

TARGETING THE ANTI-APOPTOTIC BCL-2 FAMILY MEMBERS FOR THE TREATMENT OF CANCER

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Most cells express a variety of both anti-apoptotic and pro-apoptotic Bcl-2 proteins and the interaction within this family dictates whether a cell survives or dies. The dysregulation of the anti-apoptotic Bcl-2 family members is one of the defining features of cancer cells in comparison to normal cells, and significantly contributes to the resistance of cancer cells to current treatment modalities. This anti-apoptotic subfamily of proteins is now a major target in the development of new methods to improve treatment outcomes for cancer patients. Several drugs directed at inhibiting Bcl-2 and related anti-apoptotic proteins have been developed with some showing considerable promise in the clinic. This Review presents the current knowledge of the role of the anti-apoptotic Bcl-2 family in cancer cells, as well as current and future perspectives on targeting this subfamily of proteins for therapeutic intervention in human malignancies. This article is part of a Special Issue entitled "Apoptosis: Four Decades Later".

Key Words: apoptosis, Bcl-2 family, cancer, BH3 mimetics.

INTRODUCTION

The term apoptosis originates from the Greek expression "the falling of leaves from a tree" and refers to an evolutionary preserved mechanism of controlled cell deletion. It was first introduced into scientific literature in 1972, when John Kerr et al. published a detailed description of the distinct morphological features of dying cells; chromatin condensation, nuclear fragmentation and cell shrinkage [1]. Cell death via apoptosis plays a role in many diverse fundamental processes. Apoptosis enables the removal of superfluous or damaged cells from the body of multicellular organisms during embryogenesis and contributes to cellular homeostasis [2–4]. Apoptosis is also essential in the defence against infectious microorganisms and the removal of cancerous cells [5].

The demolition of a cell during apoptosis is primarily achieved through the cleavage of numerous cellular proteins by the proteolytic caspase (cysteine aspartate proteinases) enzymes [6]. Two major pathways lead to caspase activation in mammalian cells: the extrinsic or death receptor pathway and the intrinsic mitochondrial pathway [7]. The extrinsic apoptotic pathway can be induced by the association of death

receptors belonging to the tumour necrosis factor (TNF) receptor superfamily, such as Fas or TNF-R1 and their respective ligands, FasL and TNF-alpha [8, 9]. Such association results in the recruitment of adaptor proteins and either procaspase-8 or procaspase-10 to form the death inducing signalling complex (DISC) [8]. Depending on the cell type, extrinsic apoptotic signalling can proceed via two pathways: in type I cells, active caspase-8 cleaves and activates executioner caspase-3, directly leading to nuclear fragmentation and ultimately cell death [10, 11]. In type II cells, active caspase-8 cleaves the BH3-only protein Bid to form truncated Bid (tBid), which activates the intrinsic mitochondrial apoptotic pathway [12, 13]. The intrinsic apoptotic pathway is primarily induced by developmental cues and diverse cytotoxic events including DNA damage and exposure to drugs or radiation during cancer treatment, leading to changes in Bcl-2 family interactions, which converge on the outer mitochondrial membrane culminating in pore formation [14]. Mitochondrial outer membrane permeabilisation (MOMP) results in the release of various mitochondrial intermembrane space proteins, such as cytochrome *c* [7]. Released cytochrome *c* binds to apoptotic protease-activating factor 1 (Apaf-1), thereby inducing the oligomerisation of Apaf-1 and the formation of the apoptosome [15]. In the presence of (d)ATP, initiator procaspase-9 is recruited to the complex and activated [16]. Active caspase-9 in turn triggers the activation of executioner caspases, caspase-3 and -7, leading to a cascade of caspase-mediated cleavage reactions that lead to cell death (Fig. 1) [16].

