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Review

The BCL-2 family of proteins and mitochondrial outer membrane permeabilisation

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ABSTRACT

Apoptosis is a form of programmed cell death critical for the development and homeostasis of multicellular organisms. A key event within the mitochondrial pathway to apoptosis is the permeabilisation of the mitochondrial outer membrane (MOM), a point of no return in apoptotic progression. This event is governed by a complex interplay of interactions between BCL-2 family members. Here we discuss the roles of opposing factions within the family. We focus on the structural details of these interactions, how they promote or prevent apoptosis and recent developments towards understanding the conformational changes of BAK and BAX that lead to MOM permeabilisation. These interactions and structural insights are of particular interest for drug discovery, as highlighted by the development of therapeutics that target pro-survival family members and restore apoptosis in cancer cells.

Contents

1. Introduction to apoptosis 152
2. BCL-2 protein family sequence and structure 154
3. Pro-survival BCL-2 family proteins 154
4. Pro-apoptotic BH3-only proteins 155
5. Pro-apoptotic effector proteins and pore formation 156
6. Models for BCL-2 family regulation of apoptosis in mammals 156
7. BCL-2 family across species 157
8. Permeabilisation of the mitochondrial outer membrane 158
9. Conclusions 159
Funding 159
Conflict of interest 159
Acknowledgements 159
References 159

1. Introduction to apoptosis

A defining feature of multicellular organisms is their ability to control the destiny of individual cells in order for the organism to develop and evade irregular cellular function that may pose a threat. Multicellular organisms have adopted multiple strategies to initiate targeted removal of appropriate cells, and whilst there are many types of programmed cell death, apoptosis represents one of the principle pathways in mammals [1]. Apoptotic pathways result in destruction of the target cell with minimal inflammatory response and disruption to surrounding tissue. This is reflected in the use of apoptosis to remove webbing between digits and in the shaping of organs during development [2]. Apoptosis can proceed through one of two pathways: mitochondrial or extrinsic. Mitochondrial apoptosis (also referred to as intrinsic or stress induced

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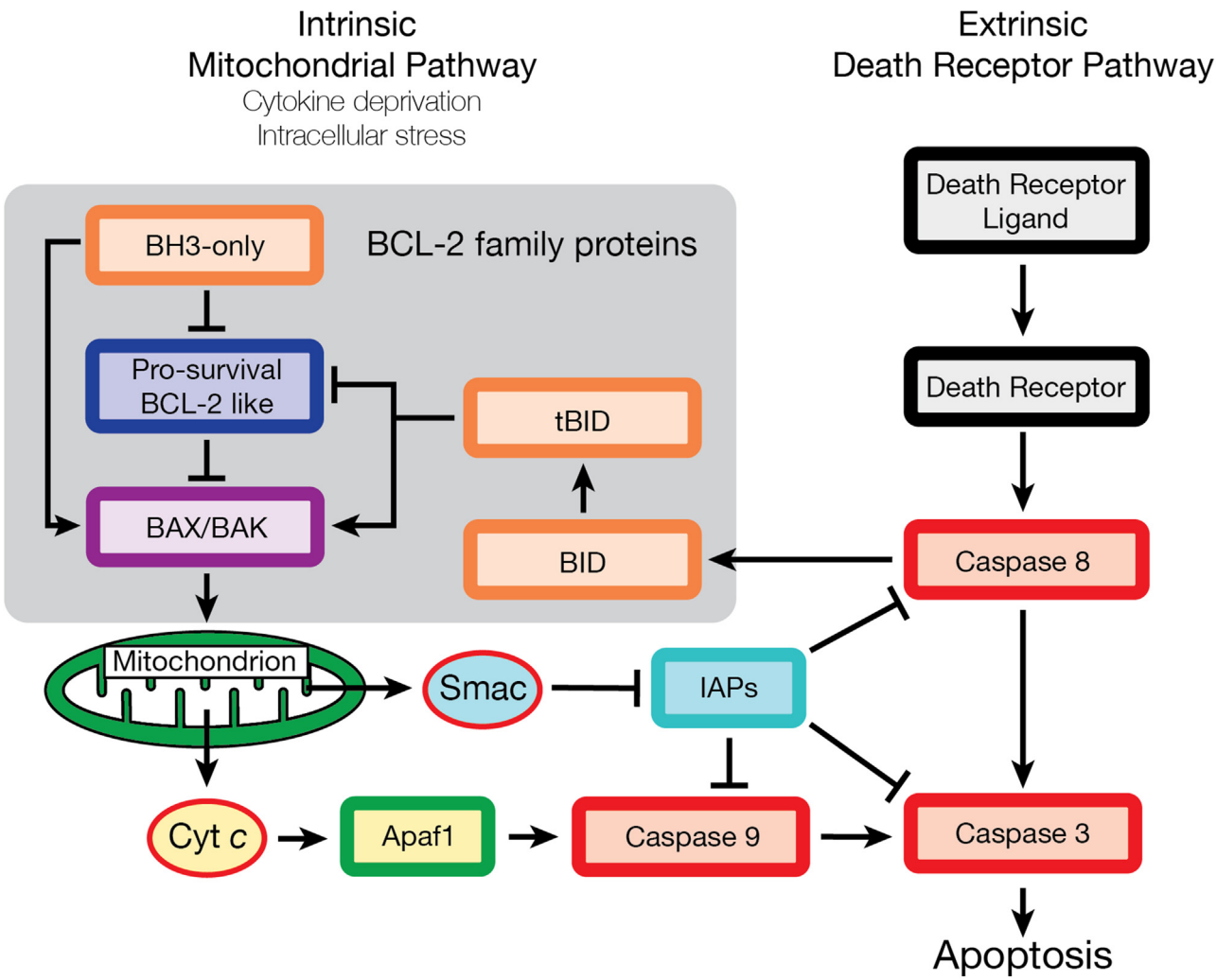

Fig 1. Overview of mammalian apoptotic pathways. Arrows indicate activation and progression towards apoptosis, bars indicate inhibitory interactions that prevent apoptosis. Pro-survival proteins include: BCL-2, BCL-X_L, BCL-W, MCL-1, BFL-1 and BCL-B. BH3-only proteins include BAD, BID, BIK, BIM, BMF, HRK, Noxa and PUMA. Smac is also called DIABLO and inhibits the IAPs (eg. XIAP, c-FLIP), preventing their inhibition of caspases.

apoptosis) results in triggering of the apoptotic pathway from internal stimuli, for example excessive DNA damage [3]. In contrast extrinsic apoptosis results from sensing of an external stimuli, for example signalling by the tumour necrosis factor (TNF) receptor superfamily (eg. TNF-R1, Fas/CD95 or TRAIL-R) [4] (Fig. 1).

