Pharmacological manipulation of Bcl-2 family members to control cell death

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The commitment to programmed cell death involves complex interactions among pro- and antiapoptotic members of the Bcl-2 family of proteins. The physiological result of a decision by these proteins to undergo cell death is permeabilization of the mitochondrial outer membrane. Pharmacologic manipulation of proteins in this family appears both feasible and efficacious, whether the goal is decreased cell death, as in ischemia of the myocardium or brain, or increased cell death, as in cancer.

The Bcl-2 family of proteins

Bcl-2 was initially cloned from the breakpoint of the t(14;18) chromosomal translocation found in the vast majority of patients with follicular lymphoma, an indolent B cell non-Hodgkin lymphoma (1–3). Expression of Bcl-2 blocked cell death following numerous cell insults (4, 5). As a test of its oncogenic function, a minigene bearing the *bcl-2*–immunoglobulin gene fusion, after a period of follicular hyperplasia (4, 5), induced lymphoma in transgenic mice (6). While previously characterized oncogenes shared the ability to increase cellular proliferation, Bcl-2 established a new class of oncogenes: inhibitors of programmed cell death.

Since the cloning of Bcl-2, an entire family of proteins related by sequence homology and participation in the control of apoptosis has been identified. Certain proteins share Bcl-2's ability to oppose programmed cell death: Bcl-x_L (7), Bcl-w (8), Mcl-1 (9), and Bfl-1 (A1) (10). These proteins share sequence homology in 4 α-helical Bcl-2 homology (BH) regions, BH1–BH4. Bax (11) and Bak (12), which promote cell death, share only the BH1–BH3 domains. Later, a third class of protein was discovered (13). These include Bid, Bad, Bik, Puma, Noxa, Bmf, and Hrk, which are called "BH3-only" proteins and demonstrate homology only in the BH3 region (14, 15). Like Bax and Bak, the proapoptotic BH3-only proteins require an intact BH3 domain to promote apoptosis (14, 16).

Bcl-2 proteins control mitochondrial permeabilization. Complex interactions among Bcl-2 family members govern mitochondrial outer membrane permeabilization (MOMP), the final common endpoint for execution of death signals by the Bcl-2 family (17) (Figure 1). Data show that activation of either Bax or Bak is required for MOMP (18–20), suggesting that Bax and Bak are the effectors in the Bcl-2 family most proximal to MOMP. In healthy cells, inactive Bax monomers reside either in the cytosol or in loose association with the mitochondrial outer membrane (21), while monomeric Bak is inserted in the outer membrane. Activation of Bax and/or Bak is accompanied by an allosteric change detectable by conformation-specific antibodies (22–24). Following activation, Bax inserts into the membrane, Bax and Bak homo-oligomerize, and then MOMP occurs (19, 25–27). Permeabilization releases proapoptotic factors, including cytochrome *c* (28), omi/htra2 (29), Smac/DIABLO (30, 31), endonuclease G (29), and AIF (32), to the cytosol. Released cytochrome *c* participates in a holoenzyme complex with Apaf-1 and caspase-9 that activates effector caspases by cleavage, resulting in widespread proteolysis. An extrinsic pathway of caspase activation initiated by cell surface death receptor signaling, which operates independently of the mitochondrion and Bcl-2 family members, exists but is beyond the scope of this discussion (33). While oligomers of Bax can form pores in artificial membranes that permit the passage of cytochrome *c* (34) or high–molecular weight dextran (35, 36), it is not clear whether activated Bax and/or Bak independently form pores in vivo, or whether Bax and/or Bak cooperate with other factors (17).

Activation of Bax and/or Bak. The mechanism of Bax and/or Bak activation has been controversial. Recent evidence supports activation of Bax and/or Bak via interaction with select BH3-only proteins. Bid protein and BH3 domains from Bid and Bim, but not other BH3-only proteins, induce MOMP in a Bax and/or Bak–dependent fashion and induce Bak and Bax oligomerization (19, 37, 38). Induction of these apoptotic changes requires an intact BH3 domain. The ability of Bid, Bim_S, or their BH3 domains to stimulate Bax oligomerization and pore formation required no other proteins in a defined synthetic liposomal system; this supports a direct interaction (35, 36). It has been hypothesized that Bid performs primarily as an inhibitor of Bcl-2. However, a Bid mutant that lacks the ability to interact with Bcl-2 but maintains interaction with Bax is still potently proapoptotic, which suggests that interaction with Bax and/or Bak is important in Bid's function (14).

Complexes of Bid or Bim with Bax or Bak have been difficult, but not impossible, to isolate (14, 39–41). Interactions between BH3 domains and Bax and/or Bak may be transient, with the BH3 domain leaving after allosteric activation of Bax and/or Bak in a "hit and run" model. While the BH3 domains of Bid and Bim are necessary for interaction with Bax and Bak, their most efficient presentation to Bax and Bak may require conformational changes, post-translational modifications, and/or certain isoforms of the entire protein (39, 40, 42–45). In addition, proteins outside of the Bcl-2 family bind and modulate function of Bax (46–48) and Bak (49).

