Cell death and the mitochondria: therapeutic targeting of the BCL-2 family-driven pathway

Abbreviated title: Therapeutic targeting of the BCL-2 family proteins

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Summary

The principal biological role of mitochondria is to supply energy to cells, though intriguingly, evolution has bestowed another essential function upon these cellular organelles: under physiological stress, mitochondria become the cornerstone of apoptotic cell death. Specifically, mitochondrial outer-membrane permeabilization (MOMP) allows cell death factors such as cytochrome *c* to be released into the cytoplasm, thus inducing caspase activation and the eventual destruction of essential cellular components. The BCL-2 family proteins control the tightly regulated pathway that causes MOMP. The equilibrium between pro-survival and pro-apoptotic members of the BCL-2 family dictates the fate of cells, the homeostasis of organs, and by extension, the health of whole organisms. Dysregulation of this equilibrium is implicated in a large number of diseases such as cancer, auto-immunity and neuro-degenerative conditions. Modulating the activity of the BCL-2 family of proteins with small molecules or peptides is an attractive but challenging therapeutic goal. This review highlights the latest developments in this field and provides evidence that this strategy will likely have a positive impact on the treatment of still poorly addressed medical conditions.

Keywords: apoptosis, BCL-2 family, protein-protein interactions, cancer, neuro-degenerative diseases.

Abbreviations

Apaf-1: Apoptotic protease activating factor 1; BAD: BCL-2-associated death promoter protein; A1 or BCL2A1: BCL-2 related protein A1; BAK: BCL-2 homologous antagonist/killer protein; BAX: BCL-2–associated X protein; BCL-2: B-Cell lymphoma 2 protein; BCL-X_L: B-cell lymphoma-extra large protein; BH: BCL-2 homology domain; BID: BH3 interacting-domain death agonist; BIM: BCL-2 Interacting mediator of cell death; CLL: Chronic lymphocytic leukemia; MCL-1: myeloid cell leukemia sequence 1 protein; MEF: Mouse Embryonic Fibroblasts; MOM: Mitochondrial outer-membrane; MOMP: Mitochondrial outer-membrane permeabilization; ; PAGE: Polyacrylamide gel electrophoresis; PUMA: p53 upregulated modulator of apoptosis protein; PS: phosphatidyl serine.; SAHB: stabilized α-helices of BCL-2 domains; SCLC: small cell lung carcinoma.

Introduction

'Apoptosis' is a form of genetically programmed cell death, which is both evolutionally-conserved and tightly-regulated at a molecular level. The process plays a key role in embryonic development and in the destruction of diseased, damaged or unwanted cells. From the first coinage of the term in 1972, a poetic adoption of a Greek word used to describe how leaves are dropped from a tree (Kerr *et al.*, 1972), it was foreshadowed that defects in the process might play a role in multiple disease states in which normal regulation of cell number becomes perturbed, such as cancer or autoimmunity (survival of unwanted cells), or developmental/degenerative disorders (inappropriate killing of vital cells).

Cells may be triggered to undergo apoptosis *via* either an 'extrinsic' (death receptor) or 'intrinsic' (mitochondrial) pathway; the latter of which is regulated by proteins of the BCL-2 family and proceeds *via* key steps that lead to permeabilization of the mitochondrial outer membrane (MOMP). Both pathways converge in the activation of a cascade of downstream caspases (cysteine-aspartic proteases), which catalyze the process of cellular demolition. This results in the phenotype characteristic of apoptotic cells: DNA laddering, cell shrinkage, apoptotic body formation, chromatin condensation and plasma membrane changes such as blebbing and externalization of phosphatidyl serine (PS), which rapidly signals for the ultimate engulfment and digestion of the dying cell by macrophages. Understanding the key molecular interactions between BCL-2 family members that regulate the intrinsic apoptotic pathway leading to MOMP has paved the way for the development of new therapies that modulate apoptosis.

This review focuses on the intrinsic BCL-2 family-driven pathway to apoptosis. It summarizes the biological context to targeting these proteins and describes recent advances in therapeutic approaches with compounds directly interacting with BCL-2 family proteins, including an outlook as to their clinical potential.

The BCL-2 family: members and interactions

The BCL-2 family of proteins comprises two functionally opposing subsets, the pro-survival proteins and pro-apoptotic proteins. The relative proportions of these two subsets control the fine balance between cell survival and death *via* the intrinsic apoptotic pathway. In mammals, pro-survival proteins (e.g. BCL-2, BCL-X_L, BCL-W, MCL-1, and A1) act as gatekeepers to block apoptosis by inhibiting their pro-apoptotic counterparts (Adams and Cory, 2007). The pro-apoptotic proteins are divided into two further sub-groups: the so-called BH3-only proteins (including BIM, tBID, BAD, PUMA, NOXA) act as initiators and are invoked in response to sensing discrete cellular apoptotic stimuli (such as growth factor withdrawal, DNA damage, anoikis) and the multidomain BAK/BAX proteins which directly facilitate permeabilization of the mitochondrial outer membrane (MOMP, Figure 1A)) (Youle and Strasser, 2008).

In response to stimuli, BH3-only protein activity can be up-regulated by increased expression, activation by proteolytic cleavage, or post-translational modification. These BH3-only proteins then trigger apoptosis either by directly activating BAK/BAX (Cartron *et al.*, 2004; Certo *et al.*, 2006; Deng *et al.*, 2007; Gavathiotis *et al.*, 2008; Kuwana *et al.*, 2005; Letai *et al.*, 2002), or by disrupting complexes between pro-survival proteins and activated BAK/BAX (Oltvai *et al.*, 1993; Willis *et al.*, 2007). The BAK/BAX molecules go on to oligomerize on the outer-mitochondrial membrane (OMM, Figure 1B-C). This oligomeric assembly triggers MOMP (although whether this occurs due to formation of a pore or by some other mechanism is yet to be determined), allowing the release of cytochrome *c* and other apoptosis-inducing factors, from the mitochondrial inter-membrane space. Cytosolic cytochrome *c* interacts with apoptotic protease-activating factor-1 (Apaf-1) to form a structure known as the apoptosome. The apoptosome activates caspase-9, thus initiating a caspase cascade which ultimately leads to demolition of the cell (Bratton and Salvesen, 2010). MOMP is likely to constitute an irreversible step in the pathway as the amplification of the caspase activation cascade (upstream caspases activating downstream caspases) is difficult to interrupt.