There are a range of characteristic morphological changes that occur during apoptosis [5]. Initially the cell decreases in size creating a dense cytoplasm, and a characteristic condensation of chromatin (pyknosis) occurs. As the process develops the membrane blebs and the nucleus fragments (karyorrhexis), resulting in the distribution of the chromatin fragments throughout the cytoplasm [1,5,6]. The cell then further fragments into smaller apoptotic bodies of 1–4 μm [7]. Apoptotic cells are rapidly phagocytosed by neighbouring cells, principally macrophages, and in a healthy setting this might be so rapid that it occurs before fragmentation into apoptotic bodies. Phagocytosis results in the removal of the target cell, degrading the redundant material. The plasma membrane is maintained during apoptosis and thus the degrading cellular contents are encapsulated and cytokine release is prevented, resulting in a minimal immune response to the event. In contrast, the plasma membrane is ruptured in other types of cell death, causing release of damage associated molecular patterns (DAMPs), resulting in inflammation and a strong immune response [8]. Thus there are morphological distinctions between apoptosis and other forms of programmed cell death, characterised by shrinkage and blebbing of target cells. Immunological tolerance further differentiates apoptosis and other cell death processes.

Initiation of mammalian apoptosis results in activation of caspases, cysteine proteases that are expressed in proenzyme forms [9]. Upon activation the proenzymes are cleaved rendering them functionally active and capable of cleaving their target substrates. The two different apoptotic pathways function through discrete initiator caspases but culminate with the activation of common effector caspases (Fig. 1). When the intrinsic apoptotic pathway is induced, for example by excessive DNA damage, a signalling pathway ensues, that ultimately results in disruption of the mitochondrial outer membrane (MOM) [3,10]. This facilitates the release of cytochrome *c* (cyt *c*) from the mitochondrial intermembrane space into the cytosol. Cyt *c* release initiates formation of the apoptosome protein complex. The adaptor protein, apoptotic protease activating factor 1 (Apaf1), binds cyt *c* and activates caspase 9 in an ATP dependent manner [11–14]. Activated caspase 9 then cleaves its targets including procaspase 3, the point of convergence with extrinsic apoptotic mechanisms (Fig. 1). Activation of the caspase cascade results in fragmentation of DNA, degradation and crosslinking of proteins, and formation of apoptotic bodies [6]. Permeabilisation of the MOM also enables mitochondrial release of the larger 100 kDa protein Smac/DIABLO (second mitochondria-

derived activator of caspases/direct IAP binding protein with low pI) [15–17]. Smac/DIABLO then interacts with inhibitor of apoptosis proteins (IAPs) including X-linked IAP (XIAP) and cellular FLICE inhibitory proteins (c-FLIP), to prevent IAP inhibition of caspase activation [18–20]. Apoptosis is characterised by changes at the plasma membrane and exposure of markers, principally translocation of phosphatidyl serine (PS) from the inner to outer leaflet [6,21]. This provides a label for other cells to recognise the apoptotic body for phagocytosis [22]. In addition, it provides a useful biochemical tool to label apoptotic cells [23]. MOMP is widely regarded as the point of no return in the mitochondrial pathway to apoptosis. In mammals MOMP is directly regulated by the BCL-2 (B cell lymphoma 2) family [24]. This review will focus on the mitochondrial pathway, with particular emphasis on the BCL-2 family of proteins and their role in mitochondrial outer membrane permeabilisation (MOMP), a pivotal step towards cell death by this process.

2. BCL-2 protein family sequence and structure

The first member of the BCL-2 family of proteins, BCL-2 itself, was first described in the 1980s [25]. The gene for BCL-2 was first identified in cells from follicular lymphoma patients with a translocation of chromosomes 14 and 18 (t14;18), resulting in an increase level of protein expression. Subsequent work demonstrated that BCL-2 contributed to tumour development by keeping cells alive [26], this was in contrast to other oncogenes that drove cell proliferation. Discovery of the BCL-2 sequence allowed rapid identification of related proteins through sequence homology, in particular that of the conserved BCL-2 homology (BH) domains (Fig. 2a), although motifs is a more appropriate description for these short stretches of amino acid homology. There are four BH domains in total, numbered according to their date of discovery (Fig. 2a, b) [27]. Aside from BH domains, the family shows striking divergence in sequence identity, often below 30%, implying key functional roles for the BH domains.