Bcl-2 blocks Bax and/or Bak–dependent MOMP. Bcl-2 and the related antiapoptotic proteins Bcl-x_L, Mcl-1, Bcl-w, and Bfl-1 inhibit MOMP by binding pro-death family members. Like the proapoptotic multidomain proteins Bax and Bak, the antiapoptotic proteins possess a hydrophobic pocket made from the α-helices BH1, BH2, and BH3, where the hydrophobic face of amphipathic α-helical BH3 domains from proapoptotic members binds

Nonstandard abbreviations used: BH, Bcl-2 homology (domain); MOMP, mitochondrial outer membrane permeabilization.

Conflict of interest: The author has declared that no conflict of interest exists.

Citation for this article: *J. Clin. Invest.* **115**:2648–2655 (2005). doi:10.1172/JCI26250.

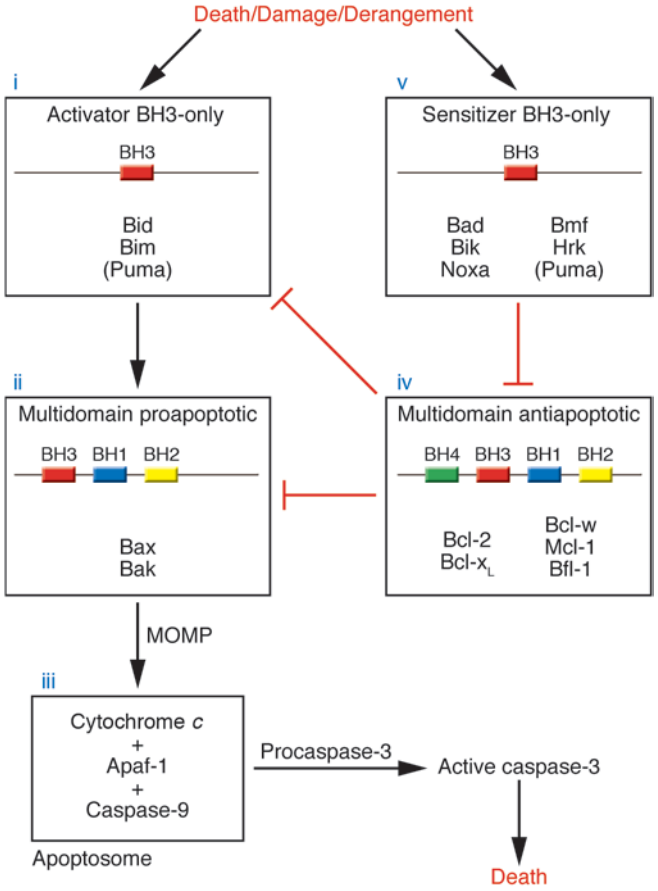

Figure 1

A model of Bcl-2 family member control over programmed cell death. In response to myriad death, damage, or derangement signals, BH3-only family members are activated (i). Activator BH3-only proteins interact with multidomain proapoptotic Bax and/or Bak (Bax/Bak), inducing their oligomerization (ii) and thus resulting in MOMP, release of cytochrome *c*, apoptosome formation, and caspase activation (iii). Bcl-2 and other multidomain antiapoptotic proteins interrupt the death signal by binding and sequestering activator BH3-only family members, and perhaps also Bax/Bak (iv). Bcl-2 antiapoptotic function may be antagonized by the competitive displacement of activator BH3-only molecules by sensitizer BH3-only proteins (v).

(50–53). While earlier work focused on the ability of antiapoptotic proteins to bind Bax or Bak, a Bcl-x_L mutant lacking Bax or Bak binding, but still binding BH3-only proteins, retained the majority of its antiapoptotic function. This result suggests that binding and sequestration of BH3-only family members prior to their interaction with Bax and Bak is also an important function of the antiapoptotic proteins (26, 54). It is also consistent with the finding that Bcl-2 inhibits apoptosis upstream of Bax and/or Bak conformational change, membrane insertion (in the case of Bax), and oligomerization (26, 37, 55, 56). By binding and sequestering activator BH3 domains like Bid and Bim, the antiapoptotic proteins inhibit Bax and/or Bak activation and subsequent MOMP.