The majority of anti-cancer treatments act by inducing stress signals that can activate the intrinsic mitochondrial pathway of apoptosis in tumour cells [17]. This Review presents the current knowledge of how cancer cells overcome such treatment strategies, with particular reference to the role of the anti-apoptotic Bcl-2 family in this process, as well as current and

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Abbreviations used: A1 – Bcl-2-related gene A1; Apaf-1 – apoptotic protease-activating factor 1; Bad – Bcl-2-associated death promoter; Bak – Bcl-2 homologous agonist killer; Bax – Bcl-2-associated-x protein; Bcl-2 – B cell lymphoma-2; Bcl-x_L – Bcl-2-related gene long isoform; BH – Bcl-2 homology; Bid – BH3 interacting-domain death agonist; Bik – Bcl-2-interacting killer; Bim – Bcl-2 interacting mediator of cell death; Bmf – Bcl-2 modifying factor; Bok – Bcl-2 related ovarian killer; caspase – cysteine aspartate proteinase; DISC – death inducing signalling complex; FasL – Fas ligand; Hrk – Harakiri; Mcl-1 – myeloid cell leukemia-1; MOMP – mitochondrial outer membrane permeabilisation; PUMA – p53-upregulated modulator of apoptosis; tBid – truncated Bid; TNF – tumour necrosis factor; TNF-R1 – tumour necrosis factor-receptor 1.

future perspectives on targeting the anti-apoptotic Bcl-2 family of proteins for therapeutic intervention in human malignancies.