BCL-2 family members are typically α-helical globular proteins composed of 9 helices, although most BH3-only proteins are largely unstructured (Fig. 2b) [2,28]. Eight of the nine helices (α1-8) form the canonical BCL-2 fold. The 9 helices are predominately amphipathic (both hydrophilic and hydrophobic faces), with the notable exceptions of the α5 and α9 helices that are hydrophobic. The α9 helix is typically separate from the globular core and inserts into the MOM providing a transmembrane tail-anchor. In contrast the α5 helix forms the core of the globular fold protected from the aqueous environment by the surrounding amphipathic helices. The Bcl-2 fold provides a structural context for the BH domains; domain in this instance refers to primary amino acid sequence and not the conventional structural definition of small independent units of tertiary structure. For multi-BH domain proteins the BH4 domain is in the α1 helix, BH3 in the α2 helix, BH2 bridging the α4-5 helices and the BH1 spanning the α7-8 helices. The pertinent feature of the BCL-2 family fold is the hydrophobic BH3 binding groove composed of the α2-5 helices. The base of the groove is formed primarily by the α5 helix. The α2 and α3 helices kink over the top of the α5 helix, the polypeptide then loops around to the α4 helix, at an acute angle and anti-parallel to the α3 helix (Fig. 2b). This hydrophobic groove provides a site on the protein into which an amphipathic helix can bind, primarily BH3 domains from other Bcl-2 family proteins. This ability to bind an incoming helix forms the principle functionality of the BCL-2 fold. Hydrophobic pockets within the groove provide anchor points for hydrophobic residues on the incoming BH3 domain ligand, canonically pockets (p) 1–4 [29] but additionally p5 on some pro-survival proteins [30,31] and p0 on Bax [2,32,33] (Fig. 2c). In addition, a conserved arginine residue in the α5 helix (BH1 motif) forms a salt-bridge with an aspartate found on all wild-type BH3 domains [29]. The BCL-2 family can be subdivided into three categories based on function: pro-survival, pro-apoptotic BH3-only and pro-apoptotic effector. Interactions between the different family groups occur through these groove-helix interactions and regulate progression to apoptosis.

3. Pro-survival BCL-2 family proteins

The mammalian pro-survival BCL-2 family proteins include BCL-2, BCL-X_L, BCL-W (BCL2L2), MCL-1, BFL-1 (known as A1 in mice) and BCL-B (also known as BCL2L10 or BOO/DIVA in mice) [26,34–37]. Pro-survival proteins are also present in other multicellular organisms including CED-9 in *Caenorhabditis elegans* [38]. The mammalian pro-survival proteins contain all 4 BH motifs and adopt the canonical BCL-2 fold (Fig. 2b). The pro-survival BCL-2 members are typically localised to the MOM by their α9 helix with the globular BCL-2 fold exposed to the cytoplasm [10,28]. The pro-survival members sequester pro-apoptotic proteins and prevent MOMP and subsequent apoptosis. The pro-survival proteins can inhibit apoptotic progression by two modes (Fig. 3a) [39]. Binding to the BH3 domain from a pro-apoptotic BH3-only protein is often termed mode 1 (Fig. 3a). Alternatively, binding to a pro-apoptotic effector protein (BAK or BAX) can be referred to as mode 2 (Fig. 3a). Different pro-survival proteins have differing specificities for the range of BH3-only and BAK or BAX binding partners allowing for a complex interplay and multiple redundancies to ensure appropriate apoptotic outcomes (Fig. 3a).

The inhibition of apoptosis by pro-survival BCL-2 family proteins plays a prominent role in disease. Many viruses encode pro-survival BCL-2 fold proteins to maintain cell viability during infection. Examples include proteins from members of the herpes family such as BHRF1 from Epstein-Barr virus and the vaccinia virus F1L protein [40–42]. These proteins share the canonical BCL-2 fold but in some cases have very little sequence homology to mammalian BCL-2 family proteins and unidentifiable BH motifs. Expression of these proteins results in inhibition of apoptosis by sequestering pro-apoptotic proteins, thus allowing the virus to evade defence mechanisms that would normally cause destruction of the infected cell.

Evasion of apoptosis is also one of the hallmarks of cancer [43,44], and pro-survival proteins are overexpressed in a variety of tumours as typified by the discovery of BCL-2 [25]. The resultant sequestration of active pro-apoptotic proteins provides an insurmountable barrier to MOMP and subsequent cell death. As a consequence, BCL-2 family proteins have been the subject of drug design strategies that have led to the development of BH3 mimetics, small molecules that bind to and inhibit pro-survival family members (Fig. 3b). This includes ABT-737 (Fig. 3b) and its drug like equivalent navitoclax (ABT-263) [45,46]. Both ABT-737 and navitoclax inhibit BCL-X_L, BCL-2 and BCL-W. Targeting BCL-X_L results in thrombocytopenia, due to a reliance on BCL-X_L for platelet survival [47,48]. This creates dose limiting toxicity for the use of this drug as a cancer therapeutic. Nevertheless, targeting pro-survivals is a valid strategy as typified by the recent FDA approval of venetoclax (ABT-199, Venclexta^{TM}). Venetoclax is a BCL-2-selective BH3 mimetic and as a consequence evades the platelet associated issues of navitoclax [49]. The selectivity of venetoclax is achieved by exploiting subtle differences in the P4 pocket of BCL-2, specifically including an aza-indole moiety. This results in an affinity for venetoclax that is 1000-fold more selective for BCL-2 over BCL-X_L, thereby evading the platelet sensitivity observed for navitoclax. Venetoclax is currently approved for some cases of chronic lymphocytic leukemia (CLL) [50]. BCL-X_L selective inhibitors have also been developed but these have not yet entered the clinic [51–53]. More recently an MCL-1 selective BH3 mimetic (S63845) has been reported [54]. The