Elevated levels of one or more pro-survival proteins, as observed in many tumors, can block apoptosis. This block can occur through sequestration of activator BH3-only

proteins, or capture and restraint of active forms of BAK/BAX, or both (Llambi *et al.*, 2011). Pro-survival proteins have been shown to be capable of forming heterotypic interactions with the BH3-domain of both BH3-only proteins and BAK/BAX (Czabotar *et al.*, 2011; Liu *et al.*, 2003; Sattler *et al.*, 1997). Moreover, as direct activation of BAK/BAX by BH3-only proteins has been proposed to occur *via* a 'hit-and-run' mechanism, structural elucidation of this mechanism remains challenging, although there have been notable recent insights into how this process might proceed (Czabotar *et al.*, 2013; Gavathiotis et al., 2008; Moldoveanu *et al.*, 2013). In either case, the development of agents able to selectively inhibit pro-survival proteins (or to modulate pro-apoptotic BAK/BAX activation/oligomerization) offers significant therapeutic potential, which has been greatly assisted by a detailed structural understanding of these interactions.

Structural features of the BCL-2 family of proteins

All proteins forming part of the BCL-2 family share one or more so-called 'BCL-2 Homology' (BH) domains, named by reference to the founding member BCL-2. The pro-survival proteins and BAK/BAX each share four BH domains (BH1-4) (Kvansakul *et al.*, 2008), whereas the BH3-only proteins share only their namesake the BH3 domain and are otherwise structurally diverse (Figure 2A) (Hinds *et al.*, 2007). It is worth noting that whilst early reports claimed that BAX and BAK possessed only BH domains 1-3 (Zha *et al.*, 1996), BH4 signature motifs are evident from later sequence and structural analyses (Kvansakul et al., 2008).

Interestingly, despite their opposing function, both the pro-survival proteins as well as BAK/BAX share remarkably similar tertiary structures (Figure 2B). Structures solved of pro-survival proteins BCL-X_L (Muchmore *et al.*, 1996), BCL-2 (Petros *et al.*, 2001), BCL-W (Denisov *et al.*, 2003; Hinds *et al.*, 2003), MCL-1 (Day *et al.*, 2005), A1 (Herman *et al.*, 2008) as well as BAK (Moldoveanu *et al.*, 2006) and BAX (Suzuki *et al.*, 2000) reveal in each case an 8 α-helical bundle which folds to form a conserved hydrophobic surface groove (the 'canonical groove'). The BH3-only protein BID is exceptional amongst the BH3-only proteins in that it also shares this

fold, albeit with a shorter and shallower surface groove (Chou *et al.*, 1999; McDonnell *et al.*, 1999).

Numerous structural and functional studies have demonstrated that the canonical grooves on multi-domain BCL-2 family members are capable of binding to BH3 domains with varying specificity and affinity. The BH3 domains are essential for the killing activity of the BH3-only proteins: these BH3•groove interfaces represent key interactions that can be targeted with small molecules (Certo et al., 2006; Chen *et al.*, 2005; Simonen *et al.*, 1997).

Thus, the BH3-domain appears to be the endogenous 'ligand' for the native hydrophobic binding groove located on the surface of pro-survival proteins and BAX/BAK. In all BH3•pro-survival structures solved, the BH3 domain forms an amphipathic α-helix upon binding. Key interactions have been shown to be conserved: a set of four hydrophobic residues (h1-h4) project into corresponding binding pockets along the groove (p1-p4) (Figure 2B, right-hand panel) and a salt bridge formed between an Asp conserved among BH3 domains and an **ARG** present on all pro-survival proteins. Despite these commonalities, subtle variations between the BH3 domain ligands and the hydrophobic binding grooves impart differential binding selectivity between BH3 domains and pro-survival partners (Chen et al., 2005). While BIM, BID and PUMA display tight binding affinities for all pro-survival proteins, BAD and NOXA are more selective (the former binding to only BCL-2, BCL-X_L and BCL-W and the latter to MCL-1 and A1). This diversity in binding profile accounts partly for the complex interactions between BCL-2 family proteins. From a drug discovery standpoint, this intrinsic selectivity offered the promise of developing small molecule "BH3 mimetics" which might selectively antagonize one or more pro-survivals in the same manner as a BH3 domain. As the binding groove is relatively shallow and hydrophobic, this endeavor has required significant medicinal chemistry efforts. The recent successes of several drug discovery programs, due to unexpected plasticity in the canonical hydrophobic groove (see below), has demonstrated the feasibility of this approach.

The BCL-2 family proteins exert their activity at the mitochondrial outer-membrane

One further important structural feature of the majority of the BCL-2 proteins are C-terminal hydrophobic extensions, often removed to solve these structures as they impair protein solubility. These C-terminal portions act as membrane-anchoring domains to direct sub-cellular localization; for example, in BAK, this region is important for its constitutive localization to the outer-mitochondrial and endoplasmic reticulum membranes (Breckenridge *et al.*, 2003). In the case of BCL-W and BAX, this C-terminal α-helix (α9) can bind intramolecularly into the canonical hydrophobic groove (Figure 2B) (Petros *et al.*, 2004; Suzuki et al., 2000). This characteristic apparently allows for the normal localization of BAX as a cytosolic monomer, which is then translocated to the outer mitochondrial membrane for apoptosis to occur (Hsu *et al.*, 1997). However, the precise mechanisms by which BAX translocates to and inserts into the outer mitochondrial membrane and how BAX and BAK become activated and oligomerize to cause MOMP are still not fully understood (Westphal *et al.*, 2011).