Antagonism of Bcl-2 by sensitizer BH3-only proteins. While all BH3-only proteins are proapoptotic, it is likely that only a subset interacts with Bax and Bak. Using a series of BH3 peptides, BH3 domains have been divided into 2 classes: the activators (including Bid and Bim), which can induce Bax and/or Bak oligomerization and MOMP, and the sensitizers, which cannot (37). The ability of p53 to activate Bax suggests there may be other "cryptic" activator molecules outside the Bcl-2 family (57). The sensitizer BH3 domains interact with the antiapoptotic molecules and only indirectly induce Bax and/or Bak activation by competitive displacement of activator BH3 proteins from the Bcl-2–binding cleft (37). These so-called sensitizer BH3 domains are thus prototypes of selective inhibitors of the antiapoptotic proteins (see below) (37, 58). Binding of a given BH3 protein to antiapoptotic proteins is not necessarily promiscuous. For instance, while BH3 domains from Bid, Bim, and Puma interact with all of the antiapoptotic proteins tested, the remainder interact only with select antiapoptotic partners, suggesting that antiapoptotic proteins have biophysically distinct binding pockets (36, 37, 59, 60). In theory, therefore, individual antiapoptotic family members can be selectively targeted by small molecules that mimic sensitizer BH3 domain behavior.

BH3-only proteins' response to death stimuli. There remains the question of how BH3-only molecules are triggered to respond to death stimuli (61). In some cases, activation of BH3-only molecules is transcriptional. Noxa and Puma are p53-inducible genes that are transcriptionally induced in response to numerous DNA-damaging agents (62–66). While Bim can be transcriptionally regulated (67, 68), its regulation may also depend on cytoskeletal interaction (69) and phosphorylation, which may affect interaction with Bax (40) as well as ubiquitination and proteosomal degradation (70–72). In response to death signals that activate the extrinsic apoptotic pathway, Bid is first cleaved by caspase-8 (42, 43), and then the new amino terminus is myristoylated to facilitate targeting to the mitochondria (44). Bad is controlled, at least in part, by phosphorylation that mediates its sequestration by cytoplasmic 14-3-3 protein (73). Interaction of Bad with members of the glycolytic pathway suggests a role for Bad in glucose homeostasis (74). It appears that BH3-only family members serve individual but overlapping roles in sensing different types of cellular derangement and communicating these to the core death pathway.

To summarize, conduction of a death signal by the Bcl-2 family members begins with activation of the BH3-only proteins, which act as sentinels of myriad damage signals. Activator BH3 domains then trigger allosteric activation of Bax and/or Bak, which oligomerize at the mitochondria, inducing MOMP. Proapoptotic factors are released, including cytochrome *c*, which forms a holoenzyme complex with Apaf-1 and caspase-9, termed the apoptosome. This complex then catalytically cleaves effector caspases like caspase-3, resulting in widespread proteolysis and commitment to cell death. This pathway can be interrupted by Bcl-2 and related antiapoptotic proteins that bind and sequester the activator BH3 molecules. Sensitizer BH3-only molecules can further assist the progression of a death signal by competitive displacement of activator BH3 signals from the Bcl-2 pocket (Figure 1).

Alternative models of the control of apoptosis by Bcl-2 family members exist. In one, Bcl-2 regulates caspases, directly or via an as-yet undiscovered mammalian adaptor (13, 61, 75). In another, Bcl-2 tonically inhibits oligomerization of a Bax and/or Bak that is ready to induce MOMP without BH3 interaction (76). In a variation of this model, combinations of particular Bcl-2–like proteins must be neutralized by BH3 ligands to allow Bax and/or Bak activation (60). In another, Bax and/or Bak inhibit a dominantly acting Bcl-2 to induce death (13). However, recent results that emphasize the centrality of Bax and Bak (18, 20) and their activation by activator BH3 domains (19, 26, 35–37) to MOMP

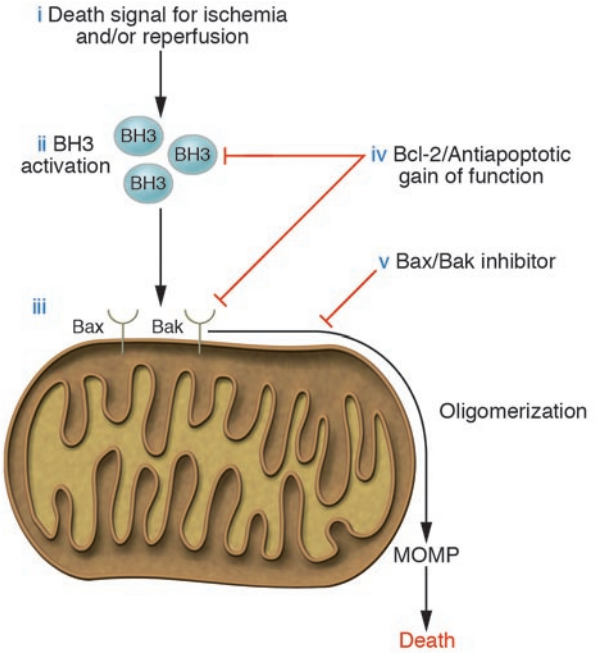

Figure 2
Model of interventions to reduce ischemic and ischemia/reperfusion injury. (i and ii) Following ischemia or ischemia/reperfusion, cell death signals are initiated (i) and conducted to the intrinsic pathway via activated BH3-only family members (ii). (iii) These BH3 family members interact with Bax/Bak, inducing oligomerization, MOMP, and commitment to cell death. (iv) Treatment with a viral vector expressing Bcl-2 or other antiapoptotic gain of function might prevent BH3-only activation of Bax/Bak and/or oligomerization of Bax/Bak. (v) Treatment with a Bax/Bak inhibitor might prevent Bax/Bak induction of MOMP.

may call into question the consistency of the above alternatives with mounting experimental data.