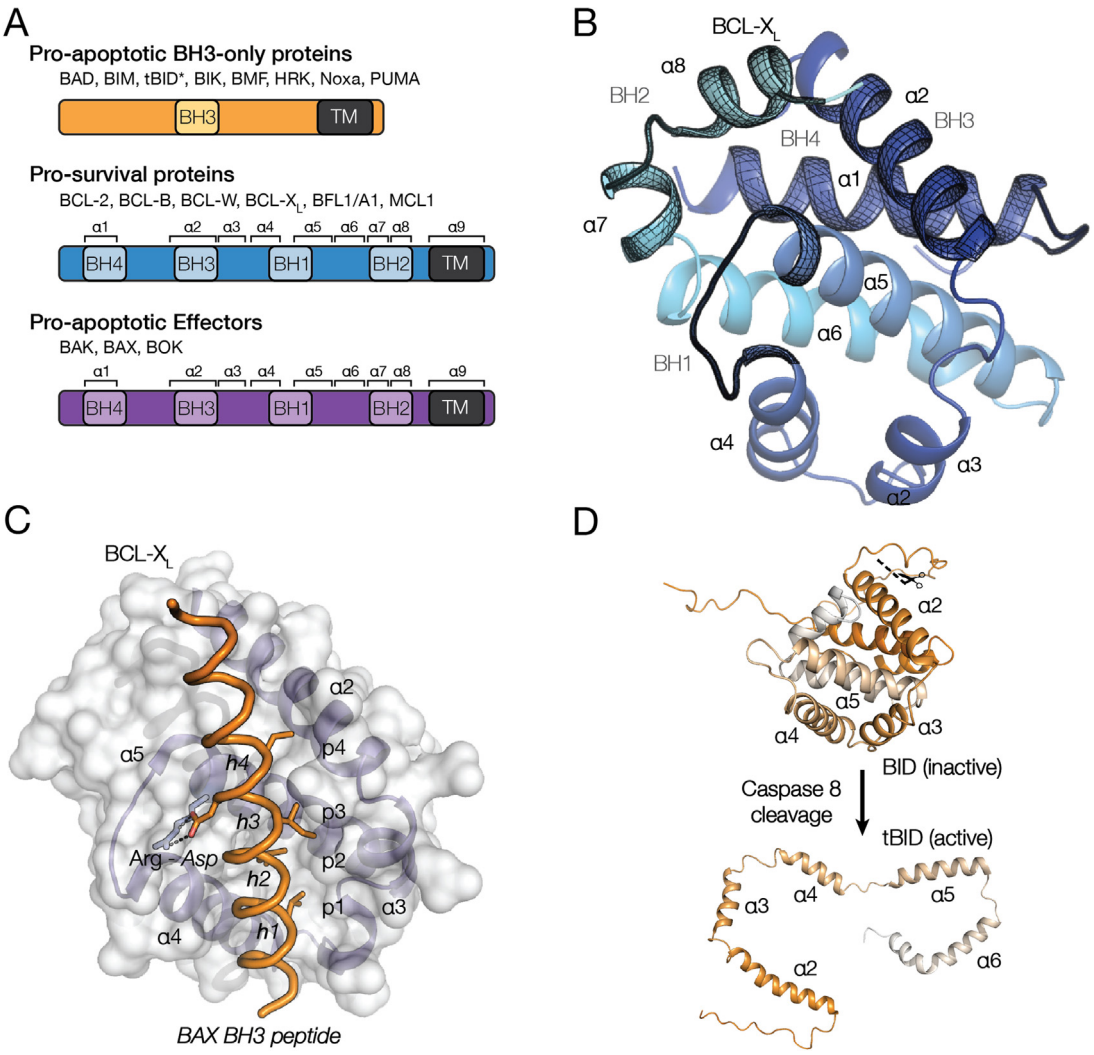

Fig. 2. BCL-2 protein family features. ***A***. Scheme for the three BCL-2 family subtypes: Pro-apoptotic BH3-only, pro-survival and pro-apoptotic effector. Positioning of BCL-2 homology (BH) domains, transmembrane domains and α-helices are indicated starting from the N-terminus at the left. The α-helices for BH3-only proteins are not indicated as they are typically unstructured, with the exception of BID (see Fig. 2D). ***B***. The canonical BCL-2 fold as represented by BCL-X_L (PDB id: 1MAZ [28]), with BH domains highlighted in black outline and colour transitioning from dark to light blue moving from the N- to C-terminus of the protein. ***C***. Crystal structure of the BAX BH3 peptide (orange) in complex with BCL-X_L (blue and white) (PDB id: 3PL7 [30]). The BCL-X_L surface and α2-5 helices lining the BH3 binding groove are shown. Key BH3 domain residues h1-4 are represented in stick representation and their corresponding hydrophobic pockets p1-4 indicated. The conserved Arg-Asp salt-bridge is indicated by dotted lines. ***D***. Solution structures of BID (top, PDB id: 2BID [153] and tBID in a lysophosphatidylglycerol micelle (bottom, PDB id: 2M5I [154]), showing the unfolding of BID upon caspase 8 cleavage and membrane association. The caspase 8 cleavage site is indicated by a dotted line with scissors.

MCL-1 gene is frequently upregulated in cancers, resulting in an increase in MCL-1 expression [54–56]. Trials are ongoing for Venetoclax in cancers other than CLL, and it is hopeful that other BH3 mimetics will also enter the clinic in due course.

4. Pro-apoptotic BH3-only proteins

The pro-apoptotic family members include the BH3-only protein subgroup and the effector proteins BAK, BAX and BOK – the latter three will be discussed independently. There are eight mammalian BH3-only proteins: BAD, BID, BIK (BLK), BIM, BMF, HRK (DP5), Noxa and PUMA [3,57,58]. BH3-only proteins can be grouped into activator or sensitiser phenotypes according to how they promote apoptosis. The activators are BID, BIM and PUMA as they can both directly activate BAK and BAX, and interact with pro-survival proteins to promote MOMP [2,59,60]. In contrast the sensitiser proteins only interact with the pro-survival proteins and do not activate BAK and BAX. However, some studies indicate that most BH3-only proteins have some capacity to activate BAK and BAX [61,62], and thus there appears to be some overlap between these two categories.

BH3-only proteins are present in other invertebrate organisms, for example EGL-1 from *C. elegans* interacts with the pro-survival CED9 protein [63]. BH3-only proteins are typically disordered providing only a single α-helix BH3 domain for interacting with other BCL-2 proteins and a C-terminal transmembrane tail-anchor for membrane targeting and insertion [64]. The exception is BID, which possesses a complete BCL-2 fold (all 8 α-helices) in its native state (Fig. 2D) [65,66]. Upon sensing appropriate apoptotic stimuli BH3-only proteins become active through transcriptional and post-translational mechanisms. For example, PUMA expression is upregulated by p53 in response to DNA damage [3,67]. In contrast BID is cleaved by caspase 8 upon activation of the TNF/Fas receptor resulting in the activated form of BID – tBID [68,69]. Upon activation, BH3-only proteins are targeted to the MOM by a variety of mechanisms. For example, BIM possesses a hydrophobic tail sequence that allows it to interact with the MOM [70,71] and Noxa contains a mitochondrial targeting sequence at its C-terminus that