Structural studies in this area have been hampered by the membrane-localization of these events and the difficulties of preparing full-length active forms of BAX and BAK. However, biochemical trapping and cross-linking experiments of BAX or BAK at various points in the oligomerization process suggest that key conformational changes occur: exposure of the BH3 domain of BAK/BAX (in the α2 helix), exposure of an N-terminal segment, and in the case of BAX additional exposure of the C-terminal α9 for membrane insertion. Additionally, the BH3 domain of BAX is known to be required for its homo-oligomerization (Wang *et al.*, 1998). Initial activation and translocation of BAX has been proposed to be either a spontaneous process (Edlich *et al.*, 2011; Schellenberg *et al.*, 2013), or to be caused by direct and transient binding of an "activator" BH3-only protein (such as BIM or BID) to BAK or BAX (Certo et al., 2006; Kuwana et al., 2005; Letai et al., 2002). For example, it has been suggested that α-helices of the BH3-domains of BIM or BAX stabilized through staples (SAHB) can bind transiently to a trigger site located near the N-terminus (α1/α6) of BAX and cause its activation (Gavathiotis *et al.*, 2010; Gavathiotis et al., 2008). This interaction may act to displace the BAX α9 helix from the canonical groove in order to initiate translocation to the outer-mitochondrial membrane (Figure 1B and 2B lower left panel). Once at the membrane, the canonical groove is free to interact with activator BH3 domains, which induce further conformational changes

(Czabotar et al., 2013). BAK is primarily located at the mitochondrial outer-membrane and similarly binds activator BH3-domains within its canonical hydrophobic groove (Leshchiner *et al.*, 2013; Moldoveanu et al., 2013).

Recent advances have provided significant insights into the nature of the BAX and BAK oligomers that facilitate MOMP. The actual size of the oligomers remain unresolved with some liposome studies suggesting that at least 4 BAX molecules are required to release cytochrome *c* (Saito *et al.*, 2000) or 20 to release other important proteins (Lovell *et al.*, 2008), but as many as a few hundred subunits have also been proposed (Zhou and Chang, 2008). Crosslinking studies suggest that activated BAK and BAX both initially form a symmetrical BH3•groove homodimer (Dewson *et al.*, 2008; Dewson *et al.*, 2012; Oh *et al.*, 2010) and that a second lower affinity interaction, on which the larger oligomer builds, occurs in the region of the *α*6 helix (Dewson et al., 2008; Dewson et al., 2012) – indeed, protein analysis by native PAGE suggests BH3-in-groove dimers and *α*6:*α*6 disulphide linkage is sufficient to stabilize higher-order BAK oligomers (Ma *et al.*, 2013). Other models suggest an asymmetric "daisy chain" (or nose-to-tail) arrangement of monomers, by which the BH3 domain binds either into a site involving the *α*6 region or a "rear pocket" (Gavathiotis et al., 2010; Leber *et al.*, 2010; Zhang *et al.*, 2010). However, such models are inconsistent with a symmetric dimer within the larger oligomer that involves the BAX BH3 domain. The structure of such a symmetric BH3-in-groove dimer interface has recently been solved (Czabotar et al., 2013) and an elaborated model proposed for the conversion of BAX from its monomeric form to its active oligomeric state. In this model, binding of activator BH3-only proteins to the canonical groove of membrane-bound BAX initiates release of a 'core' domain of BAX (*α*2-*α*5 and possibly also *α*1) from a 'latch' domain (*α*6-*α*8) and leads to destabilization of *α*2 (the BH3 domain of BAX) (Czabotar et al., 2013). Once this event has occurred, two neighboring BAX molecules with exposed BH3 domains can come together to form a BH3-in-groove symmetric dimer as the basic unit on which the larger oligomer builds (Dewson et al., 2012; George *et al.*, 2007). The model is consistent with a previous study, which found that the *α*2-*α*5 of BAX is the minimal domain sufficient for oligomerization, and that when fused to the *α*9, this portion is able to cause MOMP (George et al., 2007).

Therapeutic potential of BCL-2 family inhibition

Agents modulating apoptosis by targeting members of the BCL-2 family offer significant potential for the development of new therapies for diseases involving aberrant cell accumulation or cell loss. The best illustration of this concept is found in the development of selective inhibitors of pro-survival BCL-2 family proteins as novel cancer therapies. Such tantalizing prospect was initially proposed from the discovery that elevated levels of BCL-2 alone in follicular lymphoma, due to a chromosomal translocation, prevented these tumor cells from undergoing apoptosis (Vaux *et al.*, 1988). Many subsequent *in vitro* and *in vivo* studies have shown that elevated levels of pro-survival proteins are frequently observed in cancer (e.g. BCL-X_L and MCL-1 (Beroukhim *et al.*, 2010)) and can contribute to cancer phenotype (Adams and Cory, 2007; Miyashita and Reed, 1993; Sentman *et al.*, 1991). Down-regulation of apoptosis is now considered a key step for the initiation and maintenance of cancer (Hanahan and Weinberg, 2000, 2011). Moreover, BCL-X_L overexpression in particular has been strongly correlated with resistance to traditional anti-cancer chemotherapies, which often rely on triggering death *via* the apoptotic response (Amundson *et al.*, 2000). Small molecule BH3-mimetics, which functionally replicate the pro-apoptotic effect of BH3-only proteins and can therefore counter-balance the over-expression of pro-survival proteins, offer the possibility of significantly impairing cancer cell growth and combatting chemo-resistance. Yet, compound-induced apoptosis may raise the specter of therapeutic window especially considering the importance of the pro-survival proteins in a wide range of normal biological processes (e.g. immune system, platelet life span, spermatogenesis, cardiac function,…) (Youle and Strasser, 2008). Despite this, the examples of small molecules detailed below, some of which in the clinic, demonstrate that this approach is viable. A possible explanation for the excellent efficacy and manageable toxicities observed so far in patients may well be the strong apoptotic pressure exerted on cancer cells as compared with normal cells (Certo et al., 2006). BH3-mimetics might therefore be re-instating a pathway already primed to be unraveled in cancer cells.

