

BH3-only proteins, which promote apoptosis either directly by binding

and oligomerizing Bax and Bak or indirectly by neutralizing anti-

apoptotic family members [9–12]. When the balance between these

Bcl-2 family members tips in favor of cell death, Bax and Bak form olig-

omers that permeabilize the mitochondrial outer membrane (MOM),

resulting in release of cytochrome c and many other mitochondrial

Apoptosis plays an essential role in development, immune response and tissue homeostasis [14,15]. Moreover, dysregulation of apoptosis is thought to play a critical role in degenerative diseases and cancer [16].

20 years ago [1,3], extensive progress over the past 3–4 years has provided new insight into its critical molecular processes. Structural studies





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Abbreviations: AID, activation-induced cytidine deaminase; AML, acute myelogenous leukemia; BH, Bcl-2 homology; CLL, chronic lymphocytic leukemia; CR, complete response; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; MM, multiple myeloma; PR, partial response; R/R, relapsed or refractory; SNV, single nucleotide variant; TM, transmembrane

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Fig. 1. Overview of the mitochondrial pathway [1,2,4]. The signature event of the mitochondrial or intrinsic death pathway is MOMP, which results in release of cytochrome c and other mitochondrial intermembrane proteins to the cytoplasm [13]. Once in the cytoplasm, cytochrome c facilitates the interaction of the cytoplasmic scaffolding protein Apaf-1 and procaspase 9, activating a caspase cascade that leads to cleavage of hundreds to cellular caspase substrates to generate the apoptotic phenotype [3,4]. As described in the text, Bd-2 family members regulate this pathway upstream of MOMP. While Bax and Bak are directly responsible for breaching the outer membrane, their oligomerization and activation are modulated by other family members. BH3-only proteins such as Puma, Bim, tBid, and Noxa, which are termed "activators," transiently bind Bax or Bak [39], facilitating their activation. BH3-only proteins such as Bad, which are unable to directly bind Bax or Bak, are termed "sensitizers" because they bind and neutralize anti-apoptotic Bcl-2 family members.

of Bax and Bak have begun to illuminate their oligomerization and function. Moreover, new understanding of anti-apoptotic Bcl-2 family member regulation has emerged. Here we summarize these recent developments, focusing particularly on Bax/Bak activation and Bcl-2 regulation. For more comprehensive reviews, readers are referred to [9–12].

2. Advances in understanding Bax/Bak activation

2.1. Bax/Bak oligomerization: some answers, more questions

Despite the strong correlation between Bax/Bak oligomerization and MOMP [22,23], mechanisms of Bax/Bak activation and MOMP remain incompletely understood. Earlier studies showed that homooligomerization of Bak and Bax requires their BH3 domains [24]. More detailed mutagenesis and crosslinking assays implicated two interaction interfaces in Bak oligomerization. One is a BH3 domain:BH3 binding groove interaction in which the BH3 domain (α 2 helix) of one Bak molecule interacts with the BH3 binding groove (α 3– α 5 helices)

of another [25]. The second interface implicated in oligomerization involves Bak α 6 helices [26,27].

Several models have been advanced to account for Bak oligomerization [26,27]. First, Bak monomers could form head-to-tail oligomers. Along these lines, an octamer "asymmetric-single conformer" model of oligomerization has been derived from molecular dynamic simulations [28]. Alternatively, it has been proposed that Bak monomers could form symmetric dimers, with the BH3 domain of one monomer inserted into the BH3 binding groove of its partner and the opposite face of each molecule in the dimer involved in protein-protein interactions leading to higher order oligomerization [10,26]. A similar model has been proposed for Bax [29]. One potential problem with models based on symmetric dimers is that C-terminal transmembrane (TM) domains of the two Bax or Bak monomers in each homodimer would face in opposite directions, i.e., with one transmembrane domain pointed into the membrane and the other oriented away from it. Nonetheless, recent crystal structures of Bax or Bak fragments fused to EGFP (to facilitate crystallization) provide strong evidence for symmetric dimers [30,31]. Understanding in greater detail how assembly of these dimers contributes to formation of active Bax or Bak oligomers and MOMP is the next major challenge in this area.

2.2. BH3-only proteins and Bax/Bak activation: direct activation, indirect activation or both?

There is general agreement that formation of Bax or Bak oligomers requires BH3-only proteins [32]. The exact role of the BH3-only proteins in Bax/Bak oligomerization, however, has been contentious, with three different models proposed.

According to the direct activation model, BH3-only proteins are divided into direct activators and sensitizers [33–35]. Direct activators include Bim, Puma and tBid, which can directly bind to Bax and/or Bak to induce their oligomerization [36–39]. Accordingly, Bid^{-/-}Bim^{-/-} Puma^{-/-} triple knockout mice show some of the same developmental defects as Bax^{-/-}/Bak^{-/-} double knockout mice [40], although other defects appear to be absent [41]. Moreover, Bim^{-/-}Puma^{-/-} double knockout cells [42] and Bid^{-/-}Bim^{-/-} Puma^{-/-} triple knockout cells display extensive, albeit incomplete, resistance to most apoptotic stimuli, suggesting that these proteins play a predominant role in Bax/Bak activation [40]. Although Bid and Bim can both activate Bax and Bak, it has been reported that Bim preferentially activates Bax, while Bid preferentially activates Bak [43].