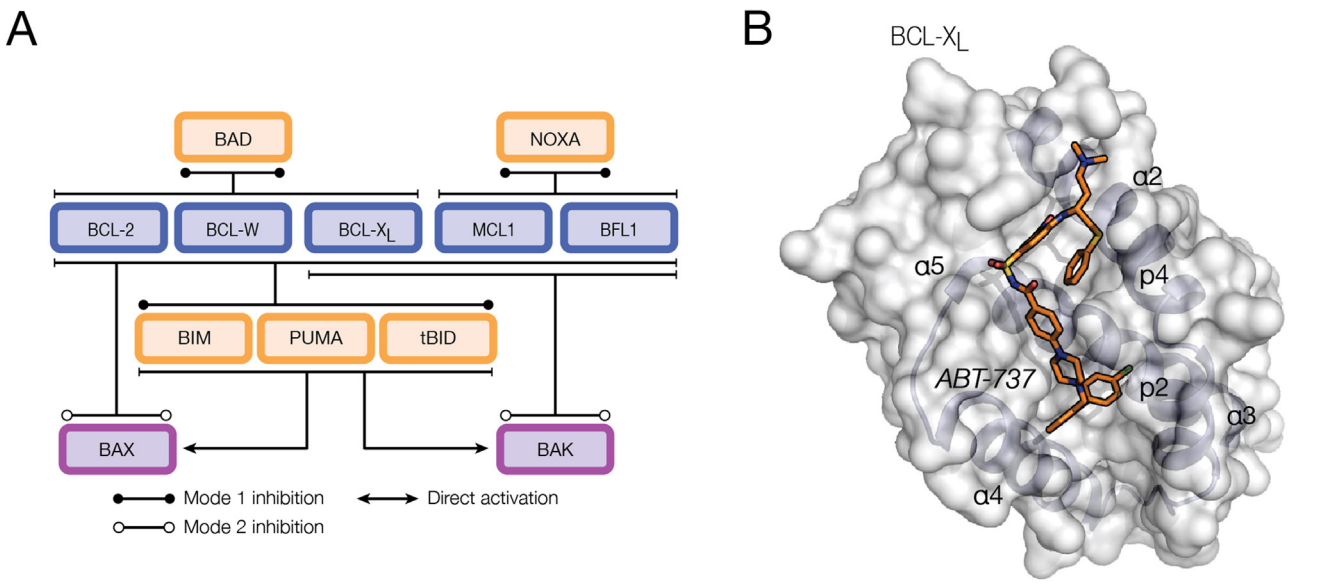

Fig. 3. Interactions of the BCL-2 protein family. ***A***. Scheme for reported inhibition (open and closed circles) of apoptosis by the pro-survival proteins (blue), indicating specificities for individual pro-apoptotic BH3-only (orange) or effector (purple) proteins. Potent BH3-only activators of BAK and BAX are indicated by arrows. ***B***. Crystal structure of the BH3 mimetic ABT-737 (orange; stick representation) bound to BCL-X_L (blue and white) (PDB id: 2YXJ [155]). The BCL-X_L surface and α2-5 helices are shown, with the hydrophobic pockets (p2, p4) exploited by ABT-737 indicated.

is found in non-BH3-only proteins [72,73]. Truncation of BID to tBID by caspase 8 causes the protein to partially unfold, losing its compact globular structure, exposing hydrophobic regions that interact with the MOM (Fig. 2D) [71,74]. The BH3-only proteins initiate apoptosis through their interactions with pro-survivals and the pro-apoptotic effector proteins BAK and BAX as described below.

5. Pro-apoptotic effector proteins and pore formation

Mammals possess three pro-apoptotic BCL-2 proteins with the capacity to permeabilise the MOM – BAK, BAX and BOK. All three share the canonical BCL-2 fold and all 4 BH motifs [40,75], however, their routes to effecting MOMP differ slightly due to their cellular localisations. BAK localises to the MOM, tethered by its hydrophobic α9 helix [76]. Prior to activation BAK forms a complex at the MOM with the outer membrane β-barrel VDAC2 [77,78]. BAX predominantly localises to the cytosol, in a state of flux shuttling on and off the MOM [79–82], BAK might also undergo such retrotranslocation with the rate heavily favouring localisation at the MOM [83]. The BAX α9 helix contains polar residues not present in BAK, reducing the energetic penalty for maintaining this region in a hydrophilic environment. Solubility in the cytosol is achieved structurally through sequestration of the α9 helix in the BH3 binding groove [81]. BAK and BAX activity is regulated through interactions with pro-survival BCL-2 proteins and activator BH3-only proteins. Upon activation BAK and BAX form dimers that subsequently form higher order oligomers (Fig. 4a) [84–89].

BOK shows sequence similarity to BAK and BAX but is less well understood functionally. In contrast to BAK and BAX, BOK is predominately localised to the endoplasmic reticulum (ER) and golgi apparatus [90–92]. It has been reported that at the ER BOK is associated with IP_3 receptors (predominately IP_3R1 and IP_3R2 and to a lesser extent IP_3R3), in an inhibited complex [91,92] and that free BOK not in complex with IP_3 receptors is rapidly ubiquitinated and degraded by the proteasome [91,93]. The role of BOK in MOMP is unclear given it is predominately ER associated and readily expressed in a variety of tissues in steady state [90,91,94–96]. It has been reported that BOK can form pores in membranes, can facilitate Cyt *c* release from the MOM and can kill cells in the absence of Bax and Bak [93,97]. However, it has also been reported that Bok cannot release Cyt *c* in the absence of BAK and BAX under physiological conditions [98]. Thus BOK, akin to BAK and BAX, can permeabilise membranes, however its precise role in apoptosis is currently unclear.

