

REVIEW

The Bcl-2 apoptotic switch in cancer development and therapy

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Impaired apoptosis is both critical in cancer development and a major barrier to effective treatment. In response to diverse intracellular damage signals, including those evoked by cancer therapy, the cell's decision to undergo apoptosis is determined by interactions between three factions of the Bcl-2 protein family. The damage signals are transduced by the diverse 'BH3-only' proteins, distinguished by the BH3 domain used to engage their pro-survival relatives: Bcl-2, Bcl-x_L, Bcl-w, Mcl-1 and A1. This interaction ablates pro-survival function and allows activation of Bax and Bak, which commit the cell to apoptosis by permeabilizing the outer membrane of the mitochondrion. Certain BH3-only proteins (e.g. Bim, Puma) can engage all the pro-survival proteins, but others (e.g. Bad, Noxa) engage only subsets. Activation of Bax and Bak appears to require that the BH3-only proteins engage the multiple pro-survival proteins guarding Bax and Bak, rather than binding to the latter. The balance between the pro-survival proteins and their BH3 ligands regulates tissue homeostasis, and either overexpression of a pro-survival family member or loss of a proapoptotic relative can be oncogenic. Better understanding of the Bcl-2 family is clarifying its role in cancer development, revealing how conventional therapy works and stimulating the search for 'BH3 mimetics' as a novel class of anticancer drugs.

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Introduction

Metazoa eliminate redundant, damaged or infected cells by apoptosis, a stereotypic program of cell suicide during which cells shrink, undergo inter-nucleosomal DNA cleavage and break up into vesicles that are rapidly engulfed by other cells. Apoptosis is vital for embryogenesis, tissue homeostasis and defence against pathogens. Notably, its deregulation can lead to cancer, as well as to autoimmune and degenerative diseases (Cory and Adams, 2002). Elucidation of the underlying regulatory mechanisms is relevant not only to disease etiology, but also to treatment. In particular, as cytotoxic therapies for

cancer rely heavily upon inducing apoptosis, a deeper understanding of apoptosis regulation in normal and neoplastic cells offers great promise for developing more selective and efficacious therapeutic agents.

The first apoptotic regulator to be identified was Bcl-2, the oncoprotein activated via chromosome translocation in human follicular lymphoma (Cory and Adams, 2002). Our laboratory discovered that Bcl-2 acts by promoting cell survival rather than by driving cell proliferation and proposed the concept, now widely accepted (Hanahan and Weinberg, 2000; Johnstone *et al.*, 2002), that impairment of apoptosis is a critical step in tumor development (Vaux *et al.*, 1988; Strasser *et al.*, 1990). Presumably, apoptosis normally eliminates most cells whose cell cycle control is disturbed by oncogenic mutations. Impaired apoptosis would preserve not only such preneoplastic cells but also those bearing mutations that ablate their need for trophic factors, allow their breach of normal tissue barriers or facilitate their metastasis to foreign sites. As well as potentially driving malignant progression at multiple steps, a wealth of observations, for example (Sentman *et al.*, 1991; Strasser *et al.*, 1991a; Schmitt *et al.*, 2000), indicates that overexpression of Bcl-2 and its close relatives is a major component of chemo-resistance. For example, with the National Cancer Institute (NCI) panel of 60 diverse cancer cell lines, the expression level of Bcl-x_L strongly correlates with resistance to most chemotherapy agents (Amundson *et al.*, 2000).

Here we discuss new insights into how the interactions between three factions of the Bcl-2 protein family determine whether the cell commits to apoptosis. We also sketch how these proteins normally regulate tissue homeostasis, briefly address their role in cancer development and describe recent advances in delineating how different modes of cancer therapy work through this family. Finally, we discuss the exciting progress towards creating novel anticancer agents to target this family, which may represent the Achilles' heel of the tumor. Some of these issues are discussed more extensively in earlier reviews (Strasser *et al.*, 2000; Cory and Adams, 2002; Adams, 2003; Cory *et al.*, 2003; Danial and Korsmeyer, 2004; Fesik, 2005; Strasser, 2005).

The two major pathways to apoptosis

Apoptosis is executed by intracellular cysteine proteases called caspases (Adams, 2003). The dozen or so caspases in mammalian cells are synthesized as largely inactive

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zymogens, and two principal pathways activate those involved in apoptosis (Figure 1). The more ancient, evolutionarily conserved 'stress' pathway, which is triggered by developmental cues and diverse intracellular stresses, activates caspase-9 on a scaffold formed by Apaf-1 in response to cytochrome *c* released from damaged mitochondria. This pathway, also termed 'mitochondrial' or 'intrinsic', is primarily regulated by the Bcl-2 family. The 'extrinsic' pathway, on the other hand, is induced when the so-called 'death receptors' on the cell surface are engaged by cognate ligands of the tumor necrosis factor (TNF) family. This pathway instead activates caspase-8 (and caspase-10 in humans), through adaptor proteins that include Fas-associated death domain protein (FADD). Once activated, caspase-9 or -8 (-10) activates downstream 'effector caspases' (i.e. caspases-3, 6 and 7), which provoke cellular destruction by cleaving several hundred cellular proteins. The two pathways are largely independent, as overexpressed Bcl-2 does not protect lymphocytes from apoptosis induced by death receptor ligands (Strasser *et al.*, 1995; Huang *et al.*, 1999). In certain other cell types (e.g. hepatocytes), however, the two pathways intersect, because caspase-8 can process the pro-apoptotic Bid into its active truncated form (tBid) (Figure 1). To prevent catastrophic unscheduled cell death, both pathways are tightly regulated, at multiple steps.

The Bcl-2 family, which includes 17 or more members in mammalian cells (Cory and Adams, 2002), functions

as a 'life/death switch' that integrates diverse inter- and intracellular cues to determine whether or not the stress apoptosis pathway should be activated. The switch operates through the interactions between the proteins within three subfamilies. Whereas the pro-survival subfamily (Bcl-2, Bcl-x_L, Bcl-w, Mcl-1, A1 and also Bcl-B in humans) protects cells exposed to diverse cytotoxic conditions, two other subfamilies, many members of which were identified as Bcl-2-binding proteins, instead promote cell death. Members of the Bax-like apoptotic sub-family (Bax, Bak and Bok) are very similar to Bcl-2 in sequence, particularly in three conserved 'Bcl-2 Homology' regions (BH1, BH2 and BH3), and both Bax and Bak have structures that closely resemble their pro-survival relatives (Suzuki *et al.*, 2000; Moldoveanu *et al.*, 2006). The other proapoptotic subfamily, the 'BH3-only proteins', includes at least eight members: Bik, Bad, Bid, Bim, Bmf, Hrk, Noxa and Puma. These proteins are largely unrelated in sequence to either Bcl-2 or each other, apart from the signature BH3 domain, which is essential for their killing function (Huang and Strasser, 2000; Willis and Adams, 2005).