In this model, the BH3-only protein Bad is a sensitizer, i.e., a protein that neutralizes anti-apoptotic Bcl-2 family members to release activators, which subsequently induce apoptosis [34]. Consensus regarding the role other BH3-only proteins has been more difficult to achieve. For example, while some studies have demonstrated direct activation of Bak by Noxa protein [39,44], others have reported that Noxa BH3 peptide cannot directly activate Bax or Bak [30,31,45]. These different results might reflect, in part, use of full-length protein in some studies versus BH3 domain peptide in others.

Other aspects of BH3-only protein interactions with Bax or Bak also remain unresolved. While transient binding of BH3-only proteins to the canonical BH3 binding groove of Bax or Bak has been implicated in triggering Bax/Bak activation in some studies [30,31,39,46], a secondary site for triggering Bax activation has been identified in others [37,47,48]. Because the peptide derivative used in the latter studies is much more effective than native Bim BH3 peptide in binding and activating Bax [49], detection of the secondary site might reflect unique properties of the ligand.

According to the alternative indirect activation model, the major role of BH3-only proteins is to neutralize anti-apoptotic Bcl-2 family members, causing them to release Bax and Bak, which are then immediately competent to permeabilize the MOM [50,51]. An earlier argument for this model was the lack of direct evidence for complexes between BH3-only proteins and Bax or Bak in intact cells [50]. However, interactions between BH3-only family members and Bak are now known to be transient [39,45]; and structural studies have also confirmed interactions between BH3-only proteins and Bax or Bak [30,31]. While the indirect activation model has fallen out of favor, the possibility that Bax and Bak are present in a preactivated state in cells – one of the postulates of this model – has not been fully explored and might have important implications for Bax and Bak function.

A more recent, unified model combines elements of both prior models by suggesting that anti-apoptotic Bcl-2 family members inhibit MOMP both by sequestering direct activators and by binding activated Bax and Bak [52]. Consistent with this model, a number of biochemical [38,53–55], cellular [56–58], and genetic [59] studies now agree that anti-apoptotic Bcl-2 family members have dual functions in inhibiting apoptosis.

2.3. Once activated, how do Bax and Bak permeabilize the MOM?

Once BH3-only proteins trigger oligomerization of Bax and/or Bak, it is still unclear how MOMP occurs. Structural studies of Bcl- x_L [60], Bax

[61] and Bak [62] have revealed similarity between Bcl-2 family proteins and channel-forming domains of bacterial toxins such as diphtheria toxin [63] and colicin E1 [64], raising the possibility that all of these proteins may utilize similar mechanisms to permeabilize membranes. By analogy to the toxins, which are thought to insert their two hydrophobic core domains as hairpins into membranes to form pore like structures [65], a "hairpin insertion model" has also been proposed for Bax [66]. In particular, Bax α 5 and α 6, together with α 9, are thought to insert into the MOM, while the other α helices from different Bax monomers interact to promote oligomerization at the MOM cytoplasmic surface [66–69].

On the other hand, recent crystal structures of activated Bax and Bak suggest that conformational changes during activation involve separation of α 5 and α 6 rather than hairpin formation [30,31]. These observed changes, which are thought to allow Bax and Bak to assume more flexible conformations, may resolve the issue of conflicting TM domain orientation in the dimers described above. However, the observed crystal structures are not consistent with models in which Bax or Bak inserts hydrophobic hairpins into the MOM to form pores. Instead, based on further results showing that some of the residues within the putative α 5/ α 6 hairpin label with membrane-impermeable reagents and others do not, it has been suggested that α 5 and α 6 collapse onto the MOM rather than inserting into it [70]. Whether this leads to MOMP through disruption of membrane curvature [71,72] or another process still remains to be determined.

2.4. Harnessing BH3-only protein biology to predict chemotherapeutic response

Understanding the mitochondrial pathway has direct implications for cancer therapy. Over the past 20 years, this pathway has been implicated in the cytotoxicity of many chemotherapeutics [73,74], including DNA damaging agents [75], spindle poisons [76,77], and kinase inhibitors [78,79]. Conversely, chemotherapy resistant cancers often contain mitochondrial pathway defects [80,81].

As described above, BH3-only proteins have been grouped into "direct activators" and "sensitizers" [35,82]. "Priming of cells for death" was originally defined as a state in which addition of BH3 domains from sensitizer BH3-only proteins to isolated mitochondria (an assay termed "BH3 profiling") results in robust cytochrome c release [83]. This state was correlated with the presence of Bim bound to antiapoptotic Bcl-2 family proteins such as Bcl-2 or Mcl-1 as well as simultaneous expression of Bax and/or Bak on the MOM [83]. Moreover, sensitivity to the Bcl-2/Bcl-x_L antagonist ABT-737 correlated strongly with release of cytochrome c by certain BH3 domain peptides, notably Bad [83–85].

In further studies, BH3 profiling predicted cancer cell sensitivity to not only BH3 mimetics, but also chemotherapeutic agents, particularly the topoisomerase II inhibitors etoposide, daunorubicin and mitoxantrone [86]. In contrast, this assay was less predictive of sensitivity to the nucleoside analogs cytarabine and clofarabine or the hypomethylating agents azacitidine and decitabine [85–87].