Once activated, oligomerised pro-apoptotic effector proteins form pores in the MOM, enabling release of cyt *c* and other factors. There are currently two proposed models for the nature of this pore: proteinaceous and toroidal lipidic. The proteinaceous pore model proposes a pore lined exclusively by protein as seen in the membrane attack perforin complex from cytotoxic T cells [99]. In this model oligomerisation forms a pore that completely excludes lipid from the solvent channel providing a lipid-protein-solvent interface, with the protein forming a barrier between the two immiscible phases. In contrast the toroidal lipidic pore model proposes that the pore interface contains both lipid and protein interacting with the aqueous environment, with no segregation between lipid and aqueous solvent. In this model BAK, BAX or BOK destabilise the lipid bilayer by introducing local curvature allowing a pore to form. Accumulating data support that BAK, BAX and BOK form toroidal pores [100–106].

6. Models for BCL-2 family regulation of apoptosis in mammals

In the past, the mechanism by which the BCL-2 family of proteins regulate apoptosis was the subject of much debate. Arguments typically supported one of two alternate models addressing how the BH3-only proteins initiated apoptosis and whether this occurred through direct activation of BAK and BAX [32,59,60,107–111] or indirectly through competition for pro-survival family members [112–114]. It is now well accepted that both mechanisms occur in cells and that a unified model incorporating both pathways is appropriate (Fig. 3a) [39]. In the unified model, pro-survival proteins on the MOM bind to the BH3 domain of pro-apoptotic proteins, this includes BAK, BAX and the BH3-only proteins (Fig. 3b). In mode 1 a BH3-only protein interacts with a pro-survival protein, in contrast when a BAK or BAX molecule interacts it is called mode 2. Upon an appropriate apoptotic stimulus, a BH3-only protein is

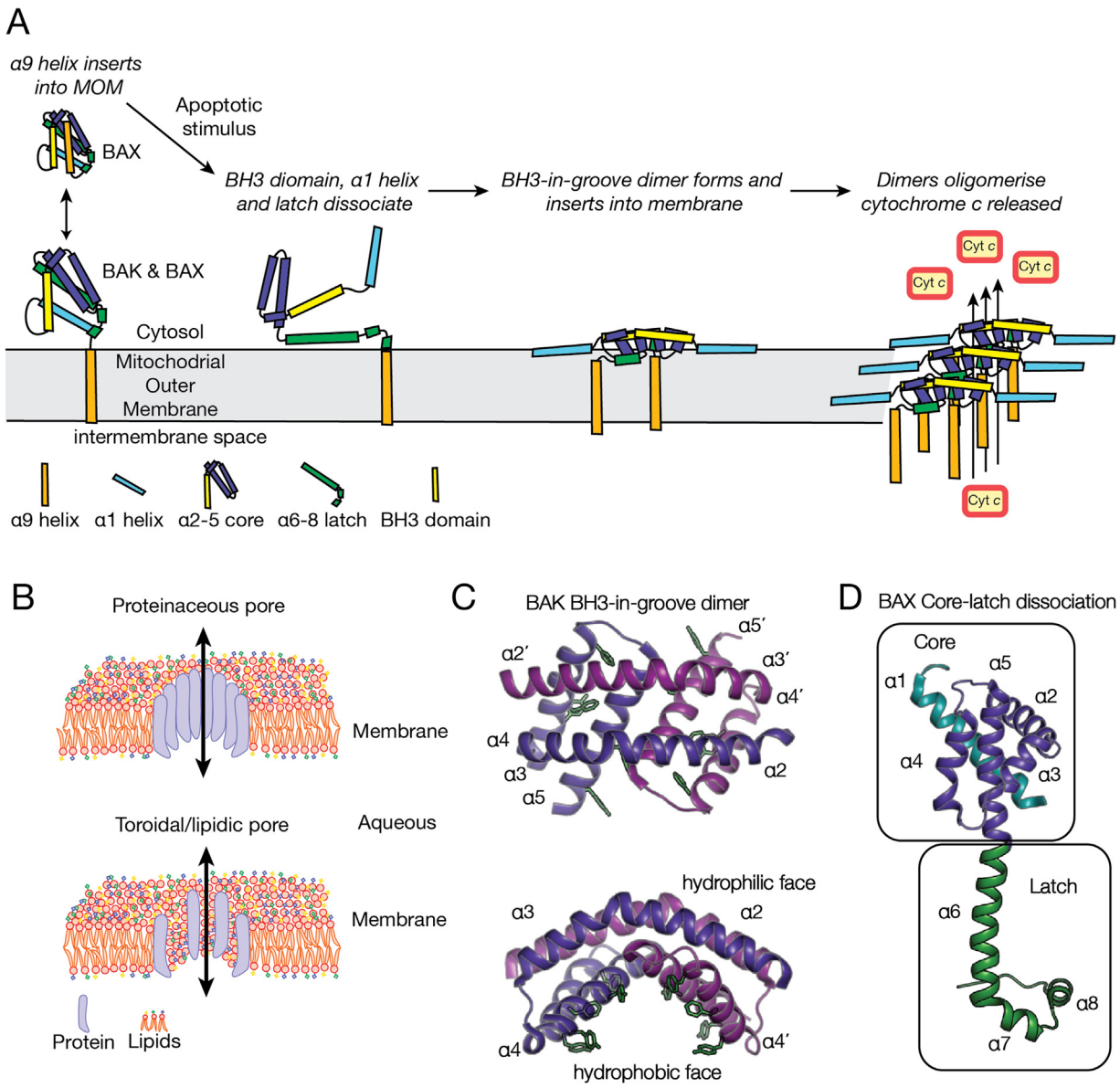

Fig. 4. Conformational changes and pore formation by the pro-apoptotic effector proteins. ***A***. Scheme for conformational changes of the pro-apoptotic effector proteins BAK and BAX at the mitochondrial outer membrane and their subsequent dimerisation followed by oligomerisation, to enable cytochrome *c* release from mitochondria. Conformational changes can be induced at the membrane by activating BH3-only proteins or by other physical stimuli including heat. ***B***. Cartoon illustrating the differences between proteinaceous and toroidal lipidic pores. Protein is represented in light blue, lipid acyl tails in orange, glycerol and phosphate in red, and head groups in dark blue, yellow and green. ***C***. Crystal structure of the BAK BH3-in-groove dimer (PDB id: 4U2V [140]). Monomers are shown in differing shades of purple and aromatic residues lining the hydrophobic α4-5 face indicated in green stick representation. ***D***. Representation of one monomer of the BAX core-latch crystal structure (PDB id: 4BD8 [32]), illustrating the dissociation of the latch domain from the core. The α1 helix is represented in teal, α2-5 core in purple and α6-8 latch in green.