Importantly, stress-induced apoptosis requires both types of pro-death proteins: the BH3-only proteins seem to act as damage sensors and direct antagonists of Bcl-2 and the other pro-survival proteins, whereas the Bax-like proteins, once activated, act further downstream (Figure 1), probably by permeabilizing the mitochondrial outer membrane and perhaps also by perturbing the endoplasmic reticulum (ER)/nuclear envelope. The crucial battle seems to be fought on these membranes and most family members either normally reside on their cytosolic surfaces, or rapidly congregate there after an apoptotic signal. Most bear a C-terminal hydrophobic sequence that targets and/or anchors them to those membranes, but full integration of the multidomain proteins probably involves insertion of additional helices in the molecules (Kim *et al.*, 2004; Annis *et al.*, 2005), and that also might hold for tBid, which is myristoylated (Zha *et al.*, 2000) but lacks a C-terminal anchor. Integration into the outer mitochondrial membrane may require components of the general import machinery such as TOM22 or TOM70 (Bellot *et al.*, 2006; Chou *et al.*, 2006).

As reviewed recently (Fesik, 2005; Hinds and Day, 2005), illuminating structural studies have revealed that the BH1, BH2 and BH3 domains in each pro-survival family member fold into a globular domain having a hydrophobic groove on its surface, to which a BH3 domain, an amphipathic alpha helix of ~24 residues, can bind (Sattler *et al.*, 1997; Liu *et al.*, 2003). This coupling neutralizes the pro-survival family member. In healthy cells, the pro-survival proteins act at least predominantly by preventing Bax or Bak from perturbing the integrity of intracellular membranes, in particular the outer mitochondrial membrane.

Bax and Bak appear to be largely redundant in function. Whereas the loss of either single gene has little effect in most cells and tissues (Bax is required for spermatogenesis and in certain neuronal cells), the

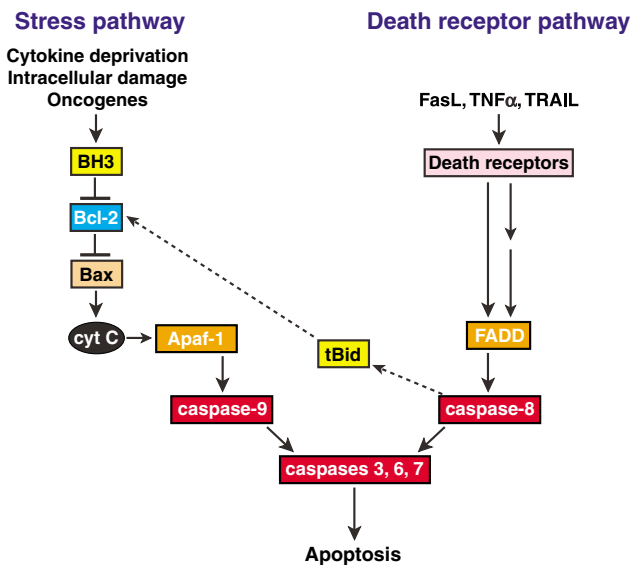


Figure 1 Pathways to cell death. The stress pathway is initiated by BH3-only proteins ('BH3'), which inactivate the Bcl-2-like proteins, keeping them from restraining Bax and Bak. Bax or Bak can permeabilize the mitochondrial outer membrane, releasing cytochrome *c*, which provokes Apaf-1 (apoptotic protease-activating factor 1) to activate caspase-9. The 'death receptor' pathway is activated when ligands of the TNF family engage with and aggregate their cognate receptors on the cell surface and activate caspase-8 via adaptor proteins that include FADD. The two pathways are largely independent, but in certain cells the death receptor pathway engages the stress pathway via a cleaved form of the BH3-only protein Bid (tBid), which can engage Bcl-2 homologs and perhaps Bax (see text).

absence of both proteins blocks apoptosis in many cell types (Wei *et al.*, 2001) and dramatically impairs developmentally programmed attrition in several tissues (Lindsten *et al.*, 2000), typically resulting in perinatal death. A notable biochemical difference between Bax and Bak is that, in healthy cells, Bax is largely cytosolic or loosely associated with mitochondria, whereas Bak is an integral membrane protein on the cytosolic face of the mitochondrion and ER. In response to cytotoxic signals, Bax translocates to those membranes, and both Bax and Bak change conformation and form membrane-associated homo-oligomers. These oligomers are thought to permeabilize the outer mitochondrial membrane, but the mechanism remains obscure (Newmeyer and Ferguson-Miller, 2003; Green, 2005). Bax and Bak can also influence the shape of the mitochondria via effects on the mitochondrial fission/fusion machinery (Karbowski *et al.*, 2006), but whether that machinery contributes to permeabilization is unclear (Parone *et al.*, 2006).

How the life–death switch operates

Previously, activated BH3-only proteins were generally assumed to bind indiscriminately to all their pro-survival counterparts. Quantitative assessment of the binding of BH3 peptides to all the mammalian Bcl-2-like proteins, however, has revealed that the affinities of different pairs vary enormously (Chen *et al.*, 2005; Kuwana *et al.*, 2005). Whereas Bim, Puma and tBid bind avidly to all the pro-survival proteins, the other BH3-only proteins associate only with subsets (Chen *et al.*, 2005). For example, Noxa engages only Mcl-1 and A1 and Bad only Bcl2, Bcl-x_L and Bcl-w (Figure 2a). Importantly, the promiscuous binders (e.g. Bim and Puma) are much more potent killers than those that cannot engage all the pro-survival proteins, but the combination of Noxa and Bad potently induces cell death (Figure 2b; Chen *et al.*, 2005). These findings indicate that efficient apoptosis requires neutralization of multiple pro-survival proteins and suggest that all the pro-survival proteins may not have equivalent function.