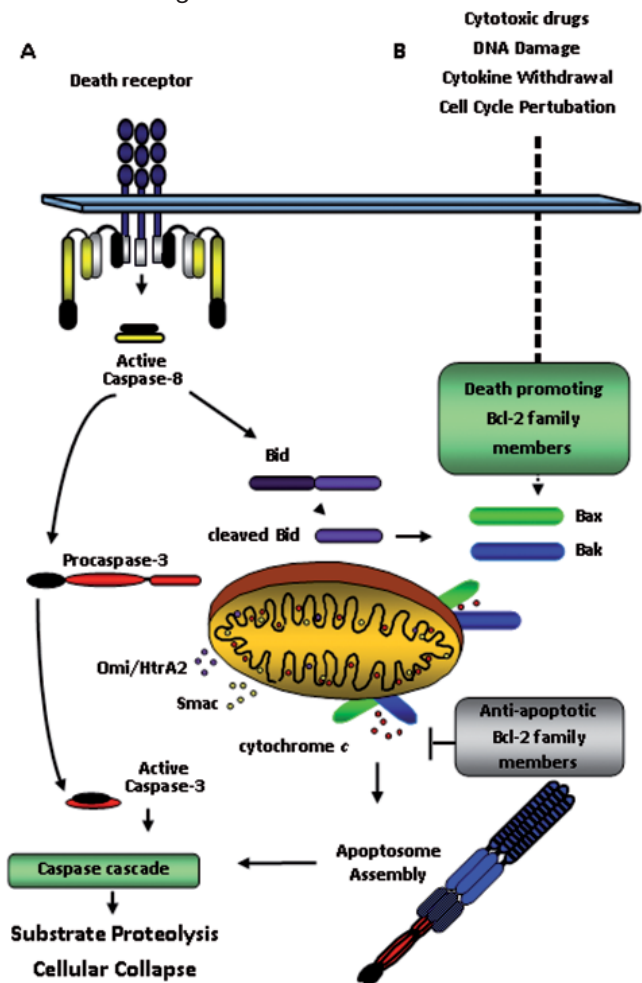


Fig. 1. Caspase Activation Pathways. (A) Engagement of plasma membrane-associated death receptors results in recruitment and activation of procaspase-8 via the adaptor molecule FADD. In type I cells, active caspase-8 can directly activate procaspase-3. Active caspase-3 can then initiate a caspase activation cascade. In type II cells, the reduced level of activated caspase-8 cleaves the BH3-only protein Bid. This 15 kDa fragment, tBid, activates the mitochondrial pathway by stimulating cytochrome *c* release via Bax and/or Bak oligomerization and insertion into the outer mitochondrial membrane. Once in the cytoplasm cytochrome *c* promotes apoptosome assembly and thus caspase activation. (B) Diverse forms of cellular stress, (DNA damage, cytotoxic drugs, cytokine withdrawal), may trigger the release of cytochrome *c* from mitochondria via the death-promoting Bcl-2 family members, such as Bax and/or Bak, oligomerization and insertion into the outer mitochondrial membrane. Cytochrome *c* release from mitochondria is inhibited by the death inhibitory Bcl-2 family members, such as Bcl-2 and Bcl-x_L. (Adapted from [110])

THE CLASSIFICATION OF THE BCL-2 FAMILY OF PROTEINS

The rapid and irreversible release of cytochrome *c* from mitochondria is generally recognised as the “point of no return” in the life of a cell [18]. Unsurprisingly therefore, the process of cytochrome *c* release from mitochondria is tightly controlled, primarily by the B cell lymphoma-2 (Bcl-2) family of proteins [19, 20]. The name of this diverse family originates from their first identified member, Bcl-2, an oncoprotein that was activated via chromosome translocation in human fol-

licular lymphoma [21, 22]. To date, there are 25 known proteins in the Bcl-2 family that can be subdivided into 3 groups, according to their pro- and anti-apoptotic effects and the presence of Bcl-2 homology (BH) domains [23]. The anti-apoptotic Bcl-2-like proteins, comprise 1 group and has amongst its members Bcl-2, Bcl-2-related gene long isoform (Bcl-x_L), Bcl-w, myeloid cell leukemia-1 (Mcl-1) and Bcl-2-related gene A1 (A1). These anti-apoptotic proteins have similar 3D structures, possess four BH domains, and all promote cell survival by inactivating their pro-apoptotic Bcl-2 family counterparts and preserving outer mitochondrial membrane integrity [23]. The pro-apoptotic Bcl-2 family members may be subdivided into 2 classes: the multidomain effector proteins and the BH3-only proteins. The multidomain members include Bcl-2-associated-x protein (Bax), Bcl-2 homologue agonist killer (Bak) and the much less studied, Bcl-2 related ovarian killer (Bok) and contain structural features of all four BH domains, similar to the antiapoptotic proteins [24]. Once activated, the effector proteins, Bax and Bak promote apoptosis by enabling pore formation within the mitochondrial outer membrane [25].

Structurally, the BH3-only proteins are homologous to the rest of the Bcl-2 family members in only one small sequence, the BH3 domain (Fig. 2).

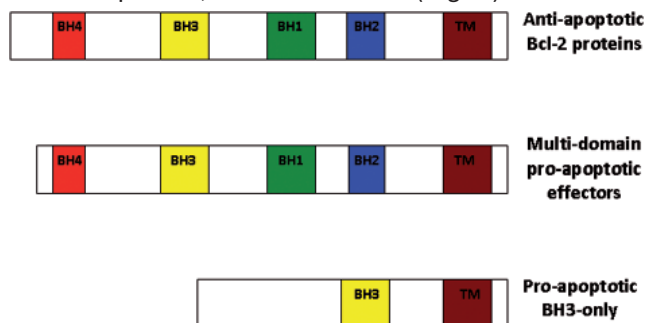


Fig. 2. Schematic representation of Bcl-2 family members. Bcl-2 family members are the key regulators of cytochrome *c* release from the mitochondria. As is illustrated, all members of the family contain at least one of 4 BH domains, designated BH1, BH2, BH3 and BH4. Some members also contain a transmembrane domain that tethers these proteins to intracellular membranes. The anti-apoptotic members most similar to Bcl-2, such as Bcl-x_L contain all 4 BH domains. There are 2 very distinct pro-apoptotic sub-families: the multidomain effectors and the BH3-only subgroup. The multidomain effectors, Bax, Bak and Bok are very similar to Bcl-2 and contain structural features of all 4 BH domains. The “BH3-only” proteins such as Bad, Bid and Bim contain a central BH3 domain that is essential for their killing activity. The “BH3-only” proteins are a very diverse family. (Adapted from [110])