Impaired apoptosis has also been implicated in autoimmunity: for example loss of BIM or the combined loss of BAK and BAX in the hematopoietic compartment leads

to a failure to eliminate autoreactive thymocytes (Bouillet *et al.*, 2002; Mason *et al.*, 2013). BH3-mimetics may thus also prove to be effective as immune modulatory agents in certain contexts such as pancreatic transplantation to treat diabetes (Carrington *et al.*, 2010).

Inhibitors of apoptosis which, block the pro-apoptotic activity of BAK or BAX, may offer potential therapies for diseases characterized by excessive cell death. Up-regulated apoptosis has been implicated in ischemia/reperfusion injury following stroke or myocardial infarction (Martinou *et al.*, 1994), neurodegeneration (such as Alzheimer's, Parkinson's and Huntington's Disease) (Galluzzi *et al.*, 2009; Lukiw and Bazan, 2010), allograft rejection, osteoarthritis, certain inflammatory disorders, or even HIV (due to depletion of T lymphocytes) (Reed, 2002). Thorough therapeutic validation of this approach remains to be established especially considering that other cell death pathways are also implicated. A number of compounds shown to block apoptosis have already been reported. However, it is unclear whether they target BAX or BAK directly or other proteins associated with mitochondrial apoptosis (Bombrun *et al.*, 2003; Hetz *et al.*, 2005; Peixoto *et al.*, 2009; Polster *et al.*, 2003; Rodrigues *et al.*, 2003). Well-validated small molecule inhibitors of BAX and/or BAK will provide much needed tools to explore this strategy.

Pharmacological inhibition of the BCL-2 family of proteins

Traditionally considered difficult targets (Wells and Mcclendon, 2007), the interfaces between BCL-2 proteins are characterized by large, shallow and mainly hydrophobic areas that generally lack anchorage points for productive interactions. The development of small molecules targeting these protein-protein interfaces has therefore been extremely difficult. Conventional drug discovery methods have in some rare cases, delivered validated BH3-mimetics. Most often, however, the success of such programs has relied on structure-guided drug discovery, NMR fragment screening and peptido-mimetic approaches.

α/β foldamers

As pointed out in the sections above, the BH3 domain is essential for the binding of BH3-only proteins to their pro-survival targets and indeed isolated 26-mer BH3

peptides retain most of the binding affinity of the full-length protein. Peptides themselves seldom represent good drug candidates as they suffer from significant pharmacological liabilities (stability, cell membrane penetration). Therefore strategies that can increase the binding affinities of short peptides (by artificially enhancing helicity) and improve their proteolytic stability have attracted significant efforts.

Incorporating β-amino acids is one approach that confers significant resistance to enzymatic degradation and enhances helicity (Johnson *et al.*, 2012). BH3-mimetic α/β-peptides utilizes combinations of α- and β-amino acids to replicate the binding interactions between endogenous BH3 domains and their pro-survival targets. Importantly for target recognition, the geometry of the backbone is maintained through "sequence-based design" with only partial modifications so as to still reproduce the side-chain projection pattern of an α-helix. This approach has been successfully applied to the design and preparation of BIM and PUMA foldamers (Boersma *et al.*, 2012; Lee *et al.*, 2011; Smith *et al.*, 2013). Initially an α/β-peptide 21-mer comprising the PUMA BH3 domain demonstrated a strong affinity for BCL-X_L but not MCL-1 (Lee et al., 2011). Previous work had demonstrated that the $\alpha\alpha\beta\alpha\alpha\alpha\beta$ pattern exploiting the heptad repeat of an α-helix maintains the helicity of the construct whilst improving proteolytic stability (Boersma et al., 2012). Importantly, the α/β-peptides maintained all of the non-covalent interactions shown to be necessary for recognition of the PUMA-BH3 by BCL-X_L (Lee et al., 2011). Interestingly, the IC_{50} values against BCL-2, BCL-W and BCL-X_L for the best peptide prepared in this study were comparable to the PUMA-BH3 peptide, albeit alongside a 50-fold decrease in binding affinity for MCL-1. The difference in binding profile between the PUMA-BH3 domain and the α/β-peptide 21-mer was suggested to be due to steric clashes involving solvent-exposed residues on MCL-1. Mouse embryonic fibroblast (MEF) cells have been shown to undergo BAK-mediated apoptosis if both BCL-X_L and MCL-1 are neutralized, providing a useful system for *in vitro* validation (Willis *et al.*, 2005). Thus, consistent with its binding profile, in particular weak MCL-1 affinity, the α/β-peptide 21-mer was found to be inactive in wild-type MEF cells but active in *Mcl-1*$^{-/-}$ MEFs. Further manipulations using structure-guided rational design were recently shown to achieve improved affinity for

MCL-1, resulting in a series of novel PUMA-BH3 based foldamers characterized by their high affinity for both MCL-1 and BCL-X_L (Smith et al., 2013).

Stapled peptides

Stapled peptides or stabilized α-helices of BCL-2 domains (SAHBs) are modified peptides incorporating covalent constraints between two amino-acid residues located on the same face on an α-helix (Walensky *et al.*, 2004). Although the term "stapling" refers chiefly to constraints installed *via* the ring-closing olefin cross-metathesis reaction (RCM), the general strategy has also been applied using amide or "click" triazole linkages (Cantel *et al.*, 2008; Kawamoto *et al.*, 2012; Skelton *et al.*, 2001; Yang *et al.*, 2004). Stapled peptides are reported to have improved pharmacokinetics through increased cell permeability and reduced enzymatic degradation (Bird *et al.*, 2008; Walensky et al., 2004). More recent reports have found that a stapled BIM-BH3 peptide affects the viability of a number of hematological cancer cell lines (Labelle *et al.*, 2012). Stapled BH3-peptides have also been used to study the direct activation model whereby activator BH3-only proteins such as BID or BIM directly interact with pro-apoptotic proteins BAX/BAK (Leshchiner et al., 2013; Walensky *et al.*, 2006). Finally MCL-1 derived constructs have also been developed and used as molecular probes for selective MCL-1 inhibition (Stewart *et al.*, 2010).