Diseases of excessive cell death

Bcl-2 proteins in acute ischemic diseases. Myocardial ischemia and cerebral ischemia are 2 of the 3 leading causes of death in the developed world. In acute ischemia, sudden loss of blood perfusion, and/or the acute restoration of perfusion, can result in extensive death in the cells of the supplied organ, as in stroke or acute myocardial infarction. In mouse models of cerebral ischemia as well as ischemia/reperfusion injury, loss of proapoptotic Bax or Bid and gain of antiapoptotic Bcl-2 function all reduced infarct size (77, 78). In murine models of cardiac ischemia/reperfusion, interference with Bax activation, genetic Bax loss-of-function, or overexpression of Bcl-2 attenuated apoptosis and reduced infarct size, while reduction of Bcl-2 levels via antisense oligonucleotides eliminated adaptive protection from injury (79–82). These studies paint a consistent picture of Bcl-2 family members controlling cell fate during ischemia and reperfusion in the myocardium and in the brain. Inhibition of the intrinsic apoptotic pathway by Bcl-2 overexpression also attenuates the phenotype in animal models of chronic neurodegenerative disease and cardiomyopathy (83–85).

Effective pharmacologic intervention would seem to require either antiapoptotic gain of function or proapoptotic loss of function (Figure 2). In an effort to use the former strategy, viral delivery of a Bcl-2 transgene to cerebral infarcts decreased infarct size (86). Pharmacologic inhibition of Bax has been reported using an assay of Bax-induced MOMP triggered by recombinant Bid (87). Whether such molecules are safe and effective in vivo remains to be reported. A pentapeptide derived from the Bax-modulating Ku70 protein also inhibits Bax-dependent apoptosis (88). In an attempt to block signaling further upstream, a class of small molecules have been described that bind to Bid and inhibit its activation of Bax in mitochondrial assays (89).

In general, the field of therapeutic apoptosis inhibition by manipulation of Bcl-2 family members is young, but mechanistic studies suggest that such a strategy may be useful and feasible. There are many additional chronic degenerative diseases involving apoptosis whose patients might benefit from apoptotic inhibition. Whether therapeutic long-term inhibition of apoptosis might predispose to cancer, as suggested by numerous mouse models, and whether such deleterious side effects might be overcome by pulsatile dosing schedules, remain to be seen.

Diseases characterized by deficient death

Bcl-2 family proteins in cancer. The third of the 3 leading causes of mortality in the developed world is cancer, characterized by a failure of programmed cell death (90). While Bcl-2 was initially identified as an oncogene in follicular lymphoma, its expression has been identified in many cancers, including melanoma, myeloma, small-cell lung cancer, and prostate and acute leukemias (58). Expression of other antiapoptotic proteins has been detected in many cancers, including Bfl-1 in diffuse large-cell lymphoma (91), Mcl-1 in myeloma (92), and Bcl-x_L in lung adenocarcinoma (93). The oncogenic EBV and human herpes virus-8 (HHV-8; also known as Kaposi sarcoma herpes virus) encode Bcl-2 homologs that oppose cell death from multiple stimuli, analogous to Bcl-2 (94, 95). EBV has been implicated in the causation of HIV-related lymphoma, Burkitt lymphoma, nasopharyngeal cancer, and post-transplantation lymphomas, and HHV-8 in the causation of Kaposi sarcoma, Castleman disease, and body cavity lymphomas. Blocking the intrinsic pathway to programmed cell death is apparently important enough in viral infection and perhaps oncogenesis for evolutionary selection for Bcl-2 homologs.

