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Review

Physiological and Pharmacological Control of BAK, BAX, and Beyond

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Cellular commitment to the mitochondrial pathway of apoptosis is accomplished when proapoptotic B cell chronic lymphocytic leukemia/lymphoma (BCL)-2 proteins compromise mitochondrial integrity through the process of mitochondrial outer membrane permeabilization (MOMP). For nearly three decades, intensive efforts focused on the identification and interactions of two key proapoptotic BCL-2 proteins: BCL-2 antagonist killer (BAK) and BCL-2-associated X (BAX). Indeed, we now have critical insights into which BCL-2 proteins interact with BAK/BAX to either preserve survival or initiate MOMP. In contrast, while mitochondria are targeted by BAK/BAX, a molecular understanding of how these organelles govern BAK/BAX function remains less clear. Here, we integrate recent mechanistic insights of proapoptotic BCL-2 protein function in the context of mitochondrial environment, and discuss current and potential pharmacological opportunities to control MOMP in disease.

Trends

Structural and biochemical studies have revealed that the BH3 domain is sufficient to mimic the proapoptotic function of full-length BH3-only proteins. Regions outside the BH3 domain are required to integrate post-translational signaling and confer secondary structure.

The involvement of mitochondria in the cellular decision to initiate MOMP has long been overlooked. No longer are mitochondria considered passive participants in apoptosis, but rather, their shape and composition directly govern proapoptotic BCL-2 family activity.

Therapeutics mimicking the BH3 domain of proapoptotic proteins function as single agents or in combination strategies to treat human diseases. Recently, the refinement of BH3 mimetics has led to the potent and specific targeting of individual BCL-2 proteins with greater efficacy and decreased side effects.

Mitochondrial Pathway of Apoptosis: Defining the Proteins

The **mitochondrial pathway of apoptosis** (see Glossary) is essential for metazoan development, tissue homeostasis, and cellular responses to therapeutics. The involvement of mitochondria in vertebrate apoptosis was first suggested when caspase activity resulted from *Xenopus* oocyte extract co-incubation with purified mitochondria. This activity was blocked by the addition of recombinant BCL-2 protein, suggesting that BCL-2 could prevent mitochondrial engagement of the cytosol by blocking cytochrome c release [1]. Since then, it was discovered that the adaptor protein apoptotic protease activating factor (APAF)-1 binds cytosolic cytochrome c, undergoes oligomerization into a heptameric complex, and recruits pro-caspase-9 to form the apoptosome. Dimerization of pro-caspase-9 within the apoptosome promotes downstream activation of executioner caspase activation (e.g., caspases-3 and -7) and ultimately commits a cell to apoptosis [2,3].

After the discovery of BCL-2, the BCL-2 family quickly expanded to include almost 20 members that are divided into two functional classes of proteins: antiapoptotic and proapoptotic (reviewed in [4]). Most cells express a variety of antiapoptotic and proapoptotic BCL-2 proteins, and through the regulation of their interactions command survival or commitment to apoptosis.

Antiapoptotic BCL-2 proteins comprise four BCL-2 homology (BH) domains (BH1–4) and are generally integrated within the outer mitochondrial membrane (OMM), but are present in other membranes like the endoplasmic reticulum (ER). BCL-2, BCL-xL, and myeloid cell leukemia (MCL)-1 are the major members of the antiapoptotic BCL-2 repertoire that preserve OMM integrity by binding and inhibiting the proapoptotic BCL-2 proteins.

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The proapoptotic BCL-2 members are divided into effectors (which also contain BH1–4) and the **BH3-only proteins**. The proapoptotic effector BCL-2 proteins **BAK** and **BAX** homo-oligomerize into proteolipid pores within the OMM and are required to promote **MOMP** and cytochrome c release. However, effectors require activation steps, upon which they oligomerize and gain the capacity to permeabilize membranes composed of mitochondrial lipids [5]. BAK and BAX activation occurs through interactions with so-called direct activators, BH3-only proteins, or by physicochemical effects of detergents, mild heat, or elevated pH. Box 1

In an issue of *Trends in Cell Biology* published in 2008 [6], we discussed a highly controversial topic in the apoptosis field: do BH3-only proteins bind BAK/BAX to induce conformational changes, revealing their membrane-permeabilizing activity, or do BH3-only proteins solely promote apoptosis by neutralization of the antiapoptotic BCL-2 proteins to release BAK/BAX? In essence, are BAK/BAX constitutively competent to promote MOMP, or, do BAK/BAX require interactions with BH3-only proteins to activate apoptosis? [6]. Since 2008, numerous biochemical, cellular, and structural studies have provided evidence that BAK/BAX require direct interactions with BCL-2-interacting domain death agonist (BID)/BCL-2-interacting mediator of cell death (BIM) to initiate membrane permeabilization and apoptosis. Furthermore, the majority of evidence supports that BH3-only proteins interact with both antiapoptotic BCL-2 proteins and effector molecules to unify these once disparate hypotheses [7].

In the above article [6], we also proposed three questions pertinent for future investigations of proapoptotic BCL-2 family function and MOMP. (i) Will our understanding of the BH3-only proteins, as determined by synthetic BH3 domain peptides, be corroborated following full-length protein analyses? (ii) What mitochondrial components regulate BAK/BAX-dependent MOMP?

Box 1. BCL-2 Ovarian Killer (BOK) Is a *Bona Fide* Proapoptotic Effector Protein

BOK is a proapoptotic member of the BCL-2 family that has puzzled the apoptosis community. BOK is considered a globular protein closely related to BAK/BAX sharing multiple BH domains, 70–80% sequence homology, and a C-terminal transmembrane motif [76]. BOK was identified using a yeast two-hybrid screen using antiapoptotic BCL-2 members as bait [76]. Exogenous BOK leads to apoptosis in numerous cell models [77], and *BOK* is deleted in a subset of human malignancies, suggesting a tumor suppressor function [78]. But what role does BOK play in the mitochondrial pathway of apoptosis?