Recent studies have highlighted that the staple, far from being just a constraining element, can affect binding affinity in both positive and negative ways (Brown *et al.*, 2012; Okamoto *et al.*, 2013; Stewart et al., 2010). With these observations, it is becoming clear that the design of novel stapled peptides must take into account the staple while surveying structure-activity relationship.

Obatoclax and Gossypol derivatives

Obatoclax (Figure 3A, GX-15-070, Gemin X Biotechnologies), derived from the natural product progidiosin, and (-)-Gossypol (AT-101, Ascenta Therapeutics, Figure 3B) are small molecules displaying low- to sub-micromolar affinities for multiple pro-survival proteins (as such, they are often referred to as pan-inhibitors) (Zhai *et al.*, 2006). Their discovery, biochemical and biological characterization have been reviewed previously (Czabotar and Lessene, 2010; Lessene *et al.*, 2008). Although a

number of studies have shown that their cell killing activity is the result of modulation of the BCL-2-driven pathway, their mode of action is still a matter of debate. For example, recent studies suggest that obatoclax is also involved in programmed necrosis (Basit *et al.*, 2013). Currently obatoclax is in multiple phase I/II clinical trials as a single agent against hematological malignancies and in combination therapy (Chiappori *et al.*, 2012; Schimmer *et al.*, 2008). (-)-Gossypol demonstrated only moderate efficacy in clinical trials as single-agent against castration-resistant prostate cancer (Liu *et al.*, 2009) and lacked efficacy as a single agent and in combination against small cell lung carcinoma (SCLC) (Heist *et al.*, 2010; Ready *et al.*, 2011).
A recent derivative of the gossypol chemical class, sabutoclax (BI-97C1, Figure 3C) binds to MCL-1, BCL-2, and BCL-X_L with IC_{50} values of 0.20, 0.32 and 0.31 μM, respectively (Wei *et al.*, 2010). Sabutoclax induces cell death in a BAX/BAK-dependent manner and displayed efficacy both *in vitro* and *in vivo* in a xenograft model using prostate cancer cell lines (Wei *et al.*, 2009a; Wei *et al.*, 2009b). In contrast to the BH3-mimetic ABT-737, which binds to BCL-2, BCL-W and BCL-X_L with high affinity, sabutoclax was able to sensitize these malignant cells to the *mdl-7*/IL24 (Dash *et al.*, 2011) due to its MCL-1 targeting. In separate studies, it was found that Sabutoclax cause regression of castrate-resistant prostate cancer cells (Jackson *et al.*, 2012).

Acylsulfonamides series

The AbbVie team (formerly Abbott) pioneered drug discovery targeting of BH3-mimetics. This team was indeed the first to translate the fundamental discoveries around the BCL-2 family of proteins to well-validated small molecule inhibitors of pro-survival proteins. This effort culminated in the disclosure of ABT-737 (Figure 3D), a potent inhibitor of BCL-2, BCL-X_L and BCL-W (Oltersdorf *et al.*, 2005), which has become a widely utilized chemical biology probes. The development of ABT-737 and of its orally available analogue ABT-263 (navitoclax, Figure 3E) has been reviewed extensively elsewhere (Czabotar and Lessene, 2010; Juin *et al.*, 2013; Lessene et al., 2008). Notably, the mechanism of action of this class of compounds has been thoroughly studied and there is now little doubt that their potent cell killing ability is mediated by direct interaction with pro-survival BCL-2 proteins (Del Gaizo Moore *et al.*, 2007; Konopleva *et al.*, 2006; Tse *et al.*, 2008; van Delft *et al.*, 2006).

Their binding mode has also been disclosed and shows that these large hydrophobic molecules bind in 2 out of 4 key hydrophobic pockets on the surface of their pro-survival protein targets (p2 and p4, Figure 2B) (Lee *et al.*, 2007; Souers *et al.*, 2013). Interestingly, these compounds induce an important remodeling of the p2 pocket. ABT-263 is now in clinical trial for the treatment of hematological tumors and small cell lung cancer (Gandhi *et al.*, 2011; Roberts *et al.*, 2012; Rudin *et al.*, 2012). As predicted from pre-clinical study results, this compound displays particularly good efficacy in patients suffering from chronic lymphoctytic leukemia (CLL) as a single agent and in combination (Campas *et al.*, 2006; Del Gaizo Moore et al., 2007; Mason *et al.*, 2009; Roberts et al., 2012; Wilson *et al.*, 2010). Conversely, the recently published phase II clinical trial data for single agent ABT-263 indicate its low efficacy in patients affected by recurrent metastatic small cell lung cancer (Rudin et al., 2012). ABT-263 induces sharp but temporary drop in circulating platelets (Roberts et al., 2012). The origin of this phenomenon lies in the exquisite sensitivity of platelets to BCL-X_L inhibition (Mason *et al.*, 2007; Zhang *et al.*, 2007). As such, these blood cells represent excellent biomarkers of on-target BCL-X_L inhibition.