BH3-only group members include, Bcl-2-associated death promoter (Bad), BH3 interacting-domain death agonist (Bid), Bcl-2-interacting killer (Bik), Bcl-2 interacting mediator of cell death (Bim), Bcl-2 modifying factor (Bmf), Harakiri (Hrk), Noxa and p53-upregulated modulator of apoptosis (PUMA). BH3-only protein signalling is essential for the initiation of the mitochondrial apoptotic pathway, but MOMP requires the presence of either Bax or Bak [25–27].

THE INTERACTIONS BETWEEN THE BCL-2 FAMILY OF PROTEINS

Most cells express a variety of both anti-apoptotic and pro-apoptotic Bcl-2 proteins and the interaction between proteins within this family dictates whether a cell survives or dies [20]. The exact mechanisms of how Bcl-2 proteins interconnect to regulate MOMP and apoptosis has been controversially discussed. The “direct activation” model proposes that BH3-only proteins Bim, truncated Bid (tBid) and maybe PUMA act as “direct activators” of Bak/Bax and the rest of the BH3-only proteins act as “sensitisers” or decoys but do not directly activate Bax/Bak. Such “sensitisers” prevent the antiapoptotic Bcl-2-like proteins from binding to the activators, thus freeing these proteins to interact with and activate Bak and Bax, leading to MOMP and cytochrome *c* release [28, 29]. The second “derepression” model suggests that Bax and Bak are always active and the anti-apoptotic proteins prevent cell death by binding to them. In this model the role of the BH3-only proteins is to target and bind the anti-apoptotic Bcl-2 family members to release active Bak and Bax. While certain members of the BH3-only family (Bim, PUMA and tBid) can bind to all anti-apoptotic proteins, other proteins, such as Bad and Noxa, only interact with a specific Bcl-2 protein family member to regulate MOMP [30, 31]. The more recently proposed “embedded together” model combines features of both. Under this model, BH3-only sensitisers are thought to displace Bax and the BH3-only activators from the anti-apoptotic proteins. Bax is then free to oligomerise and the BH3-only activators can bind and recruit additional Bax, which also oligomerises, resulting in pore formation and MOMP. These important interactions occur at the mitochondrial outer membrane [32, 33]. Although further work is required to more thoroughly elucidate the intricate interactions between the Bcl-2 family of proteins, the shared theme of all proposed models is the engagement of anti-apoptotic Bcl-2 family members by the BH3-only subfamily of proteins. Anti-apoptotic Bcl-2 family members contain a hydrophobic binding pocket, formed by the folding of their BH1, BH2 and BH3 domains and BH3-only proteins can bind into this groove via their BH3 domain [20, 26, 34]. When the abundance of active pro-apoptotic Bcl-2 family proteins exceeds the binding capacity of the anti-apoptotic Bcl-2 family proteins, MOMP occurs and the mitochondrial pathway of apoptosis proceeds [20].

THE ROLE OF THE ANTI-APOPTOTIC BCL-2 FAMILY PROTEINS IN CANCER DEVELOPMENT AND MAINTENANCE

The discovery that Bcl-2 did not drive cell proliferation, as for previously characterized oncogenes, but rather promoted cell survival, led to the realisation that the inhibition of apoptotic pathways was a critical step in tumourigenesis [35]. Indeed many studies have since highlighted that the dysregulation of Bcl-2 and other anti-apoptotic family members is one of the