The BH3-only proteins clearly act upstream of Bax and Bak, because they cannot induce apoptosis in cells lacking both Bax and Bak (Cheng *et al.*, 2001; Zong *et al.*, 2001). How they induce activation of Bax and Bak is addressed by two distinct models (Figure 3; Willis and Adams, 2005). The *direct activation* model (Figure 3a) holds that certain BH3-only proteins, termed *activators*, namely Bim and tBid, can bind to Bax and Bak directly and promote their activation (Letai *et al.*, 2002; Kuwana *et al.*, 2005; Certo *et al.*, 2006; Oh *et al.*, 2006; Walensky *et al.*, 2006). Puma has also been implicated as an activator (Carton *et al.*, 2004; Kim *et al.*, 2006a). In this model, the remaining BH3-only proteins, termed *sensitizers*, bind only to the pro-survival proteins and purportedly act by displacing any bound Bim or tBid, allowing them to directly activate Bax and Bak. The *indirect activation* model (Figure 3b), on the other hand,

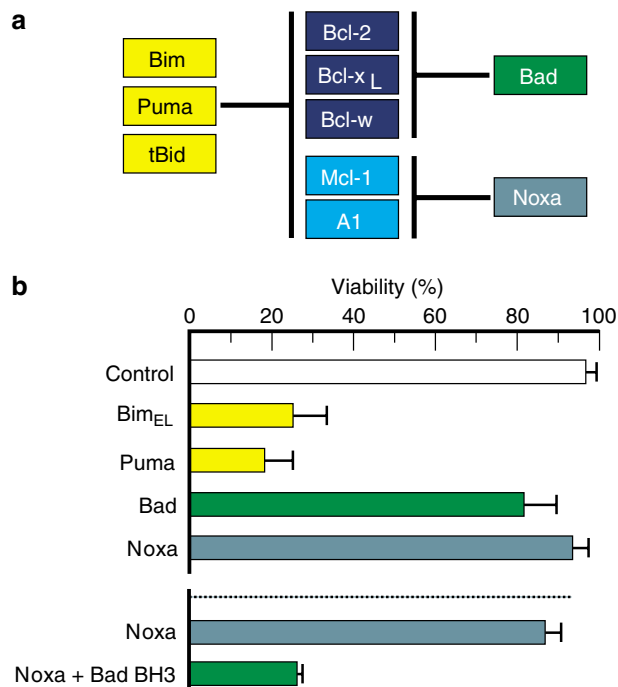


Figure 2 Differing binding profiles and apoptotic potency of BH3-only proteins. **(a)** The ability of Bim, Puma and tBid to engage all the pro-survival proteins contrasts with the selective binding of others, such as Bad and Noxa (Chen *et al.*, 2005). **(b)** Cytotoxicity assays in fibroblasts show that Bim and Puma are potent killers, but Bad and Noxa alone are much weaker. Nevertheless, Noxa in conjunction with a Bad BH3 (within an inert backbone) kills potently (Chen *et al.*, 2005).

suggests that all the BH3-only proteins engage only their pro-survival relatives and act by preventing them from countering Bax or Bak activation (Chen *et al.*, 2005; Willis *et al.*, 2005; Willis *et al.*, 2007). In this model, Bim and tBid are potent inducers of apoptosis simply because they can engage all the pro-survival proteins (Chen *et al.*, 2005).

Several recent findings strongly challenge the direct activation model (Willis *et al.*, 2007). Firstly, Bak bound to none of the BH3-only proteins. Secondly, Bax did not bind to either of the two physiologically relevant forms of Bim, namely Bim_{EL} and Bim_L, and no Bax could be co-immunoprecipitated with Bim in dying cells. Thirdly, although tBid and a very minor Bim isoform (Bim_S) did bind weakly to Bax (albeit only in detergents that alter the Bax conformation), tBid and Bim_S proteins bearing subtle BH3 mutations that prevent binding to Bax but not to pro-survival proteins killed cells as potently as wild-type tBid and Bim_S. Finally, and most tellingly, in response to several apoptotic stimuli, including imposed co-expression of Noxa and Bad, cells lacking both Bid and Bim underwent apoptosis as readily as wild-type cells. Although it has been suggested that Puma might also serve as an *activator* for Bax (Carton *et al.*, 2004; Kim *et al.*, 2006a), this has been disputed (Kuwana *et al.*, 2005; Certo *et al.*, 2006; Ming *et al.*, 2006) and downregulation of Puma by RNA interference, in cells

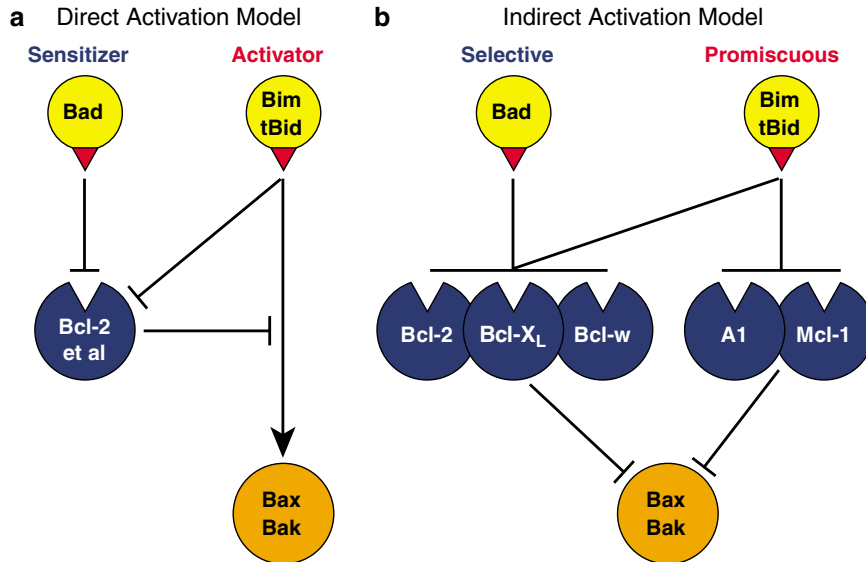


Figure 3 Contrasting direct and indirect activation models for Bax and Bak. (a) In the direct model (Letai *et al.*, 2002), the putative activators Bim and tBid bind directly to Bax and Bak to drive their activation, whereas the sensitizers only bind to the pro-survival Bcl-2 homologs ('Bcl-2 *et al.*') via the BH3 domain (red triangle). (b) In the indirect activation model (Chen *et al.*, 2005; Willis *et al.*, 2005, 2007), the BH3-only proteins activate Bax and Bak not by binding to them directly, but instead by engaging the multiple pro-survival proteins that guard Bax and Bak. In this model, Bim and tBid are more potent than Bad and other BH3-only proteins owing to the greater range of pro-survival proteins that they can engage and neutralize.