ABT-737-derived BH3-mimetics

The acylsulfonamide moiety in the compounds developed by AbbVie may represent a potential metabolic and chemical stability (Figure 3D-E). Efforts aimed at removing this group led to quinazoline derivatives (Figure 3F), which maintained low nanomolar binding affinity against BCL-2 (IC_{50} = 9 nM) and BCL-X_L (IC_{50} = 7 nM) (Sleebs *et al.*, 2011). Intriguingly, this series of isosteric analogues of ABT-737 have significantly weaker affinity for BCL-W (IC_{50} = 440 nM). X-ray crystallographic studies show that the quinazoline derivative and ABT-737 interact with BCL-X_L with similar binding modes. However, an additional polar interaction was observed between a quinazoline ring nitrogen and BCL-X_L Tyr101 (Sleebs et al., 2011). Mechanism-based cellular activity of the quinazoline compounds was also consistent with a potent dual inhibitor of BCL-X_L and BCL-2 (potent killing of MEF cells lacking MCL-1 and no effect on wild-type MEF). These quinazoline derivatives were also shown to have submicromolar activity against a panel of small-cell lung carcinoma cell lines.

More recently, a Novartis team reported a similar approach exploring the isosteric replacement of the acyl sulfonamide with heterocyclic rings (Toure *et al.*, 2013). Initial replacement of the acyl sulfonamide with a naphthyl ring led to a complete loss of binding affinity, a result consistent with the essential role of the acidic acylsulfonamide NH. Moving towards analogues containing a more acidic sulfonamide allowed the development of the piperidyl pyrimidine ring system. The best analogue (Figure 4A) has good binding affinity for BCL-2 (K_D = 7 nM, IC_{50} = 19 nM) and BCL-X_L (IC_{50} = 24 nM) translating into cell killing activity in the BCL-2-driven Toledo cell lines (LD_{50} = 0.298 μM).

Using a 3D pharmacophore template defined by key interactions between the BAD-BH3 peptide and BCL-X_L, the FDA-approved drugs Lipitor and Celecoxib were selected as starting point towards the design of new BCL-2 inhibitors (Zhou *et al.*, 2012a; Zhou *et al.*, 2012b). Modifications of the two drugs were guided by structure-based design that led to the 3,4-diphenyl-1H-pyrrole-2-carboxamide scaffolds linked to the nitrobenzene sulfonamide half of ABT-737 (Aguilar *et al.*, 2013; Chen *et al.*, 2012). This effort produced BM-957, which displays K_i values below 1 nM for both BCL-2 and BCL-X_L (Chen et al., 2012). Cell growth inhibition in cancer cell lines was observed with IC_{50} values around 20 nM against the H1147 and H146 small-cell lung cancer cell lines. BM-957 *in vivo* experiment in the H146 xenograft tumor model achieved rapid and complete tumor regression (Chen et al., 2012).

Combining previous work from Celltech (Porter *et al.*, 2009a; Porter *et al.*, 2009b) and the AbbVie acylsulfonamide series, a collaborative team between Bristol-Myers-Squibbs and Nerviano has developed novel and potent dual BCL-2/BCL-X_L inhibitors (Perez *et al.*, 2012; Schroeder *et al.*, 2012). The lead compound from this series has single digit nanomolar activity against the two pro-survival proteins.

BCL-2 selective BH3-mimetics

Since the dose-limiting toxicity in the ABT-263 clinical trials is thrombocytopenia induced by potent BCL-X_L inhibition, it was thought that BCL-2 selective compounds would considerably improve the therapeutic window of such BH3-mimetics. It has also been shown that ABT-737 and ABT-263's main target in transformed lymphoid cells is BCL-2 (Merino *et al.*, 2012; Rooswinkel *et al.*, 2012). Using fortuitous structural insight on analogues of ABT-263, AbbVie designed and developed highly

selective inhibitors of BCL-2 culminating in ABT-199 (K_i < 0.010 nM for BCL-2 and 48 nM for BCL-X_L, Figure 4D) (Souers et al., 2013). This compound engages the same binding pockets as ABT-263 (p2 and p4) but does so without a "bend-back" π-stacking arrangement observed for ABT-737 and ABT-263. Instead the aza-indole linked directly to the molecule core forms a π-stacking arrangement with the nitroaryl moiety. The structural information reported for a close analogue of ABT-199 suggests that hydrogen bonding between the aza-indole group and BCL-2 Asp103 is responsible for the enhanced selectivity (Souers et al., 2013). As expected ABT-199 has negligible effect on platelets (*in vitro* and *in vivo*) (Vandenberg and Cory, 2013). ABT-199 is currently in phase 1 clinical trials and early reports have demonstrated that this molecule does not induce thrombocytopenia while retaining potent efficacy against CLL (Souers et al., 2013). Interestingly, ABT-199 may find application beyond that of blood related tumor treatments. In particular, there is evidence that ABT-199 may be an effective agent to combat estrogen receptor-positive breast cancer in which BCL-2 is over-expressed (Vaillant *et al.*, 2013).

BCL-X_L selective BH3-mimetics

Potent inhibition of BCL-2 has also been linked to potential toxicities such as reversible neutropenia (Roberts et al., 2012). As a large number of solid tumors have been found to rely on BCL-X_L for survival (Beroukhim et al., 2010), a compound selectively inhibiting this pro-survival protein could have applications in this context. Recently, we, in collaboration with colleagues at Genentech have reported a potent and highly selective inhibitor of BCL-X_L (WEHI-539, Figure 4E) (Lessene *et al.*, 2013). Interestingly, this novel series is the first to arise in this field from high-throughput screening. A combination of classical medicinal chemistry and structure-guided design was key to the success of this work (Lessene et al., 2013; Sleebs *et al.*, 2013). WEHI-539 binds tightly to BCL-X_L with IC_{50} and K_D values close to or below 1 nM and demonstrated high selectivity for BCL-X_L over other pro-survival BCL-2 family members (at least 400 fold). Structural information showed that, like ABT-737, WEHI-539 induce a significant conformational change around p2 to accommodate its benzothiazole moiety. It was suggested that WEHI-539's selectivity for BCL-X_L may be due to an array of hydrogen bonds taking place in this enlarged p2 pocket between the benzothiazole hydrazone group and backbone NH and carbonyls. Careful analysis of WEHI-539's mechanism of action support direct engagement of BCL-X_L: in

contrast to ABT-737, WEHI-539 induces primarily BAK-mediated cell death, consistent with BCL-X_L's key role alongside MCL-1 in BAK inhibition (Willis et al., 2005). This compound also efficiently induced apoptosis in isolated mice and human platelets.