The original cytochrome c release assay employed for BH3 profiling has been replaced by a more efficient cell-based JC-1 dye binding assay [88] that measures loss of mitochondrial membrane potential [85,89, 90]. This JC-1 assay can be performed by flow cytometry, which enables examination of responses in phenotypically distinct cell types such as leukemic myeloblasts or hematopoietic stem cells (HSCs) [86]. Interestingly, this assay demonstrated that leukemic myeloblasts are more highly primed and exhibit a pattern more suggestive of Bcl-2 dependence than HSCs [86,91], leading to a phase 2 trial of the Bcl-2 antagonist ABT-199. For reasons that are still under investigation, the assay predicted far better clinical activity than was actually observed [92], raising the possibility that it might be better to assay the effects of the BH3 mimetic drugs themselves rather than effects of naked synthetic peptides. Before describing that trial, we first review recent advances in understanding and targeting antiapoptotic Bcl-2 family members.

3. Advances in understanding regulation of anti-apoptotic Bcl-2 family proteins

3.1. Structure and function: variations on a theme

Since the discovery of Bcl-2 [17,93,94] and its ability to promote tumorigenesis by inhibiting cell death [95], five structurally related antiapoptotic family members, including Bcl- x_L , Mcl-1, Bcl-w, BFL1 (A1 in mouse) and Bcl-B (Bcl2L10 in mouse), have been identified [10,11,96]. These share four conserved regions [Bcl-2 homology (BH) domains] (Fig. 2). In addition, Bcl-2, Bcl- x_L , Mcl-1, Bcl-w and Bcl-B all contain C-terminal TM domains that direct them to intracellular membranes, particularly the MOM. BFL1 lacks a classical TM but is targeted to mitochondria by its C-terminal α -helix [97–99], whereas the mouse homolog Bcl-2A1 appears in some studies to be cytoplasmic. All of these proteins possess a remarkably similar globular structure containing a so-called "Bcl-2 core" [11] consisting of eight α -helices oriented so that helices 3, 4 and 5 form a hydrophobic groove that is capable of binding the BH3 domains of pro-apoptotic family members.

Despite their similarity, anti-apoptotic Bcl-2 family members exhibit specificity for various pro-apoptotic proteins [51,100]. Most of the anti-apoptotic proteins bind most of the BH3-only family members, but Bcl-x_L and Bcl-w reportedly fail to bind Noxa; Bcl-2 does not bind truncated Bid, Bik, Hrk or Noxa well; Mcl-1 does not interact with Bad, Bik and Hrk; and BFL1/A1 fails to bind Bad and Bmf. Bcl-B, on the other hand, only binds Bim and Bik. Likewise, all of the anti-apoptotic family members bind Bax, but only Bcl-x_L, Mcl-1, BFL1 [101,102] and in some cases Bcl-2 [103] bind Bak.

The selectivity of these interactions, coupled with differences in expression of pro- and anti-apoptotic proteins under divers conditions, is thought to underlie the variable responses of cells to assorted stresses [36,83,101]. According to this view, Bcl-2, Bcl-x_L and Bcl-w bind to almost all pro-apoptotic proteins and are more potent protectors than Bcl-B, BFL1 and Mcl-1, which bind only a subset of the pro-apoptotic proteins. On the other hand, several observations also argue against this model. First, it has been observed that *Mcl1* gene deletion is lethal in a variety of cell lineages [104–109], although this might reflect an obligate role for Mcl-1 in oxidative phosphorylation within mitochondria rather than its anti-apoptotic role on the surface mitochondria [106]. Moreover, recent results suggest that the relative potencies of these proteins might reflect their relative expression levels rather than the range of pro-apoptotic proteins neutralized [110].

3.2. The role of anti-apoptotic Bcl-2 family proteins in tumorigenesis

Apoptosis not only contributes to normal development and tissue homeostasis, but also provides a barrier to cancer development. For example, c-Myc provokes changes that induce not only proliferation, but also apoptosis, limiting its ability to transform cells [81,111]. As a result, Myc-induced transformation of lymphoid cells in vitro and in vivo is augmented by overexpression of Bcl-2 [95,112] or Bcl- x_L [113]. Similar results in other neoplasms [114] have led to extensive efforts to elucidate apoptotic pathway dysfunction in various cancers.

High Bcl-2 protein levels are detected in a variety of neoplasms, including small cell lung, breast, prostate, colorectal, and bladder cancers, melanoma, and especially human lymphoid malignancies [115–117]. Elevated Bcl-2 expression has also been reported in acute myeloid leukemia (AML), particularly chemotherapy-resistant AML [118,119], although this has not been universally observed [120].

As summarized in Table 1, many mechanisms contribute to the high Bcl-2 levels observed in various neoplasms. First, the t(14,18) chromosome translocation that places the BCL2 gene next to immunoglobulin heavy chain (IGH) enhancer elements [17,93,94] is a major mechanism for elevated Bcl-2 transcription in lymphomas. Second, chromosome deletions and mutations that result in loss of miR-15a and miR-16, which target and repress Bcl-2 mRNA, occur in more than 50% of chronic lymphocytic leukemia (CLL) cases [121]. Third, Bcl-2 levels are regulated by protein ubiquitination [122-124]. The inhibitor of NF-E2-related factor 2 (INrf2) interacts with the BH2 domain of Bcl-2 and directs it to Cul3-Rbx1-mediated ubiquitination. Accordingly, inactivating mutations of INrf2 found in some lung cancer cell lines [124] can stabilize Bcl-2 and confer resistance to apoptotic stimuli. Similar mechanisms, i.e., genomic changes that enhance expression, altered regulation by miRNAs, and diminished ubiquitin-mediated turnover, contribute to upregulation of the other anti-apoptotic Bcl-2 family members in various cancers as well (Table 1).