Numerous genetic models of *Bok* deficiency have been generated, and each used a different targeting strategy to eliminate *Bok* expression [79–81]. All $Bok^{-/-}$ murine models fail to display significant differences in development or fecundity compared with wild type counterparts. Most *in vivo* data suggest that BOK is not redundant with BAK or BAX. For example, $Bok^{-/-}$ mice do not demonstrate accelerated $E\mu$-*Myc* driven lymphomagenesis, which contrasts with the genetic loss of *Bax* [80].

Transformed liver fibroblasts from one of the genetic models of *Bok* deletion demonstrate impaired apoptotic responses to thapsigargin (TG, a SERCA pump inhibitor) and bortezomib (BTZ, a proteasome inhibitor) [79]. These *in vitro* results were extended with *in vivo* studies comparing Wt and $Bok^{-/-}$ mice for sensitivity to TG-induced apoptosis in the liver. This study also suggests that *Bok* deficiency leads to decreased ER stress signaling, potentially through the regulation of calcium release [82]. More recent findings support a selective and distinguishing role for BOK in regulating the apoptotic response to ER and proteasomal stressors, and show evidence that BOK is fully competent to promote MOMP in the absence of BAK/BAX and BH3-only proteins [81,83].

In addition, proteomic analysis revealed that several components of the ER-associated degradation (ERAD) machinery interact with BOK, suggesting that BOK stability and proapoptotic function are responsive to signaling perturbations both upstream and downstream of ERAD. Remarkably, the proapoptotic activity of BOK does not seem to be negatively regulated by antiapoptotic BCL-2 members [81].

While an apoptotic role for BOK was originally limited to the ovary, BOK is now generally accepted to have a broader impact in adult tissues. Recent insights into the chemotherapeutic responses of ovarian cancer cells also point to a BAK/BAX-independent role for BOK in mediating MOMP and apoptosis [83]. At present, we have neither structural information nor any details pertaining to how mitochondria may regulate BOK.

Glossary

Antiapoptotic BCL-2 proteins: antiapoptotic BCL-2 proteins are responsible for maintaining the integrity of the OMM. They are globular proteins containing BH1–BH4 domains. The members of this subfamily include A-1 (BCL-2 related gene A1), BCL-2, BCL-xL (BCL-2 related gene, long isoform), BCL-w, and MCL-1. These proteins are generally found at the OMM, but can also localize to the ER membrane and cytosol. The antiapoptotic BCL-2 proteins preserve OMM integrity by directly binding to both classes of proapoptotic BCL-2 proteins (i.e., the BH3-only and effector proteins), which prevents them from cooperating to induce MOMP and subsequent apoptosis.

BAK and BAX activation: This phrase is used throughout the BCL-2 literature and it involves a highly regulated multi-step process: (1) structural rearrangement exposing N and C termini; (2) stable insertion into the OMM; (3) protein dimerization via the $\alpha 2$–$\alpha 5$ core; and (4) higher-order protein oligomerization resulting in MOMP. Cellular and mitochondrial contributions to this process are not well defined, but the composition and shape of the OMM is critical for BAX and BAK activation. The above multistep activation process is specific to BAX, as BAK is constitutively localized and inserted into the OMM. Biochemical requirements for BOK have not been addressed (Box 1). BAK/BAX activation is often detected using conformation-specific antibodies recognizing hidden epitopes in the nonactivated proteins.

Derepressor/sensitizer BH3-only proteins: a subset of the BH3-only proteins, including: BAD (BCL-2 antagonist of cell death), BIK (BCL-2 interacting killer), BMF (BCL-2 modifying factor), HRK (Harakiri), Noxa, and PUMA that bind antiapoptotic BCL-2 proteins, but do not efficiently activate BAK or BAX. These proteins can competitively displace direct activators from antiapoptotic BCL-2 proteins, thus promoting MOMP. Also, this class of BH3-only proteins sensitize mitochondria and cells to low levels of direct activator proteins.

Direct activator BH3-only proteins: a subset of the BH3-only proteins, including BID and BIM that

(iii) Is it possible to pharmacologically regulate proapoptotic BCL-2 proteins as an effective therapy in human disease? These questions will be approached within the context of recent biochemical, cellular, and genetic evidence to provide an updated perspective of the physiological and pharmacological control of MOMP and apoptosis (Figure 1, Key Figure).

Are BH3 Domain Peptides Appropriate Surrogates to Study BH3-Only Protein Function?

A majority of mechanism-based studies involving the BCL-2 proteins defined the intra-family interactions with the BH3-only proteins via chemically synthesized peptides derived from BH3 regions. These peptides are referred to as BH3 domain peptides, and due to the significant number of BH3-only proteins, these tools provided a rapid means to investigate key interactions and activities [8,9].

BH3 domain peptides enabled rapid and insightful 3D structures of antiapoptotic BCL-2 family proteins in complex with BH3 domains – along with providing the basis of drug discovery in the family [10]. Interactions between BH3 domains and the antiapoptotic BCL-2 members are established through the cooperation between four hydrophobic residues (h1–h4) on the BH3 domain α helix and the corresponding hydrophobic binding pocket of the antiapoptotic BCL-2 protein (Figures 2 and 3) [11]. Variations in the BH3 domain sequence and hydrophobic grooves establish binding selectivity and affinity, and stapling BH3 domain peptides into stable helices increases their relative affinities for antiapoptotic BCL-2 proteins [11]. However, has the ease of this model system prevented us from fully understanding how BH3 domains regulate apoptosis?