MCL-1 selective BH3-mimetics

Using a stapled MCL-1 peptide, a high-throughput screen led to the discovery of MIM-1, a selective inhibitor of MCL-1 (Figure 4F) (Cohen *et al.*, 2012). Despite its modest binding affinity (IC_{50} = 4.8 μM), MIM-1 is active against leukemic cell lines and synergized with ABT-737 (Cohen et al., 2012). In contrast, a recent comparison of MCL-1 inhibitors showed that MIM-1 induced apoptosis only weakly at very high concentration (Varadarajan *et al.*, 2013) suggesting that other pathways may be involved in its activity in other cell lines.

More recently, a team at Vanderbilt University reported novel MCL-1 inhibitor discovered through NMR-based fragment screening (Figure 4G) (Friberg *et al.*, 2013). Stephen Fesik, a member of this team, was one of the inventors of the 'SAR by NMR' technique that led to the discovery of ABT-737 (Petros *et al.*, 2006). The chemical class identified through this screen is reminiscent of MCL-1 selective compounds reported by AbbVie (Bruncko *et al.*, 2008; Elmore *et al.*, 2008) and suggests a privileged scaffold binding to MCL-1. The lead compound obtained in this series displays a K_i of 0.055 μM for MCL-1, and weaker binding affinity for BCL-X_L and BCL-2. Interestingly, this compound induced the same changes around p2 to fit the phenoxyalkyl extension. As discussed above, induced structural changes to this pocket are also observed in the complexes of ABT-737 and WEHI-539 bound to BCL-X_L and ABT-199 bound to BCL-2. Notably, these different compounds derive from diverse chemical starting points. This highlights the plasticity of this pocket across the pro-survival protein sub-group and indicates that it represents a hotspot for the discovery of potent and in some cases highly selective inhibitors of pro-survival BCL-2 family proteins.

Targeting the pro-apoptotic BCL-2 family proteins BAX and BAK

The development of agents targeting BAX and BAK has lagged significantly behind the development of agents targeting their pro-survival relatives. This is in part due to a scarcity of structural information on the nature of the interactions between BAX and BAK and other family members, or on the larger BAX and BAK homoligomers, the formation of which initiates outer-mitochondrial membrane permeabilization. However, recent advances in this area are providing new insights and opportunities for targeting these proteins. In particular, the structural details for interactions between BAX and BAK and activating BH3-only proteins (Czabotar et al., 2013; Moldoveanu et al., 2013) and of an interface within the larger BAX oligomer (Czabotar et al., 2013) highlight the canonical BH3 binding groove of these proteins as a potential target for therapeutics.

The BAX and BAK canonical grooves share many structural similarities with their pro-survival relatives and thus it seems likely that compounds targeting this interface on the pro-apoptotic members can also be developed. Nonetheless, there is a range of subtle differences in the nature of these interactions indicating that it may possible to develop compounds specifically targeting the BAX or BAK groove. For example, interactions between BH3 domains and BAX rely on contacts at the N-terminal region of the bound BH3 domain (Czabotar et al., 2013); such residues do not appear to be critical for interactions with pro-survival proteins.

Whilst the nature of the interactions between BH3 domains and the canonical grooves of the two BCL-2 family subgroups is similar, in a structural sense the consequence of these interactions varies wildly. In the case of the pro-survival proteins a stable complex is formed (Figure 2). In the case of BAX, and likely of BAK, interactions at this interface instead are transient and initiate widespread conformational changes that lead to homo-oligomerization (Czabotar et al., 2013). Consequently there exists the possibility of developing therapeutics targeting the canonical groove of BAX, and likely of BAK, with opposing cell death activities. For example, agents that mimic the BH3-peptide-mediated initiating event could promote apoptosis through induction of

BAX and/or BAK conformational change. Such agents may be useful in cancer settings in a similar manner to the pro-apoptotic drugs being developed against pro-survival proteins. Alternatively, agents that bind the groove without initiating conformational change but which would inhibit BH3-domain interactions could instead inhibit apoptosis. The canonical groove is also involved in BAX homo-oligomerization (Czabotar et al., 2013; Dewson et al., 2008; Dewson et al., 2012) and thus, agents with inhibiting interactions at this interface could additionally block the critical step of BAX and BAK oligomerization. Such anti-apoptotic agents might hold promise for the treatment of conditions where excessive apoptosis leads to pathology such as in neurodegenerative disorders or cardiovascular disease as described above.

A second potential therapeutic target for the development of BAX modulators is a proposed rear site on this pro-apoptotic protein (Gavathiotis et al., 2008). The relationship between this novel interaction site and the canonical groove remains unclear. As discussed above, it is possible that this rear site represents a triggering interface for initiating BAX translocation from the cytosol to the mitochondria. Alternatively this might be an alternative site for triggering BAX conformational change. In any case, the rear site has been the subject of computational screening studies for small molecule binders and compounds with pro-apoptotic activity have been reported (Gavathiotis *et al.*, 2012). However, mutation to a reportedly key residue on this interaction surface suggests that engagement of the site is not essential for apoptosis to proceed (Okamoto et al., 2013; Peng *et al.*, 2013). This holds important implications for the development of therapeutics aimed at inhibiting BAX activity as it suggests that such agents would be ineffective in inhibiting apoptosis. Nonetheless, it may be possible to target this pocket with agents aimed at accelerating apoptosis and thus be of interest for the development of cancer therapeutics.

Conclusion

Through their association with BCL-2 family proteins, the mitochondria play key role in apoptosis induction. The function of apoptosis in normal cell physiology as well as in many diseases makes it a compelling pathway to target pharmacologically. The development of small molecules targeting the BCL-2 family of proteins has however proved extremely challenging and only a handful have reached the clinic. Amongst

the preclinical and clinical compounds reported, few have been carefully characterized. The use of appropriate biochemical and biological tools (e.g. cell lines engineered to depend on particular BCL2-proteins for survival) reported in the literature is limited. The increasing number of genuine BCL-2 inhibitors, coupled with the development of robust assays, should positively impact future developments in this field.