3.3. Anti-apoptotic Bcl-2 family member phosphorylation

The anti-apoptotic functions of Bcl-2 and its kin are governed not only by changes in expression, but also by phosphorylation. In particular, Bcl-2 is phosphorylated in response to a number of stimuli, including spindle poisons such as paclitaxel, vincristine and vinblastine, as well as serum starvation [125–128]. As summarized in Fig. 2, several residues within the unstructured flexible loop domain (FLD) of Bcl-2 are phosphorylated, including T56, T69, S70, T74, and S87 [122, 128–130]. Kinases implicated in these phosphorylations include c-Jun N-terminal kinase (JNK) [131,132], c-Raf [127], protein kinase A [133], p38 MAPK [134], PKC α [135], mTOR [136] and CDK1 [137,138]. Importantly, Bcl-2 is phosphorylated on several of these residues during mitosis [139,140], suggesting a possible physiological role for Bcl-2 phosphorylation during cell division.

How phosphorylation affects Bcl-2 function has been somewhat controversial. An S70A Bcl-2 variant protects cells from paclitaxelinduced cell death better than wild type (wt) Bcl-2 does, suggesting that S70 phosphorylation inhibits Bcl-2 function [141]. Likewise, Bcl-2 T69A/S70A/S87A affords enhanced protection from Ca²⁺-dependent death stimuli [142]. However, phosphomimetic mutants with Glu in place of T69, S70 and/or S87 also exhibit enhanced anti-apoptotic effects [143–145], suggesting that phosphorylation activates Bcl-2.



Fig. 2. Bcl-2 phosphorylation. (A). Schematic representation of the Bcl-2 protein with BH domains boxed in blue and flexible loop domain (FLD) labeled in gray. Residues T56, T69, S70, T74 and S87 are located within the FLD and selectively targeted for phosphorylation. S70 is phosphorylated by CDK1 in vivo, inducing a conformation change of the FLD and enhancing the anti-apoptotic function of Bcl-2 [54]. (B). Residues S62 and S64 (labeled in red) within the FLD of Bcl-x_L and Mcl-1, respectively, are targeted for phosphorylation by CDK1 [137,154]. TM, transmembrane domain. Adapted from [54,154].

Table 1

Mechanisms of antiapoptotic Bcl-2 family member dysregulation in cancer.

Bcl-2 family	Overexpressed in	Genomic alterations	miRNA loss	E3 ubiquitin ligase loss	Other mechanisms
member					
Bcl-2	Lymphoid malignancies [115–117] and melanoma [224] as well as small cell lung [21,225], breast [226], prostate [227], colorectal [228] and bladder cancers [229].	t(14;18) chromosomal translocation in lymphomas [17,93,94]	Loss of miR-15a and miR-16 in CLL [121] Loss of miR-195, miR-24-2, miR-365-2 and miR-204 in breast, lung and colon cancer [230,231]	Loss of INrf2 [124]	
Bcl-x _L	Hormone-refractory prostate cancer [232] and mesenchymal breast cancer [233]	Gene amplification, often in concert with c-Myc, in multiple solid tumors [18]	Decrease miR-let7c in hepatocellular and lung cancers [234,235] as well as AML [236] Decreased miR-491-5P in pancreatic [237], colorectal [238], and ovarian cancer [237].	Loss of phosphoglycerate mutase 5, which is required for binding to the E3 ligase INrf2 [124]	Stat5 activation by Bcr/abl in CML [239]
Mcl-1	Relapsed acute leukemia [120], CLL [240], multiple myeloma [241–243] as well as breast [244], ovarian [245], colorectal [246], gastric [247], hepatocellular [248], and non-small cell lung cancers [18].	Gene amplification in multiple solid tumors [18]	Loss of miR-29 in many solid tumors [249], miR-125b in hepatocellular carcinoma [250,251] or miR-133b in lung cancer [252]	Increased expression of the deubiquitinase USP9X in FL, DLBCL and multiple myeloma [250] Mutation or loss of the E3 ubiquitin ligase FBW7 in a variety of cancers [253,254]	NFxB-mediated transcription, e.g., in large granular lymphocytic leukemia [255]
Bcl-w	Gastric [256,257] and colorectal cancers [258] as well as glioblastoma multiforme [259,260].	Gene amplification in CML, ovarian and colorectal cancers [261,262]	Downregulation miR-125b [251], miR-335 [263,264], miR-497 [265], miR-203 [266] or miR-122 [267]	Not known	Enhanced promoter activity by β-catenin/TGF4 signaling in colorectal cancer [268].
BFL1	ALL, CLL and DLBL [269–272].	Not known	Not known	Regulated by ubiquitination. No E3 ligase has been identified [273,274].	Transcriptional activation by NFkB [275], e.g., downstream of constitutive PI3K or ERK activation [276,277] that occurs in many solid tumors.
Bcl-B	DLBL and breast, prostatic, gastric, colorectal, and small cell lung cancers [278]. High level associates with poor prognosis.	Not known	Not reported	Ubiquitinated protein. No E3 ligase has been identified [279].	Not known

It was originally suggested that Bcl-2 phosphorylation might alter binding of proteins such as p53 or c-Myc to the Bcl-2 FLD, thereby modulating apoptosis [146,147]. However, the observation that FLD deletion completely blocks paclitaxel-induced apoptosis [132] raised the possibility that FLD modifications might modulate Bcl-2 function through a process distinct from altering protein-protein interactions involving the FLD. Consistent with this view, we recently found that mutation of T69, S70, T74 or S87 to either Glu or Ala increases the affinity of Bcl-2 for Bak and Bim [54], indicating that FLD modifications modulate Bcl-2 function through a process that does not require introduction of a negative charge. Moreover, we also observed altered protease sensitivity, suggestive of a conformational change, after mutation of Bcl-2 S70 to either Glu or Ala [54]. During paclitaxel-induced mitotic arrest, Bcl-2 was phosphorylated and more tightly bound to Bak and Bim. Collectively, these results suggest that phosphorylation of the Bcl-2 FLD leads to a conformational change in the "Bcl-2 core" that affects binding of proapoptotic proteins to the BH3 binding groove.