Early work has suggested bifurcation of the BH3 domains into direct activators and sensitizers/derepressors based on their ability to activate BAX, or inhibit antiapoptotic BCL-2 proteins [9,12,13]. Direct comparisons of recombinant proteins and BH3 domain peptides have revealed that the majority of interactions and functions are maintained between these systems. For example, BID-mediated BAX activation is potent for both BID protein and peptide [13]. A different study compared the full-length p53 upregulated modulator of apoptosis (PUMA) protein and its BH3 peptide counterpart. The findings revealed that full-length PUMA protein, like its BH3 peptide, induced MOMP through its derepressor/sensitizer role by releasing sequestered direct activator proteins from the OMM, and by sensitizing mitochondria to future direct activator exposure [12]. A more recent study comparing BID and BIM proteins and peptides suggests that while BID/BIM can activate BAK/BAX, previously unrecognized differences are observed. In three complementary model systems (i.e., cells, mitochondria, and large unilamellar vesicles; LUVs), distinct preferences for BID to activate BAK, and BIM to activate BAX was apparent [14].

Our understanding of BH3-only proteins, however, is not yet free from controversy. Several studies using both PUMA protein BH3 peptide domains have suggested that PUMA is a direct activator. A stapled version of the PUMA BH3 peptide can potently inhibit antiapoptotic BCL-2 proteins, and also directly bind and activate BAX and induce apoptosis [15]. Both the BH3 domain peptide and full-length PUMA protein are able to interact with BAK, leading to BAK homo-oligomerization and membrane permeabilization, and in a cellular context, BAK-mediated apoptosis [16]. These results obtained with full-length protein and synthesized BH3 domain peptide provide strong evidence that PUMA, like BIM and BID, is able to act as a direct BAK/BAX activator. Curiously, the combined genetic loss of *Bid*, *Bim*, and *Puma* have also suggested that Noxa can have direct activator function in cells, yet the majority of data suggest that the Noxa BH3 domain peptide is a weak direct activator [9,13,17,18]. Furthermore, a recent finding challenges the requirement for BH3-only protein in BAK/BAX activation by genome editing [19]. How do we reconcile these differences? Differences in protein and peptide preparations could be one explanation, but perhaps changes to the mitochondrial environment impact on successful activation by distinct BH3-only proteins and their BH3 domains. We should re-evaluate

transiently interact with BAK and BAX to induce their activation. PUMA and Noxa may also function as direct activators, but there are conflicting data. Direct activator proteins are often sequestered by antiapoptotic BCL-2 proteins, which consequently inhibit their direct activator function until subsequent derepressor BH3-only protein expression or activation.

Mitochondrial outer membrane permeabilization (MOMP): this event occurs immediately downstream of BAX/BAK/BOK activation and is responsible for the release of inter-membrane space (IMS) proteins, such as cytochrome c, SMAC/Diablo, EndoG, Omi/HtrA2, and AIF (but any protein residing in the IMM can be released, depending on its membrane association and solubility). Cytosolic proteins also gain access to the IMS and alter mitochondrial function; for example, cytochrome-c-activated caspases can cleave IMS proteins, which negatively impacts ATP production. Importantly, the antiapoptotic BCL-2 proteins inhibit MOMP by blocking both effector and BH3-only proteins.

Mitochondrial pathway of apoptosis: a form of apoptosis that is engaged following cellular stress, such as DNA damage or nutrient deprivation, and is inhibited by antiapoptotic BCL-2 proteins. In this pathway, proapoptotic effector BCL-2 proteins compromise the OMM, which causes mitochondria to release IMS proteins that initiate caspase activation, cellular disassembly, and phagocytosis.

Proapoptotic effector BCL-2 proteins: BAK, BAX, and BOK are the proapoptotic effector molecules of the BCL-2 family because they actively permeabilize the OMM and promote the release of IMS proteins. BAK and BAX activate, homo-oligomerize, and permeabilize the OMM in response to direct activator BH3-only protein interactions. BOK may not require activation, and may promote MOMP following after expression and protein stabilization.