While it seems clear that Bcl-2 can be important in oncogenesis, the essential therapeutic question is whether Bcl-2 is necessary for tumor maintenance. To formally test this question requires the establishing of a tumor that expresses Bcl-2 followed by the induction of loss of Bcl-2 function. To this end, a mouse model of leukemia was generated that contained a Bcl-2 transgene that could be silenced by doxycycline administration (96). To accelerate oncogenesis, mice bearing the conditional Bcl-2 expression system were bred with those bearing the Eμ-myc transgene. Mice coexpressing both c-myc and Bcl-2 developed a B-lymphoblastic leukemia in the first days of life (97). After diagnosis of leukemia, doxycycline was administered to turn off Bcl-2, and a rapid drop in wbc count, from as high as 500,000–1,000,000 cells per microliter to the normal range of 5,000–10,000 cells per microliter, was seen in a matter of days. Bcl-2 downregulation also fostered significantly longer survival (96). This in vivo experiment established the proof of principle that under certain conditions, correction of an apoptotic defect by itself could be lethal to cancer cells, and thus that Bcl-2 was a valid target for cancer therapy. An implication is that Bcl-2 antagonists may have the potential to be efficacious in cancer therapy, even as single agents.

Peptide-based Bcl-2 antagonist compounds

If Bcl-2 is a valid therapeutic target, how might it be antagonized? Studies suggest that a molecule behaving like a sensitizer BH3