Interpretation of these results would have been enhanced by structural information about the impact of FLD alterations on overall Bcl-2 conformation. Unfortunately, all published Bcl-2 structures either lack the FLD or replace it with the corresponding region of Bcl-x_L [148,149].

Phosphorylation also modulates the function of other anti-apoptotic Bcl-2 family members. Bcl- x_L is phosphorylated on T47 and S62 [150]. Phosphorylation of the latter site by JNK decreases binding of Bcl- x_L to Bax, thus diminishing Bcl- x_L anti-apoptotic function [151]. Likewise, Mcl-1 phosphorylation at S159 by glycogen synthase kinase-3 (GSK-3) diminishes Mcl-1 anti-apoptotic function, in this case by enhancing Mcl-1 binding to the ubiquitin E3 ligase β TrCP [152] and subsequent turnover [153]. In contrast, CDK- and JNK-mediated Mcl-1 phosphorylation at S64 increases Mcl-1 binding to Noxa, Bak and Bim, thereby

potentiating its anti-apoptotic activity [154]. Because phosphorylation of Bcl-2 (on its FLD) and Mcl-1 (at S64) increases the anti-apoptotic effects of these proteins, it has been suggested that these phosphorylations might contribute to chemoresistance [155–157].

4. BCL2 sequence variation

4.1. BCL2 sequence variation in clinical lymphomas

As indicated above, *BCL2* translocation to the *IGH* locus contributes to high level Bcl-2 expression in 90% of FLs and 20% of de novo diffuse large B-cell lymphomas (DLBCLs), mostly of the germinal center B-cell subtype [158]. Some of these lymphomas also contain *BCL2* mutations. Single nucleotide variants (SNVs), including some that cause amino acid substitutions and others that are silent, were demonstrated soon after *BCL2* was cloned [159,160]. Detection of similar SNVs in untreated FL ruled out chemotherapy as a cause of these *BCL2* mutations [159]. In contrast to the impact of other abnormalities involving *BCL2*, e.g., the extremely poor prognosis of so-called "double hit" lymphomas that harbor translocations involving both the *MYC* and *BCL2* genes [161], the effects of *BCL2* SNVs on DLBCL and FL have been less clear.

Recent sequencing of large numbers of primary DLBCL samples and matched germline DNA has demonstrated a high incidence of tumorassociated *BCL2* mutations and linked these changes to somatic hypermutation, an activation-induced cytidine deaminase- (AID-) driven mutagenic process that ordinarily generates antibody diversity [162–165]. Because most *BCL2* mutations in DLBCL (60–90%) are synonymous [162] and not associated with any impact on chemosensitivity or survival [165], it has been assumed that *BCL2* mutations in DLBCL are passenger mutations. Additional studies have examined *BCL2* mutations in FL. More indolent than DLBCL, FL nonetheless carries a 2–3%/year risk of transformation to a more aggressive neoplasm. Transformed FL (tFL) most often resembles DLBCL morphologically [166,167] but historically has been quite resistant to therapy [167,168]. This poor therapeutic response of tFL has provided the impetus for recent genomic comparisons of untransformed and transformed FLs [169–171]. These analyses have demonstrated that *BCL2* is mutated, at least to some extent, in the majority of FLs and tFLs [169–171]. Analysis of subclonal heterogeneity has suggested that tFL arises from the original mutant FL clone, but not always through linear progression of the clone that predominates during the indolent phase of the disease. Instead, FL transformation commonly reflects divergent evolution from a common mutated ancestor, with independent acquisition of further aberrations in the indolent FL clone and the more aggressive tFL [166,170].

4.2. Some BCL2 variants exhibit gain of function

Because *BCL2* sequence variants inhibit cell death [172] and facilitate lymphomagenesis [173], it has been assumed that *BCL2* mutations do not affect Bcl-2 protein function. Our recent studies question this assumption. In particular, we have observed that some variant Bcl-2 proteins from lymphoma cell lines exhibit enhanced activity [103, 174]. For example, the D31H/A60V Bcl-2 variant from the *BCL2*-translocated lymphoma line RL binds Noxa 20-fold more tightly than wildtype Bcl-2 and provides increased protection from the proteasome inhibitor bortezomib, which upregulates Noxa to kill lymphoma cells [174]. These observations prompted us to examine whether *BCL2* mutations in clinical lymphoma might also impact Bcl-2 function.

To address this issue, we compared the incidence, nature, and clinical implications of *BCL2* mutations in a number of lymphoid neoplasms [175]. In contrast to DLBCL, where most mutations are silent, a vast excess of amino acid altering (nonsynonymous) variants was observed in FL. Moreover, the presence of *BCL2* mutations at diagnosis was an independent prognostic factor that correlated with increased risk of death due to lymphoma [175]. The sequence context of the *BCL2* mutations in FL suggested that AID is responsible for these mutations. Consistent with this conclusion, high AID levels in FL were associated with a higher probability of *BCL2* mutation at diagnosis and a shorter interval before transformation [175].