We will conclude by highlighting some of the future important directions in chemical and drug discovery targeting the BCL-2 family of proteins. The development of BH3-mimetics to treat tumors and possibly auto-immune diseases has now reached a mature stage. Nonetheless, the development of compounds selectively targeting MCL-1 or A1, which would complement the current set of available molecules, has lagged. Such agents would not only help elucidate the role of these two key pro-survival proteins, but would also provide tools to establish the therapeutic relevance of, and safety associated with, MCL-1 or A1 inhibition. The lack of characterized inhibitors of apoptosis is also hindering major progress towards definitively establishing the link between up-regulated apoptosis and diseases such as neuro-degeneration. Recent progress linking structural and biological studies on the sequence of events leading to activation of the pro-apoptotic proteins BAX and BAK will almost certainly accelerate the discovery of compounds modulating their activity.

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Conflict of Interest Statement

The authors declare no conflict of interests.

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Figure Legends

Figure 1. The mitochondrial pathway to apoptosis**.** Apoptosis (programmed cell death) via the mitochondrial or 'intrinsic' pathway is regulated by finely balanced interactions between members of the BCL-2 family of proteins. (A) In healthy cells, pro-survival BCL-2 proteins act as guardians of mitochondrial integrity to restrain the effector molecules BAX and BAK, either by sequestering them in heterodimeric complexes or preventing their activation by certain initiator BH3-only proteins (A and B). In response to apoptotic stimuli, BH3-only proteins become upregulated/activated and overwhelm the pro-survival proteins. This allows for BAX/BAK activation by certain BH3-only proteins and relieves BAX/BAK restraint by pro-survival proteins. This allows for BAX/BAK activation by certain BH3-only proteins and relieves BAX/BAK restraint by pro-survival proteins. Following a series of conformational changes in BAK/BAX, they oligomerize on the outer mitochondrial membrane, leading to an irreversible step known as mitochondrial outer-membrane permeabilization (MOMP, B). When this occurs, cytochrome *c* is released into the cytosol (with other apoptotic factors) and together with Apaf-1 forms a structure known as the apoptosome, that activates a cascade of proteolytic caspases which demolish the cell, leading to its death.

Figure 2. (A) Proteins of the BCL-2 family. The BCL-2 family is composed of proteins that share in common at least one of the so-called BCL-2 Homology (BH) domains (BH1-4). They fall into two functional subclasses - the pro-survival proteins (eg. BCL-2, BCL-X_L) which act to restrain apoptosis, and the pro-apoptotics, which can be further subdivided into the multidomain effectors BAK, BAX (and possibly BOK) and the BH3-only proteins (eg. BIM, BAD). BH3 only proteins sense apoptotic stimuli to trigger apoptosis. This occurs either by displacing heterodimeric complexes of pro-survivals bound to active BAK/BAX; or by BH3-only proteins saturating binding to pro-survivals, allowing release of sequestered 'activator' BH3-only proteins which directly bind to and activate BAK/BAX, or a combination of both. The BH3 domain is essential for this killing function. (B) Structures and key interactions of BCL-2 family members. Both pro-survivals and BAK/BAX share domains BH1-4 as well as a similar overall fold. The X-ray crystal structure of the BH3 domain of BIM (green) bound to human BCL-X_L (white) (PDB: 1PQ1, Liu *et al.*, 2003), left figure in ribbon representation, right figure with BCL-X_L in surface representation) revealed that the BH3 domain of BH3-only proteins can form an amphipathic alpha-helix and binds along a hydrophobic surface groove on BCL-X_L (formed mostly from helices α2-5). Key binding interactions include four hydrophobic residues (h1-h4) present in all BH3 domains which bind into corresponding pockets on the pro-survival (P1-P4) and a salt bridge formed between a conserved Asp on the BH3 domain and an Arg residue on the pro-survival protein. The structure of inactive monomeric human BAX (PDB: 1F16, Suzuki *et al.*, 2000) shows a very similar structure, albeit instead with the putative TM domain α9 sequestered along the hydrophobic groove, consistent with its capacity to localize in the cytosol. Based on recent structural information (Czabotar *et al.*, 2013) we have proposed the following model for BAX activation: on direct binding by an 'activator' BH3 into the groove of BAX (after insertion of α9 into the outer mitochondrial membrane) a 'latch' domain of BAX (α6-α8, blue) becomes released from the 'core' domain (α2-α5, red) of the protein, allowing homodimerisation with the core domain of another BAX molecule to form a symmetric BH3-in-groove dimer (PDB: 4BDU, Czabotar *et al.*, 2013). This would form the starting point to nucleate further BAX oligomerization at another face, ultimately triggering MOMP leading to cell death.

Figure 3. BH3-mimetics (1). (A) Pan-selective BCL-2 inhibitor GX15-070/obatoclax; (B) Pan-selective BCL-2 inhibitor AT-101/(-)-Gossypol; (C) Pan-selective BCL-2 inhibitor BI-97C1/sabutoclax; (D) BCL-X_L, BCL-2 and BCL-W inhibitor ABT-737; (E) BCL-X_L, BCL-2 and BCL-W inhibitor ABT-263/navitoclax; (F) BCL-X_L and BCL-2 inhibitor derived from ABT-737.

Figure 4. BH3-mimetics (2). (A) BCL-X_L and BCL-2 inhibitor derived from ABT-737; (B) BCL-X_L and BCL-2 inhibitor BM-957; (C) BCL-X_L and BCL-2 inhibitor; (D) Selective BCL-2 inhibitor ABT-199; (E) Selective BCL-X_L inhibitor WEHI-539; (F) Selective MCL-1 inhibitor MIM-1; (G) Selective MCL-1 inhibitor.