expressed or activated. If this BH3-only protein is an activator it can directly promote BAK and BAX oligomerisation. If the pool of activator BH3-only proteins are bound to pro-survival proteins, up regulation of sensitiser BH3 proteins can lead to their displacement to allow direct activation of free BAK and BAX. In mode 2 BAK or BAX have their BH3 domain exposed and bound to a pro-survival protein. The activated BH3-only proteins compete with the pro-survival proteins for BAK and BAX, displacing the pro-apoptotic effectors and allowing their homo-oligomerisation and subsequent MOMP. Thus there are multiple redundancies in the mechanisms by which apoptosis can be promoted and controlled by the BCL-2 family. This redundancy allows for precise regulation of apoptosis, as inappropriate apoptotic signalling would be detrimental to the organism.

7. BCL-2 family across species

Cell death has been extensively studied in invertebrate model organisms including *C. elegans* and *D. melanogaster*. Both these systems have BCL-2 family proteins present, however, they do not have the full suite of pro-survival and pro-apoptotic members present in mammals [63]. As a consequence, the role of BCL-2 proteins in these systems is distinct from mammalian apoptosis. In *C. elegans* CED9 is the only pro-survival protein. CED9 binds to CED4, which is analogous to APAF-1 in mammals [63,115,116]. Upon an apoptotic stimulus EGL-1, A BH3-only protein, is upregulated. EGL-1 displaces CED4 from CED9. CED4 can then oligomerise and cleave the caspase CED3 to form the active caspase, allowing apoptotic progression [117–119]. In contrast to mammals, MOMP and cyt *c* release are not essential for apoptotic progression in *C. elegans*. Reflecting this

there are no pro-apoptotic effector BCL-2 proteins in the *C. elegans* genome [63]. However, this is not necessarily the case for all worm species. For example, the blood-flukes *Schistosoma japonicum* and *S. mansoni* have been shown to have a fully functioning triapartite BCL-2 family similar to vertebrates [120].

The role of BCL-2 proteins in *D. melanogaster* differs from both mammals and *C. elegans*. Although *D. melanogaster* encode two BCL-2 family proteins buffy and Debcl [63,121,122], they are not essential for apoptosis. In addition, their roles in cell death are unclear. Overexpression of both proteins individually results in cell death. However, deletion of both Debcl and buffy results in no developmental phenotype in the flies. Therefore, development in *D. melanogaster* is not thought to be regulated by induction of apoptosis by the BCL-2 family [123]. Instead *D. melanogaster* rely upon the pro-apoptotic regulators Reaper, Hid, Grim and Sickle [63,124–126]. These proteins inhibit the function of the *Drosophila* inhibitor of apoptosis 1 (DIAP1), which inhibits caspases under normal conditions [63,127]. These proteins do not share any homology with the BCL-2 family and as such are distinct.

More recently it has been shown that the freshwater hydrozoan *Hydra* (*Hydractinia echinata*) contains nine BCL-2 family members [128,129]. This includes two BAK-like proteins, seven BCL-2-like proteins and three BH3-only proteins. The diversity of the *Hydra* BCL-2 family indicates a role more similar to the mammalian BCL-2 family where a complex interplay of the different family members occurs to regulate cell death. These observations are intriguing; however little research has been published on the family since their initial discovery in 2010. In addition to *Hydra*, BCL-2 family proteins have been identified in sponges including *Geodia cydonium* [130]. These are considered to be some of the most evolutionary distant (ancient) BCL-2 family proteins from those of mammals. For a broader discussion on the evolution of metazoan BCL-2 family proteins see this recent review by Green and Fitzgerald [131].

8. Permeabilisation of the mitochondrial outer membrane

Large structural changes and oligomerisation of BAK, BAX and BOK are required for mammalian MOMP. As yet these changes are not fully understood, however progress has been made. To promote MOMP the pro-apoptotic effector proteins must translocate to the MOM. Upon expression BAK is translocated to the MOM, tethered to the membrane by its hydrophobic α9 helix and associated with VDAC2 in an inert complex [77,78]. Although BAX predominantly localises to the cytosol [80,81], its interaction with VDAC2 in the MOM is thought to determine its mitochondrial/cytosol distribution [132,133]. This difference in subcellular localisation is a fundamental difference between BAK and BAX, as BAX needs to accumulate on the MOM for permeabilisation to occur. How this translocation occurs is not fully understood, however BAX appears to cycle between the MOM and cytoplasm in healthy cells but upon an apoptotic stimulus remains associated with the MOM [79]; presumably through conformational changes of the α9 helix relative to the groove. The hydrophobic α9 helix is likely to be displaced from the BAX groove by a BH3-only protein, either through binding to an allosteric site [108] or through competition for the canonical BH3 binding groove [32]. In contrast, BAK is already localised to the MOM in complex with VDAC2. Upon binding of an activating BH3-only protein BAK can dissociate from VDAC2 and become activated. Pro-survival proteins can trap BAK or BAX at the membrane and upon displacement by a BH3-only protein release BAK or BAX in a state primed for oligomerisation.

Studies have shown that activated BAK and BAX change conformation on the MOM. This is believed to involve three key transitions: exposure of the BH3 domain, dissociation of the α1 helix and dissociation of the α6-8 helices from the core (Fig. 4a); however the order of these changes is not clear. The disassociation of α1 helix is an established marker of BAX activation. The antibody 6A7 will only bind to BAX upon exposure of an epitope at the start of the BAX α1 helix [134], the same is true of the Ab-1 and 8F8 antibodies for BAK [84,135]. Antibodies that target the α1-2 loop have been shown to activate both BAK and BAX [136] and provide further evidence that the dissociation of the α1 helix from the core is required for MOMP [137]. However, It is unclear if α1 dissociation occurs independently or simultaneously with other structural changes described below.