In FL, amino acid altering mutations are spread along the Bcl-2 protein [165,175], with the highest frequency in the FLD (Fig. 3), a region implicated in regulating affinity of Bcl-2 for pro-apoptotic Bcl-2 family members [54] as described above. Consistent with earlier studies in lymphoma cell lines [103,174], preliminary analysis demonstrated that some variant Bcl-2 proteins identified in FL exhibit increased ability to bind and sequester the pro-apoptotic Bcl-2 family members Bim and Puma [175]. Further analysis of additional variant proteins is needed to determine the prevalence of *BCL2* gain of function mutations in this region.

FL-associated mutations also occur in other regions of Bcl-2 (Fig. 3). For example, several mutations alter the BH4 domain, a region partially conserved in Bcl- x_L , Bcl-w, BFL1 and Bcl-B [10,176]. The implications of these BH4 domain mutations are currently unknown. Although multiple studies have reported that deletion of the BH4 domain abrogates Bcl-2 anti-apoptotic function [177–179], the underlying mechanism has been unclear. It has been suggested that BH4 domain deletion abrogates Bcl-2 heterodimerization with Bax [178]. Consistent with this view, binding of a stapled BH4 peptide to a unique site on Bax has recently been reported [180]. Other studies, however, have found no impact of BH4 deletion on binding of Bcl-2 to Bax, Bak, Bad, Bik or Bim [177]. Accordingly, further work is required to clarify the function of the Bcl-2 BH4 domain and assess the impact of FL-associated mutations in this region.

Comparison of sequential FL biopsies revealed a 4-fold increase in frequency of overtly detectable *BCL2* mutations at transformation compared to the same patients at diagnosis [175], suggesting that mutagenesis is ongoing in FL during the course of disease. Mutations identified at transformation had a lower frequency of classical AID signatures, possibly reflecting AID-induced mutation of cytidines outside the context of



Fig. 3. Distribution of Bcl-2 amino acid substitutions in FL. (A). Schematic representation of the Bcl-2 protein with BH domains boxed in blue and flexible loop domain (FLD) labeled in gray. Somatic variants detected in FL derived from 2 independent cohorts (black and green bars) are distributed throughout the Bcl-2 protein. Color-coded symbols depict distinct types of alterations, with purple for synonymous, white for nonsynonymous with no charge introduction, red for nonsynonymous with negative charge introduction, and blue for nonsynonymous with positive charge introduction. TM, transmembrane domain (adapted from Correia et al. [175]). (B). Affinities of Bak BH3 peptide and Bak protein for Bcl-2 variants derived from lymphoid cell lines (adapted from Dai et al. [103]). † indicates undetectable binding. (C). Kaplan Meier plots showing the impact of *BCL2* mutations on death due to lymphoma in a FL cohort (n = 128) (adapted from Correia et al. [175]).

preferred AID target sequences [181]. Moreover, some of these mutations were clearly nonfunctional, e.g., a *BCL2* variant with a premature stop codon [175], again emphasizing that not all *BCL2* mutations enhance Bcl-2 function.

In accord with studies showing that FL transformation is genetically complex and heterogeneous [166,169–171], *BCL2* mutations can suffer several fates during the course of FL. In cases where there is a linear evolution from an original malignant clone, *BCL2* mutations would be maintained throughout the course of the disease, perhaps with the acquisition of additional alterations in the same mutant gene over time. In cases where clones that give rise to the indolent FL and tFL follow divergent paths from a common precursor, mutations present in the dominant clone at diagnosis might not predominate at transformation, where new genetic alterations that confer a stronger proliferation or survival advantage could instead emerge. Conversely, in clones that lack *BCL2* mutations initially, *BCL2* mutations might emerge at transformation, as reported in our cohort [175]. Whether these are driver or passenger mutations remains to be determined.

In comparing recent studies of *BCL2* mutations in FL [169–171,175], several important methodological differences must be kept in mind. First, most of the studies were designed to identify all mutations in the *BCL2* locus. These studies not only sequenced the *BCL2* promoter and extensive intronic regions in addition to exons, but also counted *BCL2* as mutated even when a relatively small percentage of the reads were variant, e.g., as few as three reads out of 85 [169]. In contrast, our recent study, which involved Sanger sequencing of the protein coding exons, was designed to identify mutations that are i) present in predominant clones and ii) capable of altering Bcl-2 protein sequence and function [175].

Second, the studies comprehensively examining genomic changes in FL at transformation relied on the availability of paired FL pathological samples. In contrast, in order to assess the relationship between FL mutations, AID expression and clinical outcome, our recent study relied on a prospectively maintained clinical database and relatively uniform treatment practices at a single institution [175]. To have enough events for survival analysis, however, the vast majority of samples in our study predated the introduction of the anti-CD20 antibody rituximab into clinical FL treatment [182]. Retrospective analyses from the pre-rituximab era have reported a median survival of only 1–2 years after FL transformation [167,168], whereas recent studies suggest improved survival with tFL in the rituximab era [166,183], raising the possibility that rituximab might partially counter the adverse impact of genomic changes leading to transformation. Thus, it will be important in the future to assess whether *BCL2* mutation status

Table 2	
Selective list of BH3 mimetics	5.

correlates with outcome in FL treated with rituximab-containing chemoimmunotherapy.