key defining features of cancer cells in comparison to normal cells [36]. BCL-2 transgenic mice develop spontaneous tumours [37] and BCL-2 gene and protein amplification has been discovered in various malignancies, including chronic lymphocytic leukaemias [38], small cell lung cancers [39], breast carcinomas [40], non-Hodgkin’s lymphoma [41] and glioblastomas [42]. Mcl-1 overexpression predisposes mice to B-cell lymphomas [43]. In humans, Mcl-1 expression is markedly high in many cases of acute myeloid leukaemia and multiple myeloma, and diverse cancers demonstrate overexpression of Mcl-1 and BCL-x genes [44, 45]. Pertinently, it has also been demonstrated that not only does the overexpression of the anti-apoptotic members of the Bcl-2 family play a role in cancer development; their elevated expression can also be correlated with resistance to cancer therapeutics, including chemotherapy and radiotherapy [23, 46]. Miyashita and colleagues first demonstrated the link between Bcl-2 and resistance to DNA-damaging agents in various lymphoid cell lines [47, 48]. Since then overexpression of Bcl-2, Bcl-x_L or Mcl-1 has been shown to protect against many diverse anti-cancer agents, in both mice [49–52] and humans, reviewed in [23, 53]. More recent studies have extended these observations even further with evidence of “oncogene addiction”. This concept, based on work from the laboratories of the late Stanley Korsmeyer and Anthony Letai, implies that even in the absence of an anti-cancer agent, many cancer cells are addicted to the presence of Bcl-2 proteins and their survival is dependent on the activity of these oncogenes. Under these circumstances, the upregulation of proapoptotic Bcl-2 family members in response to oncogenic signals in tumour cells is not sufficient to overcome the increased antiapoptotic Bcl-2 family protein signalling within the cells [54, 55].

ANTI-APOPTOTIC BCL-2 FAMILY MEMBERS AS TARGETS FOR THE TREATMENT OF CANCERS

The outcome of these collective observations from over three decades of research on Bcl-2 family proteins is that these family members are now extremely attractive targets for the treatment of numerous cancers. As previously mentioned, structural studies have elucidated a hydrophobic groove on the surface of anti-apoptotic Bcl-2 family proteins that binds the BH3 dimerization domain of pro-apoptotic family members [20]. Thus, treatment with molecules that mimic the BH3 domain of the pro-apoptotic proteins may potentially overcome the increase in anti-apoptotic Bcl-2 proteins and thus induce cancer cell death.

The first drug developed to pharmacologically inhibit Bcl-2 was Oblimersen sodium (G3139, Genasense), an 18-mer antisense oligonucleotide designed to target the first six codons of BCL-2 mRNA [56]. Initial preclinical and clinical studies showed that the combination treatment of Oblimersen with a given anti-cancer drug increased the chemotherapeutic

effect in various types of cancers [57–60]. However, after failing to result in survival differences in a pivotal melanoma trial this agent did not obtain US Food and Drug Administration approval [61]. Factors considered as contributory to the failure of this drug included the sole targeting of Bcl-2 by Oblimersen and the potential increased expression of other anti-apoptotic Bcl-2 family members as a result of the downregulation in Bcl-2 expression. To circumvent these difficulties efforts were next directed at neutralizing a broader range of the anti-apoptotic Bcl-2 family members.

HA-141 was identified via *in silico* screens for compounds that bound the hydrophobic groove of Bcl-2 [62]. In preclinical studies it has been shown to inhibit the binding of Bcl-2 and Bcl-x to Bax and Bak [63, 64] and induce apoptosis in a wide variety of cancer cells, including glioma cells [64] and colon cancer cells [65]. Additionally, in combination with etoposide, HA14-1 has been demonstrated to slow the growth of glioblastoma *in vivo* [64]. However, the binding affinity of this compound for Bcl-2 is quite high and is significantly higher than affinities of other inhibitors for Bcl-2 [62].