Key Figure

Proapoptotic Effector BCL-2 Proteins Are Activated via Distinct Regulators

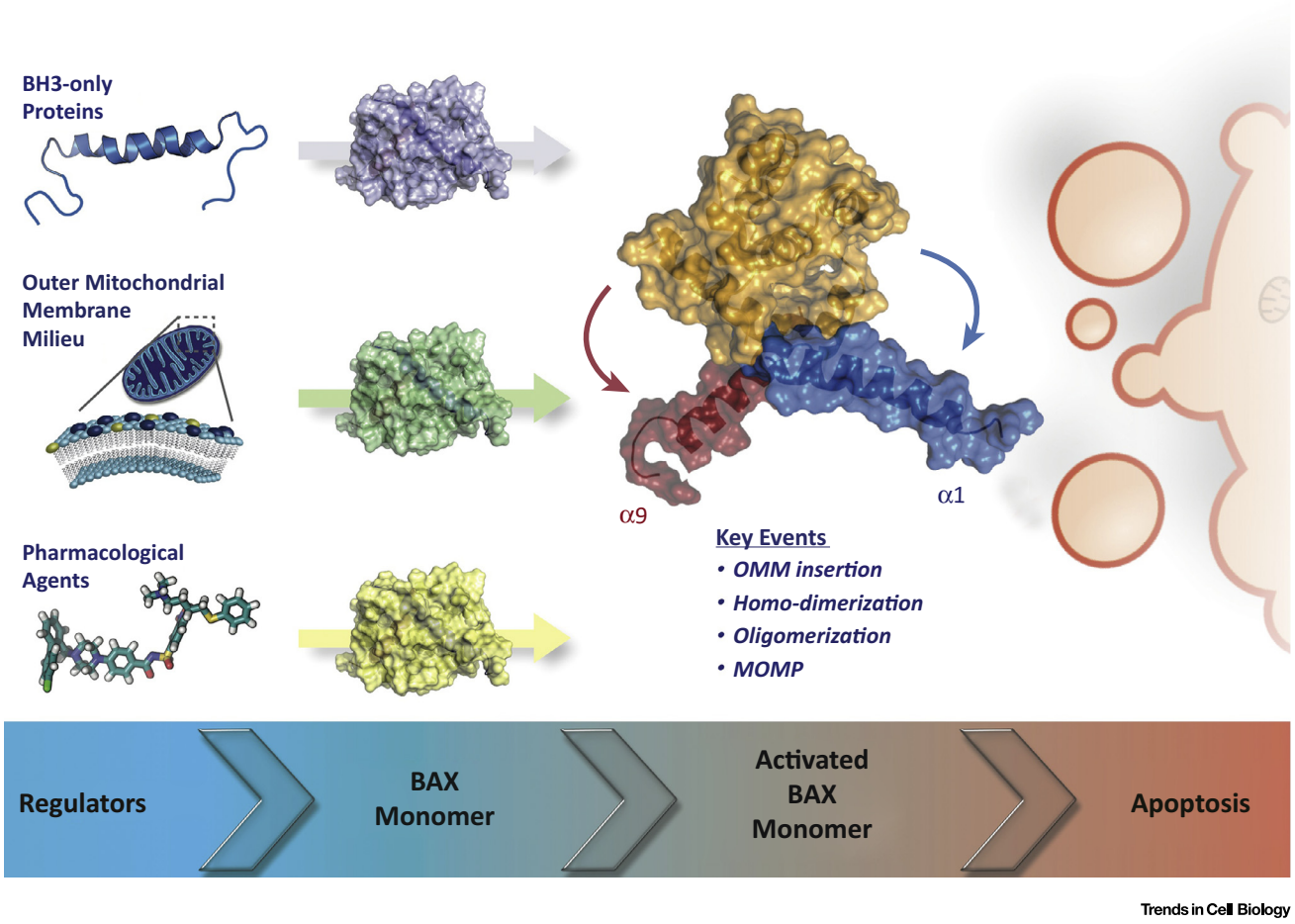

Figure 1. The proapoptotic BCL-2 homology (BH)3-only proteins (e.g., BCL-2-interacting domain death agonist, BID), the composition/shape of the outer mitochondrial membrane (OMM) (e.g., cardiolipin), and the pharmacological agents mimicking the direct activator BH3-only proteins (e.g., BAX-activating molecule 7), impact upon BCL-2-associated X (BAX) activation. Upon activation, monomeric BAX undergoes structural rearrangements in which helix α1 and the C-terminal transmembrane helix α9 moves away from the BCL-2 globular core. Afterward, several key events take place (e.g., OMM insertion, homo-dimerization, oligomerization, and mitochondrial outer membrane permeabilization; MOMP) to initiate apoptosis. An inactive BAX monomer is shown as a cartoon with surface representation in purple, green, and yellow. The conformationally active BAX monomer surface is depicted in orange representing the BCL-2 globular core; and helices α1 and α9 in blue and red, respectively (BAX PDB: 1F16). While BAX is the focus of this figure, BAK (BCL-2 antagonist killer) and BOK (Bcl-2-related ovarian killer) may share similar characteristics. BCL-2, B cell chronic lymphocytic leukemia/lymphoma-2.

the role of mitochondrial shape and composition to determine if the differences observed are reconciled based on mitochondrial biology.

Alternatively, a recent structural study revealed a surprising function for PUMA in the mitochondrial apoptosis pathway [20]. The study showed that upon PUMA BH3 domain binding with BCL-xL, a partial unfolding within the BCL-xL structure was induced. Interestingly, residue Trp71 in the PUMA BH3 domain (which is not one of the hydrophobic h1–h4 residues) plays a critical role in mediating the partial unfolding in the BCL-xL structure. Via π-stacking with residue His113 of BCL-xL, this interaction unfolds α2-3 helices within BCL-xL disrupting the interface

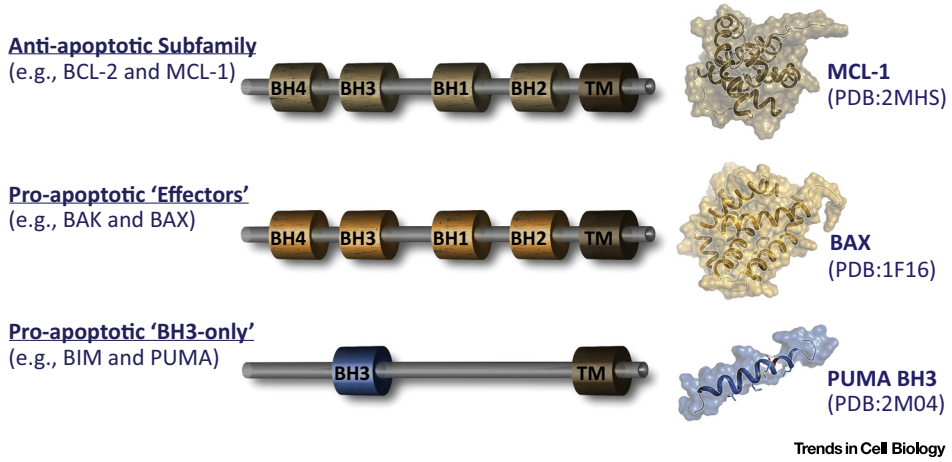

Figure 2. Overview of BCL-2 Family. BCL-2 homology (BH) domain composition of the antiapoptotic, proapoptotic effectors, and proapoptotic BH3-only proteins in beige, orange, and blue, respectively. Corresponding 3D structures of myeloid cell leukemia (MCL)-1 (PDB:2MHS), BCL-2-associated X (BAX) (PDB:1F16), and p53 upregulated modulator of apoptosis (PUMA) BH3 domain (generated by subtracting the BCL-xL from the complex structure PDB:2M04). BAK, BCL-2 antagonist killer; BCL-2, B cell chronic lymphocytic leukemia/lymphoma-2; BIM BCL-2-interacting mediator of cell death.