5. Targeting Bcl-2 as an anticancer strategy

5.1. Small molecule inhibitors of Bcl-2 family proteins

Because Bcl-2 overexpression contributes to the pathogenesis of various lymphoid neoplasms, particularly FL and CLL [17,93,94,121], and possibly solid tumors [80], there has been substantial effort to develop Bcl-2 inhibitors as therapeutic agents. A combination of nuclear magnetic resonance-based binding assays and fluorescence polarization displacement assays [184,185] has identified a number of small molecules that interfere with protein–protein interactions involving the Bcl-2, Bcl-x_L and/or Mcl-1 BH3 binding grooves [10,11,186–189], thus mimicking the effects of sensitizer BH3-only proteins depicted in Fig. 1.

For a small molecule to truly be a BH3 mimetic, its killing must depend on Bax and/or Bak, it must have a high affinity for at least one anti-apoptotic Bcl-2 family member, and it must induce cytotoxicity that correlates with binding to anti-apoptotic protein(s) [186]. Some compounds originally reported to be BH3 mimetics, e.g., BH3I class compounds, HA14-1, antimycin A, and purpurogallin, are no longer thought to kill in this manner because they exhibit poor binding to BH3 binding grooves or Bax/Bak-independent killing [186,190,191]. Other agents (Table 2) meet the criteria outlined above and have undergone preclinical and, in some cases, clinical testing. These include AT-101 [the R-(-)-enantiomer of gossypol] [192–194]; the gossypol derivatives TW37 [192], apogossypol, and apogossypolone (ApoG2) [195, 196]; and obatoclax (GX15-070) [186,197–199], although obatoclax also kills Bax/Bak-deficient cells [191,200], perhaps because it inhibits the pro-survival kinase mTOR [201].

Of particular note are ABT-737 and its orally bioavailable derivative ABT-263 (navitoclax). These agents bind the BH3 binding grooves of Bcl-x_L, Bcl-2, and Bcl-w [186,191,192,202–204] to kill cells in a Bax- or Bak-dependent manner [191,192]. Interestingly, their major toxicity, thrombocytopenia, also results from Bcl-x_L inhibition [205,206]. To avoid this side effect, the Bcl-2-selective derivative ABT-199 has been developed [207]. Cellular studies have shown that ABT-737, navitoclax and ABT-199 displace Bim from their identified anti-apoptotic binding partner(s), facilitating Bax-mediated MOMP [203,208,209]. Priming of Bcl-2 with the activator Bim increases sensitivity to ABT-737, navitoclax and ABT-199, whereas Mcl-1 overexpression favors resistance [56,191, 203,208–210]. All of these observations are consistent with the proposed mode of action of these agents.

Agent	Target	BH3 mimetic status ^a	Clinical status ^b	References
A-124077	Mcl-1	Authentic	NA ^c	[188]
ABT-737	Bcl-x _L , Bcl-2, Bcl-w	Authentic	NA	[186,191,192,202,204]
ABT-263 (Navitoclax)	Bcl-x _L , Bcl-2, Bcl-w	Authentic	Ph2 ^d	[203]
ABT-199	Bcl-2	Authentic	Ph3 ^e	[208,280]
GX15-070 (obatoclax)	Bcl-x _L , Bcl-2, Bcl-w, Mcl-1	Putative/off-target effects	Ph2 ^f	[197]
Gossypol	Bcl-2, Bcl-x _L , Mcl-1	Putative/off-target effects	NA	[184,281]
Apogossypolone (ApoG2)	Bcl-x _L , Bcl-2, Mcl-1	Putative/off-target effects	NA	[195]
TW37	Bcl-x _L , Bcl-2, Mcl-1	Putative/off-target effects	NA	[186,192,282]
AT-101	Bcl-x _L , Bcl-2, Mcl-1	Putative/off-target effects	Ph2 ^g	[192,193,280]

^a BH3 mimetic status defined as in [186].

^b Most advanced clinical testing reported at https://clinicaltrials.gov/.

^c Abbreviations used are: NA, not applicable (no clinical testing); Ph, phase.

^d Randomized Ph2 study of navitoclax + rituximab vs. rituximab alone in CLL.

^e Ph3 study of ABT-199 + rituximab vs. bendamustine + rituximab for CU.

^f Ph2 studies in acute myelogenous leukemia, small cell lung cancer (with topotecan or carboplatin/etoposide), mantle cell lymphoma (with bortezomib), Hodgkin lymphoma, multiple myeloma (with bortezomib) and non-small cell lung cancer (with docetaxel).

^g Ph2 studies in chronic lymphocytic leukemia (with rituximab), hormone-resistant prostate cancer (with prednisone/docetaxel), diffuse large B cell lymphoma (with lenalidomide), non-small cell lung cancer (with docetaxel), small cell lung cancer (alone or with topotecan), and glioblastoma (alone or with radiation).

 Table 3

 Summary of selected current ABT-199 clinical trials.

Disease	Clinical characteristics or eligibility	Regimens	Phase	Ref. ^a
AML ^b	Treatment naïve age \geq 65 ineligible for standard chemotherapy	ABT-199 + Decitabine or + Azacitidine	Ib	
AML	Treatment naïve	ABT-199 + Cytarabine	I/II	
AML	NA	ABT-199	II	[92]
B-cell NHL, DLBCL	NA	ABT-199 + rituximab or oblinutuzumab + CHOP		
CLL	R/R or treatment naïve	ABT-199 + oblinutuzumab	Ib	[221]
CLL	R/R or treatment naïve	ABT-199 + bendamustine + rituximab	Ib	[222]
CLL, NHL	R/R	ABT-199	Ι	[219]
CLL, SLL	R/R	ABT-199 + rituximab	Ib	[220]
CLL	R/R to B-cell receptor inhibitor	ABT-199	II	
CLL, 17p deletion	R/R	ABT-199	II	
CLL	R/R	ABT-199 + rituximab or + bendamustine and rituximab	III	
FL	R/R	ABT-199 + rituximab or + bendamustine and rituximab	II	
MM	Treatment with Bortezomib and dexamethasone	ABT-199	Ι	
MM	R/R	ABT-199	Ι	
NHL, MM	R/R	ABT-199	Ι	
NHL	R/R	ABT-199 + bendamustine + rituximab	Ib	[223]

^a References for ABT-199 trials that have been reported in abstract form.