A principle feature of MOMP by BAK or BAX is the formation of dimers that then form higher order oligomers. It has long been established that the BAX BH3 domain is required for this oligomerisation [138]. Furthermore, truncation of the BAX protein into just α2-5 helices, in combination with a mitochondrial targeting transmembrane domain, is reported to be sufficient for this dimerisation and MOMP [139]. Insights into the structure of this active dimer have been provided by X-ray crystal structures of both BAK and BAX (Fig. 4c) [32,140]. These structures have revealed a symmetric dimer where the BH3 domain from one monomer is exposed and extends into a groove formed from the α3-α5 helices of its dimeric partner. A rotation axis is formed between the central region of the α5 helices with its partner molecule. The dimer has an amphipathic architecture with the α2-3 helices providing a relatively polar surface and the α4-5 planar base providing a more hydrophobic surface, lined with bulky aromatic residues. This split polarity to the dimer is likely to facilitate membrane insertion with the α4-5 helices embedding into the membrane, leaving the polar α2-3 interface exposed to aqueous solvent. This 'core' or 'BH3-in-groove' dimer confirms results from cellular crosslinking experiments [85,86,88,100,105,141,142], providing compelling evidence that this is the dimeric base for higher order oligomerisation. However, it is still unclear how these dimeric units form into the larger oligomers that lead to membrane permeabilisation.

The other large re-arrangement upon activation is restructuring of the α6-8 helices. In addition to the crystal structures of core domain dimers there are domain swap or 'core-latch' dimer structures (Fig. 4d) [32,140,141]. These dimers are not believed to be biologically relevant as they are not consistent with cellular crosslinking studies [85,86,88,100,105,141,142]. These dimers are formed by activating BAK or BAX in the presence of detergent and/or activating BH3 peptide *in vitro*. The domain swap dimers fold into the canonical BCL-2 fold architecture, however the α6-8 'latch' region is supplied by the dimeric partner. This creates an extended continuous α5-6 helix in the BAX structures. In the BAK structures there is a slight kink that segregates the α5 and α6 helices, preventing the extended helix conformation, but still requiring release of the α6 from the monomeric core. These structures reveal how the BAK and BAX α6-8 latch domains could dissociate from the core, however at this stage do not reveal how this is involved in forming the MOM pore structure. Other sites on BAK and BAX can be crosslinked in the larger oligomer indicating that they lie proximal to interfaces mediating oligomerisation [85,86,100,105,136,142,143]. However, unlike the BH3-in-groove interface, cross-linking of these second sites is never saturating, suggesting that they might not represent distinct interfaces.

Studies investigating the arrangement of the α5 and α6 helices in activated BAK favour an in-plane model whereby the α5 helix is associated with the α2-4 helix core with α6 separated from this core and only partially buried into the membrane [144]. This observation disagrees with models that propose the α5 and α6 helices form a hairpin that traverses the membrane akin to a conventional transmembrane helix (like the α9 helix) [103,145–147]. In the in-plane model the α6 helix either resides on the leaflet of the MOM or potentially lines a lipidic pore through the membrane.

The precise structure of activated BAK or BAX at the membrane and how they permeabilise this barrier remains an enigma. Recent studies have used electron paramagnetic resonance spectroscopy, cysteine mutagenesis and spin labelling to extract distance restraints between residue pairs at the membrane [100,105,148,149]. These studies have revealed distances compatible with a BH3-in-grove dimer present at the membrane in the pore. These observations suggest that the BH3-in-groove dimers line a toroidal pore with the α4-5 helices embedded in the membrane and the α2-3 helices exposed to aqueous solvent. The orientation of the BH3-in-groove dimer relative to the membrane and opposing dimers is not clear. The "clamp" model proposes that the α2-3 helices are perpendicular to the membrane [105]. In this model the α6-8 helices from each monomer in the BH3-in-groove dimer lie in-plane with the membrane on opposing leaflets; this places the membrane penetrating α9 helices antiparallel in the membrane [105]. An opposing model proposes that the BH3-in-groove dimers are at an acute angle to the membrane, ~15° to the bilayer [100,148]. In this model all the α6-8 helices co-localise on the outer membrane leaflet with the α9 helices penetrating the membrane in a parallel orientation. Both models propose that the pore is lined by multiple copies of the BAK or BAX dimers, and that in addition to the BH3-in-groove dimer there are additional oligomerisation interfaces between the α6-8 regions of neighbouring dimers. An additional model has been proposed suggesting that BAX can form oligomers in solution, that can then directly insert into the MOM [150,151]. The number of BAK or BAX dimers required to create a pore is unclear. Recent super-resolution and atomic force microscopy studies have revealed that BAX can form in large lines, arcs and rings on the MOM [102,106]. These structures can range in size, with the arc and ring formations generating pores ranging from 25 nm to over 100 nm in size. These observations correlate with previous observations of BAX on vesicles [102,152]. These large pores could be maintained by relatively few (6–10) BAX or BAK molecules if they induce membrane curvature and support a toroidal pore, thus potentially explaining the heterogeneity in pore size in these studies.

9. Conclusions

The term apoptosis was coined to describe targeted cell deletion almost half a century ago [5]. Since then the field has matured into an active research domain pertinent to human disease. Since the discovery of the BCL-2 protein in the 1980s we have advanced considerably our understanding of the regulatory machinery of the mitochondrial pathway to apoptosis in mammals. This has been complemented by studies in model organisms such as worms and flies, although their mechanisms of regulation diverge from their mammalian equivalents. The years of research into the BCL-2 family of proteins is now bearing fruit in the form of BH3 mimetic drugs for the treatment of cancer. However, despite these successes, there are still significant aspects of the pathway that remain enigmatic, notably how the pro-apoptotic effector proteins permeabilise the MOM. Resolving these mysteries may lead to new strategies for developing future therapeutics for the treatment of diseases characterised by deregulated apoptosis.

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Conflict of interest

RWB and PEC are employees of the Walter and Eliza Hall Institute which has an agreement with Genentech and AbbVie and receives milestone payments related to Venetoclax.

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