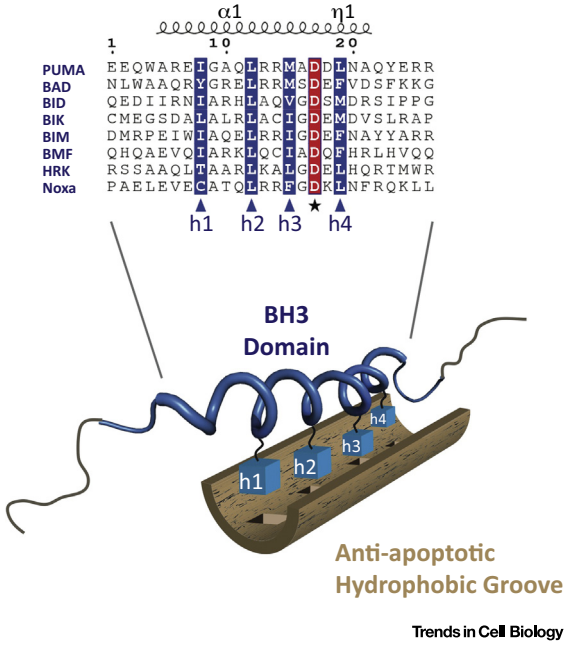

Figure 3. A BCL-2 Homology (BH)3 Domain–Antiapoptotic Hydrophobic Groove Interaction. Alignments of the α helical BH3 domains of BH3-only proteins are depicted. The hydrophobic (h) residues required for interactions with the hydrophobic groove of antiapoptotic BCL-2 family members are highlighted in blue and indicated as h1–h4. The conserved aspartic acid residue involved in salt-bridge formation is highlighted in red. A cartoon representation of the solution structure of the p53 upregulated modulator of apoptosis (PUMA) BH3 domain (PDB:2M04) is depicted in blue with the hydrophobic residues (h1–h4) as blue boxes. Motifs outside of the BH3 domain are likely regulatory regions necessary for cellular signaling modifications, stability/degradation, and secondary structure. BAD, BCL-2 antagonist of cell death; BCL-2, B cell chronic lymphocytic leukemia/lymphoma-2; BID, BCL-2-interacting domain death agonist; BIK, BCL-2 interacting killer; BIM, BCL-2-interacting mediator of cell death; BMF, BCL-2 modifying factor; HRK, Harakiri.

between BCL-xL and p53, thereby releasing the bound p53 to engage BAX, and trigger apoptosis [21]. At present it is not known if full-length PUMA protein exhibits a similar disruptive behavior towards the BCL-xL–p53 interaction or how mitochondrial biology impacts on PUMA-mediated BCL-xL conformational changes.

Is it safe to assume that the BH3 peptides function like full-length counterparts in the regulation of BAK/BAX and antiapoptotic proteins? We believe not, which then begs the question: what is the purpose of the regions outside the BH3 domain? The BH3-only protein subfamily members are intrinsically disordered proteins (except for BID) and contain both a BH3 and C-terminal transmembrane domain (Figure 2). The BH3 domain becomes ordered upon binding to a BCL-2 family member and folds into a α helix. Perhaps, the rest of the disordered regions act as an interaction platform for cellular signaling modifications to positively and negatively regulate BH3 domain function, and confer secondary structure once the BH3 domain is bound by the hydrophobic groove.

Which Mitochondrial Components Regulate BAK/BAX-Dependent MOMP?

The majority of BCL-2 family literature focuses on the interactions between individual BCL-2 proteins as the major point of MOMP regulation. More recently, however, several key studies revealed that mitochondrial composition and shape directly control BAK/BAX function and sensitivity to apoptosis. There are at least three major factors that contribute to the mechanistic control of BAK/BAX-dependent MOMP: (i) a stress-specific combination of proapoptotic BH3-only proteins; (ii) an actively maintained and regulated lipid composition within the OMM; and more recently (iii) a specific mitochondrial shape/size. The first factor is briefly highlighted above, and more detailed discussions are available elsewhere. Here, we discuss the last two factors, keeping in mind that this literature is still small, and at this point, many of the observations are not integrated into a cohesive mechanistic understanding (Figure 4). Recent discoveries reveal that mechanistic relationships between mitochondrial shape/size and the apoptosis machinery regulate stem cell identity, self-renewal, and cell fate decisions. This occurs via nuclear transcriptional responses and mitochondrial reprogramming, and it is becoming evident that mitochondrial shape/size contribute a direct role in these processes [22,23]. Indeed, earlier work first demonstrated that chronic mitochondrial fission promoted oncogenic transformation and tumor growth, suggesting that mitochondrial fission promotes apoptotic resistance and cancer signaling mechanisms [24–26].