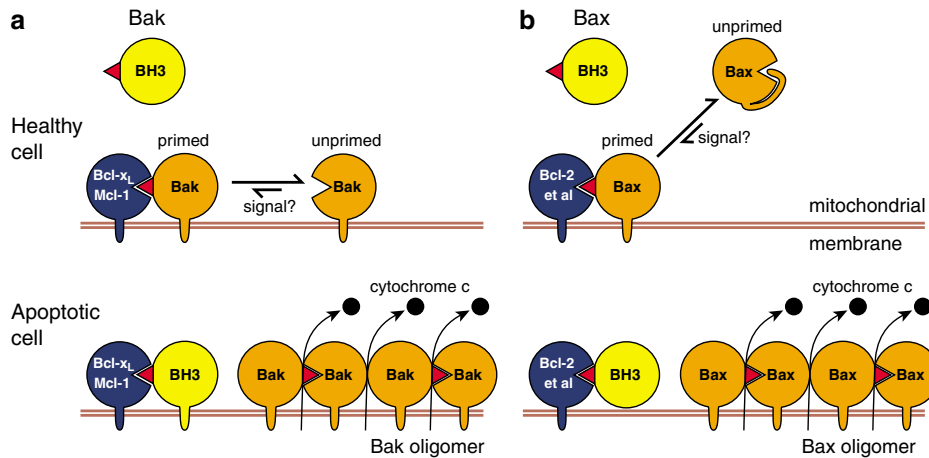


Figure 4 Model for the regulation of (a) Bak and (b) Bax by their pro-survival relatives. (a) In the healthy cell, both Bcl- x_L and Mcl-1 can bind to Bak, presumably to a 'primed' conformer with its BH3 (red) exposed (Willis *et al.*, 2007). Apoptosis is induced if and only if BH3-only proteins displace Bak from both these pro-survival guards. The free primed Bak may nucleate formation of the Bak oligomers (of unknown structure) that elicit permeabilization of the mitochondrial outer membrane and release of cytochrome *c*. (b) Bax appears to be regulated analogously (Willis *et al.*, 2007), but most of the 'unprimed' Bax is cytosolic, and all the pro-survival family members ('Bcl-2 *et al.*') appear to inhibit Bax activation. Whether a small amount of the postulated primed Bak and Bax exists in healthy cells, perhaps on the mitochondrial membrane, or instead is formed early in apoptosis by an unknown signal, is not yet established. The structures of the complexes is not known, and the integration of the proteins into the membrane probably includes regions in addition to their C-terminal regions (see text).

lacking both Bim and Bid, did not impair apoptosis driven by several stimuli (Willis *et al.*, 2007).

Collectively, these findings make it unlikely that direct activation of Bax or Bak by BH3-only proteins (Figure 3a) is the major physiological pathway to apoptosis. Nevertheless, it remains possible that direct activation can occur in some circumstances, to account for the ability of tBid or a Bid BH3 peptide with a chemically stabilized α -helix or one targeted to membranes

to cooperate with Bax in the lysis of protein-free liposomes (Kuwana *et al.*, 2002, 2005; Oh *et al.*, 2006; Walensky *et al.*, 2006).

The *indirect activation* model (Figure 3b) envisages that the pro-survival proteins function mainly by inhibiting Bax/Bak activation. Our recent evidence suggests that, at least for Bak, this occurs by direct interaction (Willis *et al.*, 2005, 2007). In healthy cells, Bak can form a complex with either Mcl-1 or Bcl- x_L

(although, unexpectedly, not with Bcl-2), and this interaction requires the Bak BH3 domain (Willis *et al.*, 2005; Figure 4a, top panel). Early in apoptosis, BH3-only proteins seem to displace Bak from these pro-survival proteins. Bak can drive cell death if and only if both Mcl-1 and Bcl-x_L are inactivated by, for example, the combination of Noxa and Bad (Figure 2). As the Bak BH3 domain is critical for Bak-mediated apoptosis (Willis *et al.*, 2005), one might speculate that this motif not only allows restraint of Bak by its pro-survival counterparts but also, once Bak is released from them, nucleates Bak oligomerization, perhaps by allowing a BH3-exposed conformer ('primed' Bak) to dimerize with an 'unprimed' receptor-like Bak conformer (Willis *et al.*, 2005; Figure 4a, bottom panel).

In view of the predominant cytosolic location of Bax in healthy cells (Wolter *et al.*, 1997), its regulation must be more complex than that of Bak. Nevertheless, very recent findings (Willis *et al.*, 2007) suggest that the indirect activation model (Figure 3b) also holds for Bax (Figure 4b). A complication has been that, although all of the pro-survival family members can bind to Bax, these interactions are generally detectable only in non-ionic detergents, which change the Bax conformation (Hsu and Youle, 1997). Furthermore, only a small proportion of Bax molecules may be required to launch the apoptotic program (Lakhani *et al.*, 2006; Zhu *et al.*, 2006). Notably, recent functional tests have demonstrated that neutralization of multiple pro-survival proteins by BH3-only proteins is necessary and apparently sufficient to allow Bax-mediated apoptosis (Willis *et al.*, 2007).

One interpretation of these results is that, in healthy cells, a small proportion of the Bax molecules exists in a 'primed' state, presumably a conformer with the BH3 domain exposed but complexed with various pro-survival relatives (Figure 4b, top panel). The putative primed Bax is likely to be located on the mitochondrial membrane (Figure 4b), because the cytosolic form is monomeric (Hsu and Youle, 1998) and has its membrane anchor buried in a groove on its surface (Suzuki *et al.*, 2000). Alternatively, the primed Bax and Bak may form early in apoptotic signalling, either spontaneously or in response to an independent cytotoxic signal. For example, Bax may be phosphorylated on one site (Kim *et al.*, 2006b) or dephosphorylated on another (Gardai *et al.*, 2004) to induce its translocation to the membrane.

In any case, the functional studies (Willis *et al.*, 2007) suggest that engagement of multiple pro-survival proteins by BH3-only proteins is sufficient to drive apoptosis via activation of either Bax or Bak (Figure 4). Studies with the BH3 mimetic drug ABT-737 (see below) are also consistent with this model (Konopleva *et al.*, 2006; van Delft *et al.*, 2006; Willis *et al.*, 2007). However, the pro-survival proteins may be able to control Bax and Bak without binding to them, because mutants of Bax and Bak have been identified that do not bind the pro-survival proteins but are not constitutively active (Kim *et al.*, 2006a; Fletcher J, DCS Huang and JMA, unpublished results).

For Bax, diverse other proteins or peptides have been proposed to contribute to its regulation (Lucken-Ardjomande and Martinou, 2005), either by restraining it in the cytosol (14-3-3 isoforms, the peptide humanin, Ku70, apoptosis repressor with caspase recruitment domain (ARC), Hsp-70 forms, crystallins) or by promoting its translocation to membranes or activity (Bif-1, p53, apoptosis-associated speck-like protein with caspase-recruitment domain (ASC), MAP-1 modulator of apoptosis-1 (MOAP-1)), but the evidence for a physiological role remains very limited. Similarly, a minor isoform of the voltage-dependent anion channel-2 (VDAC2) has been proposed to restrain Bak (Cheng *et al.*, 2003).