^b Abbreviations: AML, acute myeloid leukemia; CHOP, cyclophosphamide, doxorubicin, vincristine, and prednisone; CLL, chronic lymphocytic leukemia; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; NA, not available; NHL, Non-Hodgkin's lymphoma; MM, multiple myeloma; R/R, relapsed/refractory; and SLL, small lymphocytic leukemia.

5.2. Clinical trials of BH3 mimetics

In view of the important role of Bcl-2 in the pathogenesis of lymphoid malignancies (see above), BH3 mimetics have been extensively tested in these disorders (reviewed in [211]). Early clinical testing revealed activity of navitoclax in CLL and non-Hodgkin's lymphoma (NHL) [212,213]. With the development of ABT-199, studies of navitoclax continue in solid tumors, where it is being used to target Bcl-x_L, whereas studies in Bcl-2-dependent hematological malignancies have focused more extensively on ABT-199.

As of January 2015, 16 clinical trials testing ABT-199 either as a single agent or in combination therapy (www.clinicaltrials.gov) are ongoing. A variety of neoplasms are being studied, including CLL, aggressive NHL, FL, multiple myeloma (MM) and AML (Table 3). In preclinical studies, anti-tumor activity of ABT-199 has also been reported in T-cell ALL, chronic myelogenous leukemia, and colorectal and breast cancers [214–217].

Early clinical results suggest that ABT-199 has promising activity in relapsed or refractory (R/R) lymphoid malignancies. In particular, ABT-199 has an overall response rate (ORR) of 56% in R/R NHL [218] and 84% (20% complete response) in R/R CLL and small lymphocytic leukemia (SLL) [219]. Indeed, responses in CLL and SLL have been so brisk that tumor lysis syndrome has been a major complication of therapy. In contrast, despite tantalizing activity against AML cells ex vivo [91], ABT-199 has shown disappointing activity (12.5% complete remission rate) in R/R AML [92], perhaps because of elevated Mcl-1 in this setting [120].

Additional studies are investigating ABT-199 in combination with other therapies (Table 3). For example, Roberts et al. reported an 86% ORR (31% CR) in R/R CLL treated with ABT-199 and the anti-CD20 antibody rituximab [220]. Other studies in R/R or untreated CLL patients combined ABT-199 with obinutuzumab, a glycoengineered anti-CD20 antibody [221], or in CLL and NHL with bendamustine/rituximab [222,223]. More mature reports of these promising investigations are awaited with interest.

6. An agenda for the future

Despite improvements in understanding signaling through the intrinsic apoptotic pathway over the past 2–3 years, important questions remain. Recent studies have clarified how BH3-only proteins induce Bax or Bak activation and provided detailed descriptions of Bax and Bak homodimers, but these results provide limited insight into the mechanism by which Bax and Bak breach the MOM. Further

studies of higher order Bax and Bak oligomers, perhaps in the presence of appropriate lipids, are required for progress in this area.

Even though the Bcl-2 protein was identified almost 30 years ago, important questions about its structure and function persist. How phosphorylations in the FLD affect affinity of the BH3 binding groove for its ligands is entirely unclear. While recent studies suggest that changes in the FLD can affect Bcl-2 conformation [54], an NMR or crystal structure of Bcl-2 with its own loop intact is required to better understand this aspect of Bcl-2 regulation.

The consequences of nonsynonymous *BCL2* mutations also require further investigation. While these mutations become more prevalent during the course of FL [175], it is unclear whether they are driving FL transformation or are merely bystanders that reflect high AID activity in FLs at highest risk of transformation. Further study is also required to determine which *BCL2* mutations affect Bcl-2 protein function and how these amino acid changes, whether in the FLD or elsewhere, alter affinity of the BH3 binding groove.

Finally, a number of questions need to be answered to assure the optimal clinical development of ABT-199. In hematological malignancies, this agent currently appears to be most active in CLL, a disease driven by overexpression of wt Bcl-2. Whether the *BCL2* mutations observed in FL will affect ABT-199 sensitivity remains to be determined. Furthermore, it is presently unclear whether ABT-199 will be as active in diseases where Bcl-2 overexpression, while present, plays a less clear-cut role in pathogenesis (e.g., small cell lung cancer). Finally, additional work is required to identify optimal combinations that capitalize on the promising single-agent activity of ABT-199 observed to date.

Given the recent exciting advances, continued investigation of these questions appears warranted.

Transparency document

The Transparency document associated with this article can be found, in the online version.

Acknowledgements

We gratefully acknowledge provocative discussions with Reuben Harris, David Huang, Greg Gores, Randy Gascoyne and members of the Mayo Clinic Lymphoma Disease-Oriented Group; helpful suggestions of the anonymous reviewers; and editorial assistance of Deb Strauss. Preparation of this review was supported in part by R01 CA166741, F30 CA183507, and predoctoral fellowships from the Mayo Foundation for Education and Research.

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