The OMM environment is the predominant site for interactions between all proapoptotic BCL-2 proteins, including BH3-only–effector (e.g., BID–BAK) and effector–effector (e.g., BAX–BAX). Early insights into the mechanism of BAX-mediated pore formation suggested that mitochondrial lipids are involved in the activation of BAX, or alternatively, in the BAX-dependent membrane permeabilization process [27]. More specifically, data pointed to cardiolipin, a negatively charged phospholipid localized almost exclusively in the inner mitochondrial membrane (IMM). However, small amounts of cardiolipin are localized in the OMM, which produces a key structural junction between the IMM and OMM.

Various model systems (e.g., LUVs and outer membrane vesicles; OMVs) that mimic the OMM reveal a requirement of cardiolipin for BAX-induced permeabilization of membranes. Interestingly, LUVs composed of lipids that resemble the ER do not undergo BAX-mediated permeabilization unless cardiolipin is added [27,28]. These data suggest that cardiolipin has the potential to regulate BAX-dependent MOMP by facilitating BAX insertion or nucleating BAX-dependent pore formation. However, deletion of cardiolipin synthase in yeast mitochondrial model systems expressing human BAX had no effect on BAX-dependent MOMP and the subsequent release of cytochrome c [29]. This has been confirmed *in vitro*, where BAX-mediated permeabilization of cardiolipin-free LUVs treated with specific proteins or lipids were added

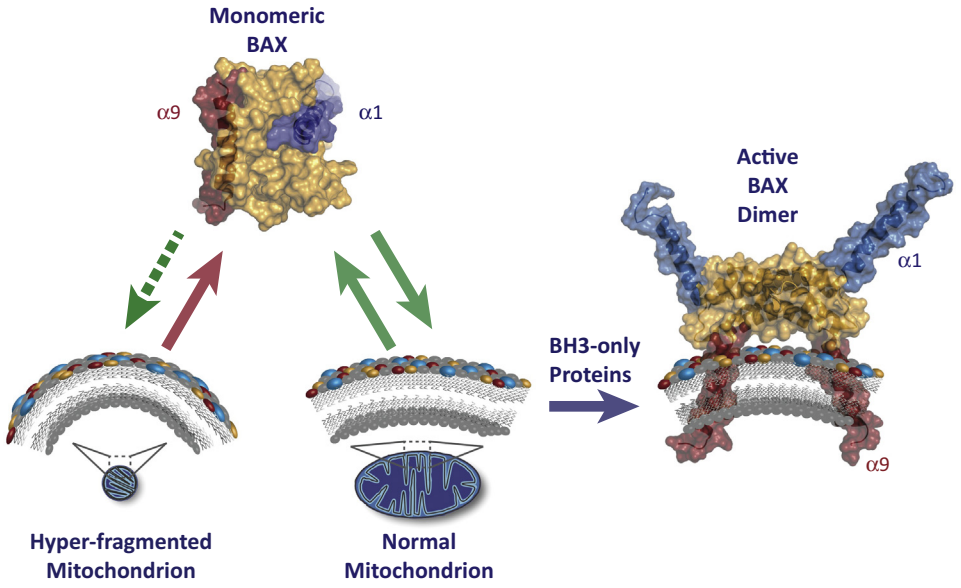

Figure 4. The Shape and Composition of the Outer Mitochondrial Membrane (OMM) Govern BCL-2-associated X (BAX) Activation. Monomeric BAX dynamically and reversibly interacts with the OMM, which causes transient exposure of both α1 and α9. When mitochondria are hyperfragmented (i.e., high membrane curvature), the dynamics between BAX and the OMM are markedly reduced, which lowers the probability of stable activation by BCL-2 homology (BH)3-only proteins. In addition to shape, hydrophobic components within the OMM engage BAX to also regulate membrane interactions and successful activation by BH3-only proteins. Cholesterol, cardiolipin, and *2(E)*-hexadecenal are depicted in yellow, blue, and red spheres, respectively. The BAX monomer and dimer are shown with surface representations: the BCL-2 globular core in orange, α1 helix in blue, and α9 helix in red (BAX monomer PDB:1F16 and BAX dimer PDB:4BDU).

(discussed below) [30,31]. In addition to BAX, the activated form of BID (cleaved by caspase-8; cBID) is also regulated by cardiolipin. Although cBID does not require cardiolipin for the initial binding to a membrane, cardiolipin may promote conformational changes in cBID that enhance BAX activation; Mtch2, a carrier protein localized to the OMM, may facilitate a similar and/or redundant mechanism [32,33].

Mitochondrial cholesterol has also emerged as potential regulator of MOMP in multiple model systems [28,32,34]. Cholesterol hinders BAX-mediated membrane permeabilization due a combination of reduced interactions with BH3-only proteins within a membrane, and reduced kinetics of BAX insertion into membranes. The mechanism by which high levels of cholesterol block BAX-dependent membranes remains controversial. There are reports that cholesterol can reduce membrane fluidity that indirectly impacts on BAX dynamics with membranes. However, there is also a putative cholesterol-binding motif on BAX that may also participate [35]. As cholesterol metabolism is often deregulated in cancer, it is tempting to speculate that high mitochondrial levels may contribute to both tumorigenesis and chemotherapeutic responses by disrupting BAX-dependent apoptosis [36].

Sphingolipids are another important class of lipids that regulate multiple aspects of BAK/BAX-dependent MOMP. Initial studies suggested that long-chain ceramides form pores in defined membranes and isolated mitochondria, and that this pore-forming activity is regulated by antiapoptotic BCL-2 proteins [37]. More recently, reports have indicated that ceramide can promote BAX-mediated permeabilization [38]. In contrast, BAK contributes to the production of long-chain ceramides following genotoxic stress [39]. Connecting BAK to ceramide generation

provides a novel link within the apoptotic pathways previously thought to function independently: does proapoptotic signaling converge via the BCL-2 effector molecules to establish a BAK-regulated mitochondrial environment that is permissive for BAX-dependent MOMP?