Thus, important questions about the apoptotic switch remain unanswered. What is the critical signal that drives Bax to the mitochondria? Is a portion of Bax and Bak molecules primed for death duty in healthy cells? What is the nature of the putative complexes between the pro-survival family members and Bax or Bak? Can the pro-survival proteins prevent cell death in a fashion independent of binding Bax or Bak? Do non-family members have any role? Finally, what is the nature of the homo-oligomers of Bax and Bak and how do they permeabilize the outer mitochondrial membrane?

Physiological regulation and roles of family members

It is likely that the absence of all pro-survival family members in almost any mammalian cell would impose 'death by default'. Most cell types express several pro-survival proteins, until trophic factors become limiting. Owing to their partially redundant function and complex expression patterns, the consequences of disrupting individual pro-survival genes vary considerably. For two, the impact appears very specific: loss of Bcl-w precludes adult spermatogenesis (Print *et al.*, 1998; Ross *et al.*, 1998), while A1 loss causes neutrophil attrition (Hamasaki *et al.*, 1998). The others, however, have more diverse non-redundant roles. Bcl-2 is essential for the survival of kidney and melanocyte stem cells, as well as mature B and T lymphoid cells (Nakayama *et al.*, 1993, 1994; Veis *et al.*, 1993) and Bcl-x_L is needed to sustain erythroid and neuronal cells (Motoyama *et al.*, 1995, 1999; Wagner *et al.*, 2000). Remarkably, Mcl-1 is obligatory for the survival and implantation of the zygote (Rinkenberger *et al.*, 2000), and its conditional elimination has revealed critical roles in B and T lymphopoiesis and early hematopoiesis (Opferman *et al.*, 2003, 2005), as well as in mature neutrophils but not macrophages (Dzhagalov *et al.*, 2006). The particularly severe defects produced by the loss of Mcl-1 or Bcl-x_L may reflect their key roles as guards on Bak (see above) (Willis *et al.*, 2005).

The levels and activity of pro-survival proteins are regulated by diverse mechanisms, including transcriptional control and protein modification and turnover. The levels of Bcl-x_L, A1 and Mcl-1, for example, are closely coupled to the supply of cytokines, which affects

both their production and stability. Bcl-2 levels may also be controlled, in part, by micro-RNAs (Cimmino *et al.*, 2005) and its activity is affected in complex ways by phosphorylation (Deng *et al.*, 2004). In cells with DNA damage, Bcl-x_L becomes deaminated on two asparagines in its flexible loop region (Deverman *et al.*, 2002), but the claim that this blocks Bcl-x_L pro-survival activity may well be erroneous (Deverman *et al.*, 2003). The Mcl-1 protein is particularly labile and is rapidly lost by proteasomal degradation early in response to several cytotoxic signals (Cuconati *et al.*, 2003; Nijhawan *et al.*, 2003). Proteins implicated in regulation of its degradation include Noxa (Willis *et al.*, 2005) and a BH3-containing E3 ubiquitin ligase called Mule (Zhong *et al.*, 2005).

The multiplicity of mammalian BH3-only proteins presumably allows more precise control over apoptosis, and indeed the regulation of these proteins is complex, involving both transcriptional and diverse post-translational mechanisms (Puthalakath and Strasser, 2002; Cory *et al.*, 2003). Puma, Noxa and Hrk seem to be regulated largely at the transcriptional level. Whereas *bim* can also be induced downstream of Akt by a Forkhead transcription factor (Dijkers *et al.*, 2000), the Bim protein is subject to several types of post-translational regulation. In some cells, its binding via the dynein motor complex to microtubules (Puthalakath *et al.*, 1999) may couple its control to the cytoskeleton (Puthalakath *et al.*, 2001), perhaps accounting for its important role in the apoptotic response elicited by paclitaxel, which affects the microtubules (see below). In some cell types, however, Bim is instead bound predominantly to pro-survival relatives on the mitochondria (Zhu *et al.*, 2004; Certo *et al.*, 2006). The level of Bim can be regulated by phosphorylation by Erk, which triggers its degradation by the proteasome (Akiyama *et al.*, 2003; Ley *et al.*, 2003; Luciano *et al.*, 2003), whereas its phosphorylation by c-Jun N-terminal kinase may potentiate its pro-apoptotic activity (Putchá *et al.*, 2003). Bad is also regulated by phosphorylation, which leads to its sequestration by 14-3-3 scaffold proteins (Zha *et al.*, 1996). Bid requires proteolytic processing, typically by a caspase (Figure 1) or granzyme B.

Mice lacking individual BH3-only proteins have provided considerable insight into their physiological roles (Strasser, 2005; Willis and Adams, 2005). BH3-only proteins trigger apoptosis not only in response to physiological stimuli such as cytokine deprivation, detachment from the cell matrix (anoikis) or antigen receptor signalling, but also in response to signals elicited by activated oncogenes, DNA damage, chemotherapy drugs and irradiation with UV light or γ -rays. Given the similar biochemical roles and widespread expression of many of these proteins, it is not surprising that most have at least partially redundant biological functions. Bim and Puma, however, have particularly notable roles, probably because they can engage all the pro-survival proteins (see above).

As reviewed recently (Strasser, 2005), Bim is a principal regulator of hematopoietic homeostasis, particularly in the lymphoid compartment (Bouillet *et al.*, 1999). It provokes apoptosis of lymphocytes following certain cytotoxic stimuli, such as cytokine deprivation or

Ca²⁺ flux, but not others such as phorbol 12-myristate 13-acetate (PMA) or etoposide (Bouillet *et al.*, 1999). Bim is also critical for shaping the immune repertoire by eliminating thymocytes that recognize self-antigens (Bouillet *et al.*, 2002) and in terminating T-cell immune responses (Hildeman *et al.*, 2002; Pellegrini *et al.*, 2003). In the differentiation of mammary epithelial cells into 3D structures, Bim appears to contribute to lumen formation, in part, by mediating anoikis of centrally localized cells (Reginato *et al.*, 2003, 2005).