Gossypol, a polyphenol derived from the cottonseed plant, was the first natural compound discovered that demonstrated inhibition of Bcl-2, Bcl-x_L and Mcl-1 [66]. Originally gossypol was studied as a male contraceptive [67], but was later shown to have potent anti-cancer effects [68, 69]. Natural gossypol is a racemic mixture and studies have found that the levo enantiomer (l-gossypol) has more potent pro-apoptotic effects than d-gossypol [70]. In preclinical studies many groups have shown gossypol's potent pro-apoptotic activity [71, 72]. However, the results of a Phase II clinical trial in which, L-gossypol (AT-101, Ascenta) was tested in patients with recurrent chemosensitive extensive-stage small cell lung cancer (SCLC) were disappointing [73]. More promising results were achieved in a Phase I/II trial, evaluating AT-101 in prostate cancer [74] and when AT-101 was administered in combination with docetaxel, in a Phase II trial of non-small cell lung cancer [75]. Further Phase I and II trials are ongoing to further evaluate AT-101 in combination with conventional chemotherapeutics across a range of malignancies, including small and non-small cell lung cancers, chronic lymphocytic leukaemia, prostate cancers and glioblastoma multiforme (AT-101, <http://clinicaltrials.gov>).

Gossypol has toxicity problems however, most likely due to two reactive aldehyde groups [76] and as a result many derivatives of gossypol have been generated, ranging from Apogossypol, the first derivative designed, to the more recent BI-97C1 (Sabutoclax). These compounds bind with even greater efficiency to the anti-apoptotic Bcl-2 family members but do not confer the same level of toxicity [77, 78]. While preclinical results are promising [79], at present there are no reports on clinical trials using such derivatives but it is expected that these derivatives will enter trials soon. Finally and of special interest are the observa-

tions by Vogler and colleagues that gossypol and its derivatives may kill even in the absence of Bak and Bax, indicating that the mechanisms of action of these drugs may in fact be independent of the intrinsic mitochondrial pathway [80]. Autophagy has been suggested as the mechanism by which gossypol induces death in cells with very high levels of Bcl-2 [81]. While not a direct focus of this review, autophagy is a second process of cell death in which the Bcl-2 family have also been described to play a role. Briefly, the BH3-only protein, Beclin-1 is essential for the initiation of autophagy and can be inhibited by binding to Bcl-2/Bcl-x_L at the endoplasmic reticulum [82–84]. Gossypol has been shown to induce autophagy by blocking this Bcl-2-Beclin 1 interaction [81].

The two Bcl-2 inhibitor drugs furthest in clinical development are obatoclax (GX-15-70) from Gemin X Biotechnologies and ABT-737 from Abbott. Obatoclax was discovered as a result of a high-throughput screen of natural compounds that disrupted protein interactions in the Bcl-2 family and was the first pan anti-apoptotic Bcl-2 protein inhibitor to be described [85]. This small molecule bipyrrrole compound has been shown to bind to Bcl-2, Bcl-x, Bcl-w and Mcl-1 *in vitro* [86]. Preclinical experiments showed that Obatoclax has pro-apoptotic effects when used alone and enhances the *in vitro* efficacy of bortezomib in human multiple myeloma [87] and mantle cell lymphoma cell lines [88]. Obatoclax has been tested in Phase I clinical trials in patients with haematological and myeloid malignancies and was well tolerated [89, 90]. In a more recent phase II study in patients with relapsed or refractory classical Hodgkin lymphoma, obatoclax displayed limited clinical activity [91] but more promising results were observed in a phase I trial of obatoclax in combination with carboplatin and etoposide in patients with extensive-stage small cell lung cancer [92]. Again, the mechanism of action of this putative anti-apoptotic Bcl-2 family inhibitor is not fully understood as it has been shown to induce cell death in the absence of Bax/Bak and caspase-9 [80]. Similar to gossypol, autophagy has also been suggested as an alternative method of inducing cell death which Obatoclax may utilize to kill cells under certain circumstances [93].