One potential answer to the above question lies with the understanding that sphingolipids are metabolized into a myriad of lipid species that influence cellular sensitivity to apoptosis. For example, ceramides are often precursors for sphingoine-1-phosphate (S1P) generation, and S1P can be degraded into the fatty aldehyde, *2(E)*-hexadecenal. Interestingly, a link between S1P and *2(E)*-hexadecenal metabolism has also converged on BAK/BAX function. Using purified mitochondria deficient in S1P metabolism, biochemical reconstitution studies have revealed that S1P and *2(E)*-hexadecenal cooperate with BAK and BAX, respectively, to promote MOMP and apoptosis [31]. While direct binding between *2(E)*-hexadecenal and BAX is observed and suggested to regulate activation-associated conformation changes within BAX, no further mechanistic insights are known.

The lipid composition of mitochondria has an impact on proapoptotic BCL-2 family function, but numerous reports suggest that mitochondrial membrane curvature and size also govern sensitivity to MOMP. Early work revealed that small LUVs (<0.2 μm diameter) resist BAX-dependent permeabilization [34]. These findings were corroborated and expanded in a recent study in which multiple OMM model systems were used to interrogate the functional contribution of mitochondrial shape in promoting BAX-mediated MOMP [26]. Specifically, *in vitro* reconstitutions of BID- and BIM-mediated BAX activation revealed that small LUVs, OMVs, and tiny mitochondria (<0.5 μm in diameter) failed to support the stable release and/or insertion of activated BAX monomers (specially, ∝ helix 9) into membranes. Indeed, the insertion of activated BAX monomers into a membrane is a key step that precedes oligomerization and MOMP [40,41].

While the majority of our discussions have focused on how mitochondrial membranes control BAK and BAX, several additional observations must be highlighted. First, mitochondrial lipids also facilitate several BH3-only proteins (i.e., BID and cardiolipin cooperate; and BIM and anionic lipids cooperate) and MCL-1 (regulated by cardiolipin and cholesterol) [32,42]. Second, BAX and cBID can independently remodel membranes, leading to alterations in tethering, stabilized membrane curvature, and the dissociation of lipid species – all of which are inhibited by the addition of BCL-xL [43]. Finally, the large GTPases responsible for mitochondrial dynamics have been shown to interact with BAX for collateral regulation of both mitochondrial fission and apoptosis. An independent role for dynamin-related protein (DRP)-1, a large GTPase required for mitochondrial fission, was described to remodel the OMM via hemifusion to promote BAX oligomerization [44]. Considering multiple signaling pathways, environmental influences, and pathophysiological conditions directly alter mitochondrial composition and shape, it is becoming increasing clear that we must continue to interrogate and integrate these pathways.

Is It Possible To Pharmacologically Regulate Proapoptotic BCL-2 Proteins as an Effective Therapy in Human Disease?

Over the last three decades, research on the mitochondrial pathway of apoptosis has positioned this mechanism as a key regulator of tumorigenesis and chemotherapeutic success [45]. Three lines of evidence suggest that BCL-2-regulated apoptosis is an excellent therapeutic target for small-molecule development to treat malignancies: (i) increased expression of antiapoptotic BCL-2 proteins confers tumorigenesis and chemoresistance; (ii) tumors constitutively sequester functional direct activator proteins that can be unleashed to promote MOMP; and (iii) the *in vitro* treatment of cancer cells with a sensitizer BH3 domain peptide reveals which tumors are apoptosis-competent and likely to respond to chemotherapeutic interventions. [46,47].

Chemically synthesized BH3 domain peptides represented a proof-of-concept that the hydrophobic groove of antiapoptotic BCL-2 proteins is a rational drug target, and significant efforts focused on discovering and refining small molecules that bind within groove [45]. In theory, these drugs release constitutively sequestered direct activator BH3-only proteins to engage MOMP (i.e., functioning as a single agent), or alternatively, this class of molecules also prevents chemotherapy-induced BH3 only proteins from being inhibited lowering the apoptotic threshold (i.e., functioning in combination strategies).

Structural analyses of BH3-only proteins in complex with antiapoptotic BCL-2 members examined within the perspective of the above observations led to the development of BH3-mimetics [48]. In this final section, we discuss recent progress in the pharmacological modulation of individual BCL-2 family proteins to selectively promote MOMP. We are focused on cancer therapeutics, but BH3 mimetics may also influence other human diseases impacted by deregulated apoptosis.

At present, there are ~20 small molecules defined as BH3-mimetics (Table 1). Some of these molecules (e.g., gossypol) were identified in high-throughput screens of natural products, while others (e.g., ABT-737) were developed using rational drug design strategies. Here, we focus on the most specific BH3-mimetics: the Abbott compounds (ABT-199, ABT-263, and ABT-737), recent MCL-1 inhibitors [MCL-1 inhibitor molecule (MIM)1 and A-1210477], and a first-in-class small molecule that activates BAX (BAX-activating molecule 7; BAM7).