It has long been debated how, in many cell types subjected to DNA damage, the wild-type p53 tumor suppressor provokes apoptosis (Vousden and Lu, 2002). Multiple targets of this transcription factor have been implicated, and it has even been proposed that p53 has a direct apoptogenic role at the mitochondrion by engaging either Bcl-x_L and Bcl-2 (Mihara *et al.*, 2003) or Bax (Chipuk *et al.*, 2004). Gene knockout studies, however, have established that the p53 transcriptional target Puma is the critical mediator of this response, particularly in the lymphoid system (Jeffers *et al.*, 2003; Villunger *et al.*, 2003). Noxa, the other BH3-only gene upregulated by p53, acts together with Puma in transformed fibroblasts, but has only a very minor role in lymphocytes. Notably, in several cell types, the protection from genotoxic damage conveyed by the loss of Puma is comparable to that conveyed by loss of p53 itself (Erlacher *et al.*, 2005). At least in these cell types, this finding strongly questions the physiological significance of any Puma-independent pathway from p53 to apoptosis, such as the postulated direct role of cytoplasmic p53 at the mitochondria.

As well as its dominant role in p53-mediated responses, Puma also has a central role in several p53-independent responses (Jeffers *et al.*, 2003; Villunger *et al.*, 2003). It mediates, for example, the cytotoxic response to phorbol 12-myristate β -acetate (PMA) and contributes to that initiated by glucocorticoids.

Tissue homeostasis requires an appropriate balance between the levels of pro-survival family members and their BH3 ligands. Overexpression of Bcl-2 or its homologs provokes an abnormal accumulation of non-cycling cells (McDonnell *et al.*, 1989; Strasser *et al.*, 1991b; Ogilvy *et al.*, 1999). Conversely, the tissue degeneration brought about by inadequate levels of these proteins can be prevented by a reduction in that of their BH3-only adversaries. Remarkably, loss of even a single allele of Bim sufficed to preclude the renal failure and immune collapse that otherwise ensues in mice lacking Bcl-2 (Bouillet *et al.*, 2001). In contrast to the BH3-only proteins, the levels of Bax and Bak do not seem to be modulated significantly. Indeed, loss of single alleles of either, or even three of their (combined) four alleles, has little physiological impact (Lindsten *et al.*, 2000).

Deregulation of the Bcl-2 life/death switch in oncogenesis

As indicated above, *BCL-2* was first implicated in cancer through its involvement in the t14;18 chromosome

translocation that hallmarks follicular lymphoma. B-lymphoid-specific transgenes (*E μ -bcl-2*) that mimicked the translocation confirmed that deregulated Bcl-2 expression was indeed oncogenic, but the tumor incidence was low, latency long and most tumors were plasmacytomas rather than follicular lymphomas (McDonnell and Korsmeyer, 1991; Strasser *et al.*, 1993). More recently, we have found that a transgene with pan-hematopoietic Bcl-2 expression (*VavP-bcl-2*) preferentially provokes follicular lymphoma, preceded by florid germinal center hyperplasia (Egle *et al.*, 2004a). As the germinal center hyperplasia was driven by an expanded helper T-cell compartment, we have suggested that autoreactivity and hypermutation in the germinal center conspire with overexpressed Bcl-2 to generate human follicular lymphoma (Egle *et al.*, 2004a).

Certain aggressive human follicular lymphomas and most tumors arising in *E μ -bcl-2* transgenic mice bear a translocated *myc* gene, suggesting that Bcl-2 might synergize with *Myc*. Evidence of collaboration first emerged from *in vitro* studies (Vaux *et al.*, 1988). Direct proof came from bitransgenic *E μ -bc-2/E μ -myc* mice, which developed tumors much more rapidly than mice expressing either transgene alone (Strasser *et al.*, 1990). The *bcl-2/myc* combination has subsequently been shown to promote malignant transformation of diverse mouse cell types (see Cory and Adams, 2002).

Myc is a transcription factor that regulates cell size, proliferation and differentiation. Under stress conditions, such as limiting cytokines, it also sensitizes cells to apoptosis; this function is thought to have evolved to eliminate cells with deregulated, and hence potentially tumorigenic, *Myc* expression. The marked synergy in oncogenesis between Bcl-2 and *Myc* therefore probably mainly reflects inhibition of *Myc*-induced apoptosis by Bcl-2 and its homologs. Many other oncogenes that perturb the cell cycle (e.g. *Ras*, adenovirus E1A) may also promote apoptosis unless countered by proteins such as Bcl-2.

In view of the oncogenic potential of antiapoptotic Bcl-2 family members, we postulated that their antagonists might be tumor suppressors (Cory and Adams, 2002). Indeed, we demonstrated that *Bim* is a potent tumor suppressor by showing that in *E μ -myc* mice loss of even one *bim* allele accelerated tumorigenesis, primarily owing to early onset of acute B-cell-leukemia (Egle *et al.*, 2004b). The reduced level of *Bim* was shown to decrease *Myc*-imposed apoptosis. Furthermore, although *Bim* does not appear to be a direct transcriptional target of *Myc*, *Bim* levels were higher in premalignant B lymphoid cells from *E μ -myc* than non-transgenic mice. Conversely, *Myc* overexpression lowers expression of Bcl-*x_L* and Bcl-2 (Eischen *et al.*, 2001; Egle *et al.*, 2004b), perhaps by blocking the activity of the MIZ transcription factor, which seems to regulate *bcl-2* expression (Patel and McMahon, 2006). We hypothesize that *Bim* is activated, and Bcl-*x_L*/Bcl-2 suppressed, when *Myc*-driven proliferation exceeds a physiologic checkpoint, such as cytokine availability. Loss of *Bim* would then permit cycling B cells to survive with the attendant risk of acquiring additional mutations (Egle *et al.*,

2004b). *Bim* is also implicated as a tumor suppressor in mouse epithelial cancer (Tan *et al.*, 2005).

In support of a tumor suppressor role for human *BIM*, examination of human lymphomas has revealed that the *BIM* gene has undergone biallelic deletion in a substantial proportion of mantle cell lymphomas (Tagawa *et al.*, 2005; Mestre-Escorihuela *et al.*, 2006) and has been silenced by promoter methylation in some other B-cell lymphomas, including the majority of Burkitt lymphomas (Mestre-Escorihuela *et al.*, 2006), which are hallmarked by *Myc* translocation. Pertinently, the translocated *MYC* gene in some Burkitt lymphomas has undergone point mutations that preclude its activation of *BIM* (Hemann *et al.*, 2005). A few other human lymphomas have a mutated *NOXA* gene (Mestre-Escorihuela *et al.*, 2006).