ABT-737 was developed using nuclear magnetic resonance to screen a chemical library for BH3-like analogues that bound with high efficiency to the hydrophobic groove of Bcl-x_L [94]. ABT-737 does not inhibit Mcl-1 but binds to and inhibits Bcl-2, Bcl-x_L and Bcl-w with nanomolar affinities, closely resembling the BH3 domain of Bad and representing a far greater potency of action than for the previously discussed compounds [80]. ABT-737 is extremely effective at enhancing the response to radiation as well as a variety of chemotherapy agents in many different cancer cell lines *in vitro*, and displayed significant activity as a monotherapeutic in two small-cell lung cancer xenograft models [94, 95]. However, drug delivery is problematic for ABT-737 and resistance is observed

in cells that express Mcl-1 [96, 97]. To overcome the delivery problems, ABT-263 (Navitoclax) was developed by Abbott for use in the clinic. ABT-263 is an oral version of ABT-737 and shares a similar binding profile and affinities to purified Bcl-2, Bcl-x_L and Bcl-w proteins as ABT-737 [98]. Furthermore, ABT-263 has demonstrated activity as a single agent in small cell lung cancer orthotopic tumour models and has been shown to enhance the activity of chemotherapy agents in cancer cell lines [98]. Several strategies are also being developed to complement the activity of ABT-737, by neutralizing Mcl-1 [99–101].

A number of Phase I and Phase II trials are currently underway or have recently been completed evaluating the efficacy of ABT-263 as both a monotherapeutic and in combination with other chemotherapeutics in patients with malignancies of lymphoid origin and solid tumours (ABT-263, <http://clinicaltrials.gov>). Thrombocytopenia, attributable to the high-affinity inhibition of Bcl-x_L in platelets, has been recognised as a dose-limiting side effect of ABT-263 in preclinical studies [98] and in Phase I trials [102–104]. Due to encouraging results of these Phase I trials, Phase II trials are proceeding [102–104]. Importantly, unlike the other BH3 mimetics discussed, ABT-737 has been shown to act in a similar manner to other BH3-only proteins and requires the presence of Bax/Bak and caspase-9 to induce apoptosis which it is hoped will lead to increased selectivity in its cytotoxicity towards cancer cells [80, 97].

PREDICTING RESPONSES TO BH3 MIMETICS

BH3 profiling was designed as a tool to understand addiction to Bcl-2 family proteins [105]. Using this technique cell lines were divided into three classes based on their specific anti-apoptotic block to the intrinsic pathway of apoptosis. A “class A” block was defined as one that arose from insufficient levels of BH3-only proteins. A “class B” inhibition block developed after significant loss of Bax and Bak. Finally, a “class C” block occurred when the cells overexpressed anti-apoptotic proteins [106, 107]. Further investigations highlighted that BH3 profiling correctly identified those cell lines that were Bcl-2 dependent based on correlation with response to the Bcl-2 antagonist ABT-737 [108]. Such experiments suggest that BH3 profiling could be a useful in the clinic as a diagnostic tool as it could potentially be used to predict patient response to an antagonist of an anti-apoptotic protein [105, 109].

CONCLUSIONS AND PERSPECTIVES

The observations that being dependent on, or addicted to an anti-apoptotic Bcl-2 family member can lead to a cancer phenotype has resulted in the development of many BH3 mimetics to treat a broad range of haematological malignancies and solid tumours. As discussed above clinical data has revealed some to be more successful than others when trialed in patients. Evidence also indicates that a single BH3 mimetic may not be sufficient as a monotherapeutic to cure

cancer patients and the best results may be achieved by appropriate drug combinations. Identifying the most suitable drug combinations may be achieved by using techniques such as BH3 profiling or a systems modelling approach examining Bcl-2 family interactions. Undoubtedly further understanding of this subfamily of proteins is needed to exploit the potential offered by their successful targeting and ultimately deliver improved therapies for cancer patients.

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CONFLICT OF INTEREST

No conflicts to disclose.

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