Table 1. Overview of Current Small Molecules Targeting the BCL-2 Family of Proteins

Compound Name	Targets	Stage	Refs
ABT-737	BCL-XL, BCL-2, BCL-W	Phase II completed	[49]
ABT-263, Navitoclax	BCL-XL, BCL-2, BCL-W	Phase I/II	[57,58]
ABT-199, Venetoclax	BCL-2	FDA approved	[59]
GX15-070, Obatoclax	BCL-XL, BCL-2, MCL-1, BCL-W	Phase I/II	[60]
AT-101, R-(-)gossypol	BCL-XL, BCL-2, MCL-1, BCL-W	Phase I/II/III	[61]
S1	BCL-XL, BCL-2, MCL-1	Phase I/II/III	[62]
Apogossypol	BCL-XL, BCL-2, MCL-1, BCL-W	Preclinical	[63]
Apogossypolone, ApoG2	BCL-XL, BCL-2, MCL-1	Preclinical	[64]
BI-97C1, Sabutoclax	BCL-XL, BCL-2, MCL-1, BCL-W, A1	Preclinical	[65]
BI-97D6	BCL-XL, BCL-2, MCL-1, A1	Preclinical	[66]
TW37	BCL-XL, BCL-2, MCL-1, BCL-W	Preclinical	[67]
BH3-M6	BCL-XL, BCL-2, MCL-1	Preclinical	[68]
BM-1197	BCL-XL, BCL-2	Preclinical	[69]
JY-1-106	BCL-XL, MCL-1	Preclinical	[70]
Marinopyrrole A, Maritoclax	MCL-1	Preclinical	[71]
MIM1	MCL-1	Preclinical	[54]
A1210477	MCL-1	Preclinical	[55]
Small molecule MCL-1 inhibitors	MCL-1	Preclinical	[72]
BAM7	BAX	Preclinical	[56]
WEHI-539	BCL-XL	Preclinical	[73]
A-1155463	BCL-XL	Preclinical	[74]
XXA1	BCL-XL	Preclinical	[75]

ABT-737 was discovered over a decade ago using NMR-based screening, parallel synthesis, and structure-based design [49]. ABT-737 and its orally available derivative ABT-263 (Navitoclax) selectively bind to BCL-xL, BCL-2, and BCL-W (Table 1). ABT-737 possesses anti-tumor activity in many different cancer models including melanoma and prostate [50,51]. ABT-737 is regarded as an experimental tool, but ABT-263 has potential clinical application. One major flaw of ABT-263 is dose-limiting thrombocytopenia, due to the dependence of platelets on BCL-xL [52]. To circumvent this limitation in tumor settings where BCL-2 is overexpressed, a BCL-2-specific antagonist was derived: ABT-199 [53]. ABT-199 has been highly successful in clinical trials and recently gained FDA approval for chronic lymphocytic leukemia.

Tumors often display dependence upon BCL-2/BCL-xL or MCL-1, so, while the Abbott compounds represented a milestone in cancer therapy, successful treatment of MCL-1-dependent tumors remained a challenge. In 2012, the first MCL-1-specific inhibitor, MIM1, was identified an inducer of apoptosis in MCL-1-dependent leukemia [54]. More recently, another MCL-1 inhibitor was discovered, A-1210477, an indole-2-carboxylic-acid-based compound, which selectively inhibits MCL-1 at low nanomolar concentrations. It induces robust apoptosis in multiple human cancer cell lines, and potently synergizes with ABT-263 to ablate a cellular antiapoptotic BCL-2 protein repertoire [55].

BH3 mimetics function by inhibiting antiapoptotic BCL-2 proteins and subsequently lowering the cellular apoptotic threshold. This is likely useful as a mechanism to increase primary responses to chemotherapeutics (both conventional and targeted), and to reduce subsequent resistance. A PubMed search for ABT-263 reveals that hundreds of studies examined the benefit of combining ABT-263 with a broad spectrum of cancer chemotherapeutics using *in vitro* and *in vivo* models of the most common human malignancies. While the preclinical results for these drugs are promising, clinical successes have been slow and few. Toxicity is one concern, but it appears that more specific BH3 mimetics – molecules that inhibit a single antiapoptotic BCL-2 protein – may have solved that problem to produce a durable clinical benefit.

While our discussions focused exclusively on how to engage apoptosis by lowering the apoptotic threshold, another class of small molecules suggests alternative strategies may also hold clinical significance. Recently, a novel class of therapeutics has been described that directly engage BAX-dependent apoptosis. Utilizing computational screening, BAM7 was identified, which interacts with the amino-terminal region of BAX to promote its oligomerization and MOMP [56]. Future investigations will likely describe more potent BAX activators, and will hopefully include compounds to selectively activate BAK.

Concluding Remarks

The utility of BH3 domain peptides as convenient and biochemically tractable surrogates for full-length BH3-only proteins has yielded critical and accurate insights into BCL-2 family function. Indeed, these tools have also enabled the exploration of how mitochondrial shape and composition impact upon the cellular decision to engage the mitochondrial pathway of apoptosis. As with all model systems, caveats pertaining to the specificity and function of BH3 domain peptides need to be recognized. While recent mechanistic insights are illuminating a path to apply the principles of MOMP to better appreciate disease etiology, prognosis, and treatment, more needs to be done to gain a broader understanding of how this large family of complex proteins interact with each other, cellular lipids, and hydrophobic compartments (see Outstanding Questions). One of the most important questions in the field has persisted for years: what is the nature (i.e., proteinaceous, lipidic, or both) of the MOMP pore created by BAK and BAX? Furthermore, as we clarify the biochemistry and cell biology of the BCL-2 proteins, their impact in human disease will surely expand, and continued investment in small molecules to target specific proteins will ultimately provide a solid foundation for therapeutics to treat human disease.

Outstanding Questions

What are the biochemical, cellular, molecular, and structural contributions of mitochondrial lipids to the entire BCL-2 family of proteins?

What is the structural nature of the mitochondrial pore formed by the proapoptotic effector proteins leading to MOMP?

Can a panel of therapeutics each targeting a specific BCL-2 family member be discovered to treat human disease?

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