The ability of wild-type p53 to elicit apoptosis is thought to be a major component of its tumor suppressor function (Vousden and Lu, 2002). Given that *Puma* is a major mediator of the p53 apoptotic response (Jeffers *et al.*, 2003; Villunger *et al.*, 2003), it is noteworthy that, unlike p53-deficient animals, *Puma*-null mice are not susceptible to spontaneous tumorigenesis (E Michalak, A Strasser and JMA, unpublished results). This may indicate that functions of p53 in addition to apoptosis (e.g. cell cycle arrest, senescence) are required for efficient tumor suppression. On the other hand, downregulation of *Puma* or its deletion does accelerate *Myc*-induced lymphomagenesis (Hemann *et al.*, 2004; E Michalak, A Strasser and JM Adams, unpublished results). Hence, the tumor suppressor role of *Puma*, and probably also *Bim*, may arise mainly in the context where cell cycle control has been disrupted, such as by enforced *Myc* expression. In keeping with that notion, the tumor suppressor role of p53 appears to be linked much more tightly to signals from activated oncogenes via p19ARF than to those from DNA damage (Christophorou *et al.*, 2006).

The redundancy of *Bax* and *Bak* would be expected to limit their roles as tumor suppressors. Indeed, *Bax*-deficient mice are not inherently tumor-prone (Knudson *et al.*, 2001), and development of epithelial tumors in one study appeared to require loss of both *Bax* and *Bak* (Degenhardt *et al.*, 2002). On the other hand, loss of *Bax* alone did accelerate development of mouse brain tumors driven by SV40-T antigen (Yin *et al.*, 1997) and *Myc*-induced lymphomas (Eischen *et al.*, 2001), and loss of even one allele accelerated mammary tumor development (Shibata *et al.*, 1999). Another indication that its tumor suppressor role may be greater in certain cell types than others is that *Bax* loss shifted the tumor spectrum in ARF-deficient mice from lymphomas towards sarcomas and carcinomas (Eischen *et al.*, 2002). Furthermore, some human colorectal carcinomas and hematopoietic tumors exhibit mutated *Bax* (Rampino *et al.*, 1997; Meijerink *et al.*, 1998) or *Bak* (Kondo *et al.*, 2000), and these alterations convey a survival advantage (Ionov *et al.*, 2000). Interestingly, several of these studies show that *Bax* loss is affecting a p53-independent pathway to tumorigenesis, while others suggest that it is acting downstream of p53. However,

Table 1 Anticancer treatments that act via BH3-only proteins

Treatment	Likely mode of action	Tumor or cell type	BH3-only protein(s) implicated	References
Glucocorticoid	Activation of transcription	ALL	Bim and Puma	Bouillet <i>et al.</i> (1999), Villunger <i>et al.</i> (2003)
Imatinib	Tyrosine kinase inhibitor	CML	Bim and Bad	Kuribara <i>et al.</i> (2004), Kuroda <i>et al.</i> (2006)
Paclitaxel	Microtubule stabilizer	Lymphocytes, epithelial cancers	Bim	Bouillet <i>et al.</i> (1999), Tan <i>et al.</i> (2005)
Genotoxic damage (via wt p53)	p53-induced transcription	Multiple cell types	Puma and/or Noxa	Jeffers <i>et al.</i> (2003), Villunger <i>et al.</i> (2003)
Bortezomib	Proteasome inhibitor	Various cancers	Bim and/or Bik	Nikrad <i>et al.</i> (2005), Tan <i>et al.</i> (2005)
Bortezomib		Melanoma, myeloma	Noxa	Fernandez <i>et al.</i> (2005), Qin <i>et al.</i> (2005)
Bortezomib		Squamous cell carcinoma	Noxa	Fribley <i>et al.</i> (2006)
Epoxomicin	Proteasome inhibitor	Tumor with wt p53	Puma	Concannon <i>et al.</i> (2006)

Abbreviations: ALL, acute lymphocytic leukemia; CML, chronic myelogenous leukemia.

Bax is unlikely to be a direct p53 target gene, because the putative p53 binding sites identified in human *BAX* (Miyashita and Reed, 1995) are not used in the mouse (Schmidt *et al.*, 1999).

Towards a more rational cancer therapy

As reviewed previously (Cory *et al.*, 2003; Willis and Adams, 2005), increasing evidence encourages us to think that most of the cytotoxic stresses imposed on a cell lead to the activation of one or more of the BH3-only proteins as essential transducers of that stress signal. Pertinently, this holds for many, if not all, of the cytotoxic agents currently used in cancer therapy (Table 1). For example, the glucocorticoids that are often successful in acute lymphocytic leukemia (ALL) rely upon Bim and Puma (Bouillet *et al.*, 1999; Villunger *et al.*, 2003), whereas the kinase inhibitor imatinib (Gleevec), which has transformed treatment of chronic myelogenous leukemia (CML), elicits apoptosis mainly through Bim and Bad (Kuribara *et al.*, 2004; Kuroda *et al.*, 2006). Similarly, paclitaxel (Taxol) requires Bim in both lymphocytes (Bouillet *et al.*, 1999) and epithelial tumor cells (Tan *et al.*, 2005). In cells with wild-type p53, the genotoxic damage elicited by etoposide or γ -irradiation proceeds via induction of Puma (alone or with Noxa), but Bim, which is not induced by p53, also plays a minor role (Erlacher *et al.*, 2005). The promising histone deacetylase (HDAC) inhibitors can also work through Bim (Zhao *et al.*, 2005), with Bmf having some role (Zhang *et al.*, 2005).

The critical mediators of the action of proteasome inhibitors seem to vary with cell type and p53 status (Table 1). In certain cancer cells, the Food and Drug Administration (FDA)-approved proteasome inhibitor bortezomib can work through Bim and/or Bik, and collaborate with TRAIL (Nikrad *et al.*, 2005; Tan *et al.*, 2005), but in melanomas and myelomas and squamous cell carcinoma, it works instead through Noxa in a p53-independent manner (Fernandez *et al.*, 2005; Qin *et al.*,

2005; Fribley *et al.*, 2006). In tumor cells with wild-type p53, however, proteasome inhibitors can instead act by driving p53-dependent expression of Puma (Concannon *et al.*, 2006).

Identification of these pathways stimulates the hope that the highly empirical nature of current cancer therapy, particularly combination chemotherapy, can be put on a more rational footing. That prospect is illustrated by a study concerning the role of Bim in the *in vivo* response of epithelial tumor cells to paclitaxel (Tan *et al.*, 2005). Paclitaxel induced Bim accumulation and Bim-dependent apoptosis. However, if the Ras pathway was activated in the tumor, no apoptosis ensued, because Bim was phosphorylated and targeted for degradation by the proteasome (Akiyama *et al.*, 2003). Nevertheless, addition of bortezomib restored the Bim levels and promoted Bim-dependent tumor regression (Tan *et al.*, 2005). Hence, combination chemotherapy of bortezomib plus paclitaxel has promise, particularly in tumors with an activating Ras mutation.