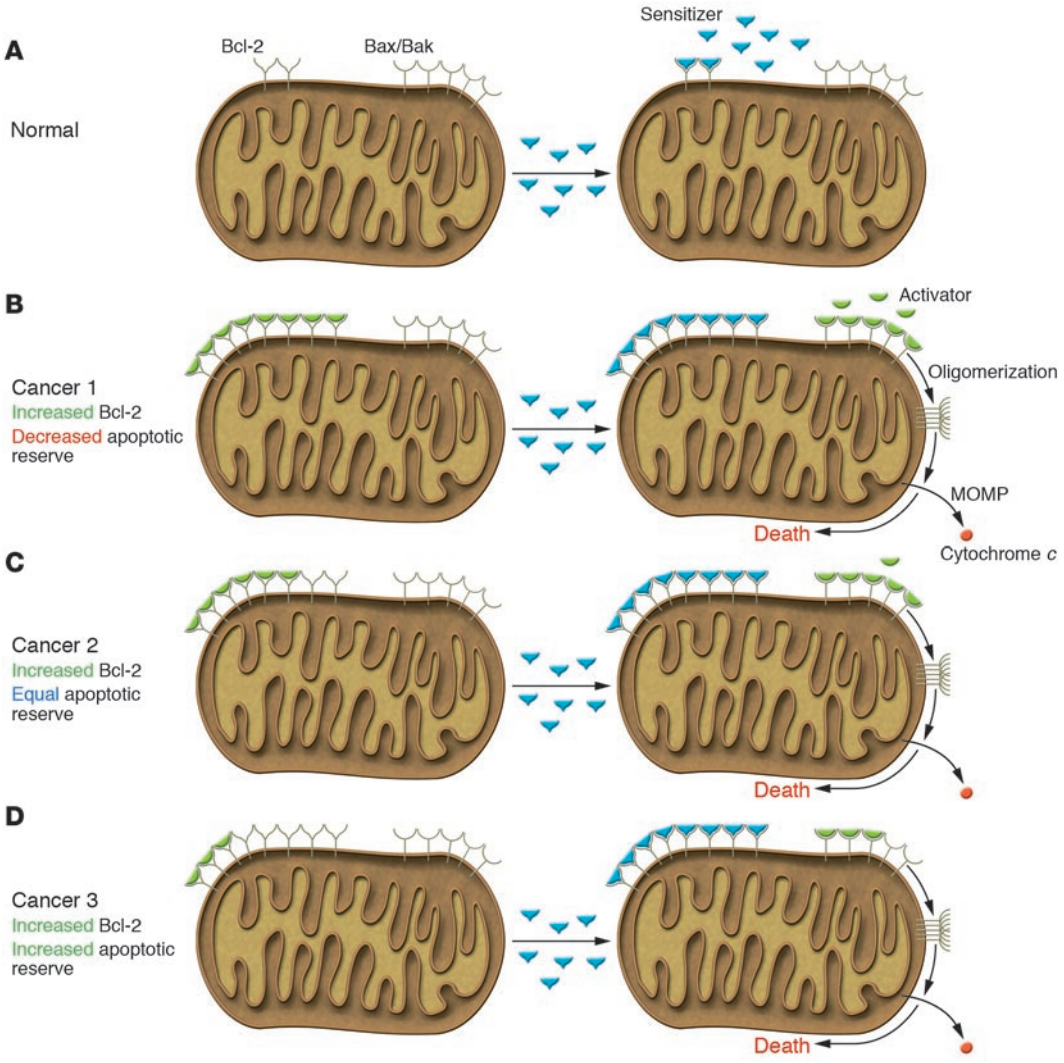

Figure 3
Model for targeting cancer cells with sensitizer BH3 mimetics. (**A**) Mitochondrion from a normal cell has some Bax/Bak and Bcl-2. Bcl-2 is unoccupied; normal cell behavior is provoking no death signals. (**B**–**D**) Mitochondria from cancer cells have equal Bax/Bak and overexpress Bcl-2 in this model. Antiapoptotic reserve is defined as the number of unoccupied antiapoptotic Bcl-2 family member binding pockets per cell. Compared with normal mitochondria, those that overexpress Bcl-2 may provide decreased (**B**), equal (**C**), or increased (**D**) antiapoptotic reserve. Because of genomic instability, oncogene activation, cell cycle checkpoint violation, or perhaps cancer-specific response to cytotoxic chemotherapy, activator BH3 domains have been triggered and are sequestered by Bcl-2. After exposure to a sensitizer BH3 mimetic (a protein, peptide, or small molecule), activator BH3 domains are displaced from cancer cells, but not normal cells, activating Bax/Bak and allowing selective cancer cell killing, perhaps even as a single agent. It can be seen why sensitizer mimetics might offer a greater therapeutic window than an activator, as an activator molecule would provide selective killing only at low doses and only for cancer cells in condition 1 (Cancer 1). At higher doses, or if the cancer cells were in condition 2 or 3, there would be killing of normal and cancer cells. It is unclear whether activator- or sensitizer-type BH3-only family members predominate in the response to conventional chemotherapy agents, and it is likely that a mixture is present. These models also speculate why certain cancers, such as follicular lymphoma and chronic lymphocytic leukemia, despite expressing higher levels of Bcl-2, are more prone to apoptosis than normal cells after DNA-damaging chemotherapy.

domain peptide, binding with high affinity to Bcl-2's pocket, would act as a competitive inhibitor of Bcl-2 function (37), which might induce selective killing of cancer cells (96) (Figure 3). Unmodified BH3 domain peptides are cell impermeant, so the problem of intracellular delivery must first be overcome. It has been shown that tagging peptides with a poly-D-arginine tag facilitates cell internalization in vitro and in vivo (98). We linked BH3 peptides from Bad, Bid, and a double point mutant of Bid to an N-terminal poly-D-arginine octomer. While r8BidBH3 killed a human leukemia cell line that expresses Bcl-2, the r8BidBH3 double point mutant did not. Furthermore, 10 μM of the r8BadBH3 peptide, which caused no apoptosis on its own, increased the killing induced by the r8BidBH3 peptide. These results suggest that the moiety did indeed facilitate internalization, and that an intact BH3 domain was necessary for killing (37). In a separate study, a 27–amino acid peptide derived from the BH3 domain of Bad was linked to decanoic acid to facilitate intracellular entry (99). This compound (cpm-1285), but not a peptide bearing a point mutation at a residue necessary

for BH3 function, induced apoptosis in a Bcl-2–expressing human myeloid leukemia line, HL-60. Furthermore, immunodeficient mice injected with HL-60 cells survived longer when treated with cpm-1285. Though intriguing, these 2 studies do not conclusively demonstrate the mechanism of action of the peptide derivatives, and it remains possible that some of the cytotoxic effects were independent of direct interaction with Bcl-2 family members.

Highlighting the difficulty in interpreting results of studies using peptides linked to cell internalization moieties, Schimmer and coworkers showed that the BH3 domain of Bad, linked to the Antennapedia internalization sequence, had considerable off-target toxicity (100). This toxicity was dependent on the presence of an α-helix, but independent of the Bcl-2 pathway. The compound was toxic to a wide variety of cells, including yeast, wherein Bcl-2 family members have yet to be identified. Others have demonstrated that BH3 peptides derived from Bax and Bcl-2 linked to an Antennapedia internalization sequence induce MOMP and apoptosis (101). These effects are impaired by neither Bcl-2 nor Bcl-x_L expression. It may be that these effects are due again to a nonspecific membrane disruption rather than to interaction with the Bcl-2 family pathway. These nonspecific toxicities may be linked to the ability of amphipathic α-helices, especially when positively charged, to interact with negatively charged mitochondrial membranes. Such interactions may disrupt the lipid matrix and membrane barrier function of biological membranes independent of Bcl-2 family protein interaction. In summary, interpretation of cell killing by BH3 peptides linked to internalization moieties is blurred by the possibility of nonspecific killing due to intrinsic biophysical properties. Further pharmaceutical development of such molecules would require considerable attention to reducing this toxicity, which would likely affect normal as well as cancer cells.

Stabilizing the α-helical conformation of BH3 peptides. Others have tried to improve peptide function by stabilizing the α-helical conformation of BH3 peptides, which generally show less than 25% α-helicity in aqueous solution. Grafting a Bak BH3 domain to a helix-stabilizing miniprotein improved affinity for Bcl-2 (102). Synthesizing BH3 peptide analogs with covalent molecular bridges also stabilized the α-helical conformation (103). Perhaps the most dramatic example of the potential of α-helix stabilization was provided by a Bid BH3 peptide stabilized by an all-hydrocarbon "staple" (104). This modification enhanced α-helicity, affinity for Bcl-2, cell entry, protease resistance, and leukemia cell line toxicity in vitro and in vivo. Mice bearing leukemia cell line xenografts enjoyed statistically significant survival improvement of 6 days; treatment duration was limited to 7 days by sufficiency of material. Normal tissues seemed unaffected as measured by histological analysis. It is important to note that this molecule does not behave as a selective Bcl-2 antagonist. As expected, since it was modeled on a Bid peptide previously shown to be an activator (37), the compound was able to directly induce cytochrome *c* release in a Bak-dependent fashion in vitro. While this molecule behaved as an activator in vitro, it was still able to exploit an apparent therapeutic window between the tumor xenograft and the normal tissues (Figure 3). Subsequent attempts using sensitizer BH3-based compounds may provide an even greater therapeutic window.

Small-molecule Bcl-2 antagonists

Small molecules that bind to antiapoptotic Bcl-2 family members have been identified using structure-based computer screening. The molecules isolated displaced the Bak BH3 peptide from Bcl-2 with an IC_{50} of 1–14 μM. The K_d for the Bak BH3 peptide is approximately 200 nM, which suggests that the K_d for binding of these molecules to Bcl-2 would be significantly higher, likely greater than 1 μM. One of these molecules proved toxic to 4 cell lines tested at concentrations of 10–20 μM. Toxicity correlated with amount of Bcl-2 expressed (105, 106).

Screens of chemical libraries have also been used. Degterev et al. identified 2 molecules out of 16,320 screened that disrupt a Bcl-x_L/Bak BH3 complex (107). Toxicity to a leukemia cell line was observed in the 10- to 90-μM range. Tetrocarcin A, derived from *Actinomyces*, was isolated from a screen of a library of natural products for its ability to counteract Bcl-2 protection of anti-Fas/cycloheximide–treated HeLa cells at concentrations in the micromolar range (108). Binding assays were not reported. Antimycin A, an antibacterial agent with antitumor properties in experimental systems, was identified from a screen for inhibitors of mitochondrial respiration in mammalian cells (109). Further characterization demonstrated antimycin A's interaction with Bcl-2 and Bcl-x_L. Increasing cellular levels of Bcl-x_L correlated with increasing toxicity of antimycin A, a known inhibitor of electron transport at mitochondrial respiratory chain complex III. A methoxy derivative of antimycin A binds to Bcl-2 with a K_d of 0.82 μM (110).

Kutzki et al. used a nonpeptide terphenyl scaffold to design molecules that mimic the binding of BH3 domains in the hydrophobic pocket of Bcl-x_L, one of which had a K_d as low as 114 nM (111). An investigation of the properties of extracts of green tea revealed, by NMR spectroscopy, that certain polyphenols containing a gallate group were able to bind to Bcl-x_L (112). In addition, these compounds displaced a BH3 domain from Bcl-x_L and Bcl-2 in the submicromolar range. A screen of a small library of natural products identified 2 molecules, purpurogallin and gossypol, that inhibit binding of a BH3 domain (resembling that of human Bad) to Bcl-x_L (113). Chemical modification of purpurogallin did not lower the IC_{50} of peptide displacement below 2.2 μM of the parental compound. A racemic mixture of the (+) and (–) isomers of gossypol displaced the BH3 peptide with an IC_{50} of 0.5 μM. The fact that gossypol kills HeLa cells less efficiently when they overexpress Bcl-x_L is taken as evidence that gossypol targets Bcl-x_L. Removal of 2 aldehyde groups from gossypol was performed after molecular modeling suggested that this might reduce steric hindrance in binding the hydrophobic pocket of Bcl-x_L (114). The so-named apogossypol, however, demonstrated inferior binding to Bcl-2 family members.

Validation of antagonists of antiapoptotic family members

Many of the studies cited above provide solid evidence that a particular molecule binds with reasonable affinity (K_d roughly 1 μM or less) to Bcl-2 or Bcl-x_L. Mechanistic evidence demonstrating that killing of living cells depends on specific targeting of Bcl-2 or Bcl-x_L is generally lacking, however. Studies lacking a defined Bcl-2–dependent system can be difficult to interpret. This is evident in that different studies have been interpreted to show, variously, that (a) the compound is working specifically via Bcl-2 or Bcl-x_L inhibition because cells expressing Bcl-2 or Bcl-x_L are *more* sensitive (106, 109) or (b) the compound is working specifically via Bcl-2 or Bcl-x_L inhibition because cells expressing Bcl-2 or Bcl-x_L are *less* sensitive to compound treatment (113). While, in fact, either statement is potentially true of selective Bcl-2 inhibition, detailed, controlled understanding of the cellular context is necessary for proper interpretation.