Therapeutic potential of BH3 mimetics

There is now great interest in the prospect of developing drugs that mimic the action of the BH3 domain by binding to one or more of the Bcl-2-like proteins and triggering the apoptotic program (Cory *et al.*, 2003; Fesik, 2005). The appeal of such 'BH3 mimetics' is that the majority of tumors have defects in the p53 pathway and many overexpress Bcl-2 or a close relative, such as Bcl-x_L (Amundson *et al.*, 2000), but the core apoptotic machinery remains intact (Figure 5). Hence, a drug that could bind to the groove on the pro-survival proteins and inactivate them could be a more effective antitumor agent than drugs that act far upstream. Furthermore, the finding that particular BH3 domains preferentially bind particular pro-survival proteins (Chen *et al.*, 2005) enhances the likelihood that it will be feasible to target specifically the Bcl-2-like protein(s) most important for maintaining a particular tumor type. That specificity

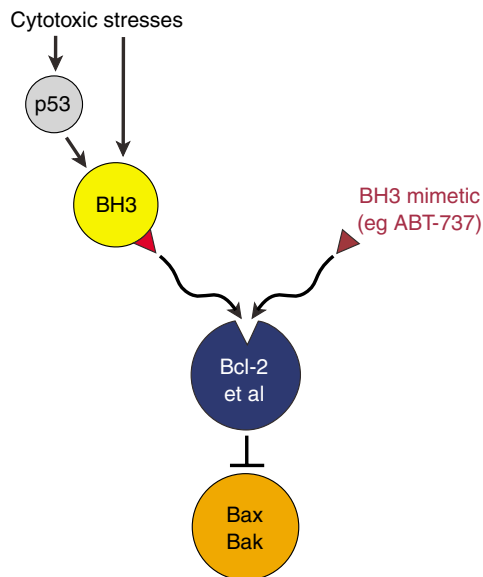


Figure 5 Potential of BH3 mimetic anticancer drugs. Because the upstream signals that induce apoptosis (particularly the p53 pathway) often are compromised in cancer cells, a drug that behaves like a BH3-only protein by binding to one or more of the Bcl-2 homologs to free Bax or Bak should be a more effective way of killing cancer cells.

offers the prospect of drugs that efficiently kill tumor cells, but have reduced cytotoxicity for normal cells.

In principle, the BH3 mimetic might be a BH3 peptide modified to have more suitable pharmacological properties, although the development of peptides as drugs has often failed. Nevertheless, BH3 peptides constrained to maintain an α helix by a chemical staple (Walensky *et al.*, 2004, 2006), and peptides where the α -helical conformation is mimicked by unnatural amino-acid residues (Sadowsky *et al.*, 2005, 2006), which should be more stable *in vivo*, are showing promise. Furthermore, the ability of a stapled Bid BH3 peptide to activate Bax, at least *in vitro* (Walensky *et al.*, 2006), raises the possibility that Bax (or Bak) could be targeted, although that should promote more apoptosis in normal cells than a compound engaging selected pro-survival proteins.

At present, small organic molecules that bind to the groove, identified through high throughput screening and/or molecular design seem to have more potential. Although interference with a protein/protein interface is challenging, Abbott Laboratories have recently used a structure-based approach to develop a very promising BH3 mimetic, ABT-737 (Oltersdorf *et al.*, 2005). This compound binds strongly (low nM affinity) to Bcl-2, Bcl-x_L and Bcl-w but not to Mcl-1 or A1. It proved cytotoxic on its own with many B lymphoid tumor cell lines, primary cells from human follicular lymphoma, most chronic lymphocytic leukemia samples and even many cell lines from small cell lung cancers. Notably, ABT-737 induced stable regression of human lung cancer in a mouse xenograft model, with minimal side effects (Oltersdorf *et al.*, 2005). The drug also effectively killed acute myeloid leukemia cells but not normal

hematopoietic progenitor cells *in vitro* and also reduced leukemia growth in a xenograft model without overt effects on normal mouse tissues (Konopleva *et al.*, 2006).

To evaluate how a BH3 mimetic might best be used, the mechanism of action of ABT-737 and six other putative BH3 mimetics has been explored (van Delft *et al.*, 2006). Of the seven, only ABT-737 failed to kill cells lacking both Bax and Bak and therefore only it behaved as an authentic BH3 mimetic. Notably, cells expressing Mcl-1, which the drug does not bind, proved resistant to ABT-737, whereas those with little Mcl-1 were highly sensitive (Konopleva *et al.*, 2006; van Delft *et al.*, 2006). Down regulation of Mcl-1 by several strategies, however, conferred sensitivity to ABT-737, and the drug was effective even in cells with a high level of Bcl-2 or Bcl-x_L (Konopleva *et al.*, 2006; van Delft *et al.*, 2006). A1, which ABT-737 also fails to bind, also promoted some resistance (van Delft *et al.*, 2006). Hence ABT-737 should prove effective on its own in tumors with low Mcl-1 and A1, and may prove more widely efficacious when combined with agents that prevent Mcl-1 synthesis, promote its degradation or induce BH3-only proteins that can inactivate it.

BH3 mimetics may also prove to have an important adjunct role in conjunction with conventional therapy. ABT-737 markedly sensitizes cells to diverse chemotherapy agents (Oltersdorf *et al.*, 2005; Chauhan *et al.*, 2006; Konopleva *et al.*, 2006; van Delft *et al.*, 2006), as does another compound (A-385358) that predominately interacts with Bcl-x_L (Shoemaker *et al.*, 2006). Conceivably, inclusion of the BH3 mimetic might allow lower doses of the conventional agent or more durable responses, unless substantial adverse effects arise. Another potential application is in imatinib-resistant CML. Bim and Bad are important mediators of imatinib action, but their activation was reduced in cells from some patients who were refractory to the drug (Kuroda *et al.*, 2006). Interestingly, however, the resistance to apoptosis caused by loss of Bim could be overcome by co-treatment with ABT-737, which effectively supplanted its role. Thus, ABT-737 might render imatinib even more effective in some patients.

Collectively, all these preclinical findings with ABT-737 provide strong proof of principle that targeting Bcl-2 pro-survival proteins will have significant benefit in cancer therapy, and the results of the clinical trials just initiated will be eagerly awaited. Indeed, the Bcl-2-guarded life/death switch may well prove to be the Achilles' heel of many tumors.

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