The essential problem of assaying Bcl-2 function is that the endpoint of cell death is shared by the targeting of countless cellular pathways, and Bcl-2 lacks quantifiable enzymatic activity. Some researchers find the use of defined Bcl-2–dependent systems indispensable for demonstration of specific Bcl-2 inhibition. One useful example is the murine FL5.12 cell line, which is dependent on IL-3 for survival. Programmed cell death following IL-3 removal is prevented by overexpression of a human Bcl-2 transgene (5). Therefore, the IL-3–starved Bcl-2–overexpressing FL5.12 cell is a model of dependence on Bcl-2 for survival. A compound working selectively via Bcl-2 inhibition should kill IL-3–starved, but not unstarved, Bcl-2–overexpressing cells; critically, the parental FL5.12 cells should also be spared death following treatment (115). Testing can be extended to isolated mitochondria, with cytochrome *c* release as the readout of MOMP (37). Peptides derived from the BH3 domains of Bad or Noxa, while cell impermeant, may then be used as validated positive and negative controls of Bcl-2 antagonism. Insensitivity of mitochondria and cells deficient in both Bax and Bak to compound treatment would provide further support for the test compound's operating through the mitochondrial pathway controlled by Bcl-2 family members (20, 37).

Bcl-2 antagonists in clinical development

Antisense strategy. Oblimersen (Genasense; Genta Inc.) is an 18-mer phosphorothioated oligonucleotide directed against the first 6 codons of the human Bcl-2 open reading frame (116). It has advanced through clinical trials with tolerable side effects, mainly thrombocytopenia, fatigue, back pain, weight loss, and dehydration (117). Demonstration of efficacy has been difficult. In a randomized phase III study of patients with metastatic melanoma, treatment with dacarbazine and oblimersen showed no significant benefit in overall survival compared with dacarbazine alone (274 vs. 238 days, $P = 0.18$). There was significant benefit in progression-free survival (74 vs. 49 days, $P = 0.0003$), but since overall survival was the primary endpoint, an FDA panel stated that the clinical benefit was inadequate to offset the increased toxicity.

In chronic lymphocytic leukemia, despite early problems with a cytokine release syndrome, a phase III trial showed improved major responses (complete response plus nodular partial responses) in patients treated with oblimersen, fludarabine, and cyclophosphamide compared with those treated with fludarabine and cyclophosphamide alone (16% vs. 7%, $P = 0.039$). A phase III trial in myeloma in which oblimersen plus high dexamethasone was compared with dexamethasone alone failed to meet its primary endpoint, which was time to disease progression. Furthermore, in another myeloma trial, response to oblimersen did not correlate with reduction in Bcl-2 protein levels.

In general, efficacy has not been overwhelming, and FDA approval of oblimersen as an anticancer agent remains in doubt. There are several reasons why this does not necessarily augur poorly for strategies targeting Bcl-2 in general. First, the cellular effects of the lowering of Bcl-2 levels by antisense oligonucelotides are likely very different from the cellular effects of functional antagonism of the protein and include possible undesirable coregulation of other Bcl-2 family members (118). Furthermore, the reductions in Bcl-2 protein levels tend to be modest, in the 10–50% range. Finally, oblimersen likely has immune system effects via its 2 CpG dinucleotides. While potentially beneficial, these may well limit its maximum tolerated dose, because of side effects including cytokine release syndrome.

Competitive antagonists of Bcl-2. Other compounds in clinical development are small molecules that bind the hydrophobic pocket of Bcl-2 analogously to our sensitizer BH3 peptides. The biotechnology company Gemin X has isolated a compound (GX01) from a high-throughput screen of chemical libraries, reported (in abstract form) to possess the ability to bind Bcl-2 and Bcl-x_L and displace BH3 domains from their binding pockets (119). There is little public information regarding its mechanism of action in living cells. It is currently in phase I clinical trials in both chronic lymphocytic leukemia (at UCSD) and solid tumors (at Georgetown University). Ascenta Therapeutics has an orally administered gossypol derivative in a phase I cancer trial. There is little publicly available information on this compound.

Using a strategy of high-throughput screening combined with iterative modulation of chemical structure based on NMR, Abbott Laboratories has developed compounds reported to displace BH3 domains from Bcl-2, Bcl-x_L, and Bcl-w with an IC_{50} of no more than 1 nM. One lead molecule, ABT-737, is reported to have significant activity in mouse xenograft models of lung cancer and lymphoma (115). This series of compounds has not yet entered clinical trials.

Conclusions

Our current understanding of the mechanisms by which Bcl-2 family members control commitment to cell death gives good theoretical backing to strategies aimed at manipulating this system for clinical benefit. Studies presented above support both the feasibility and the utility of targeting pro- and antiapoptotic proteins. Clinical trials of Bcl-2 antagonists are under way, with more to be expected in the next few years. Given that different antiapoptotic proteins demonstrate distinct binding specificities, it seems likely that in the future the other antiapoptotic proteins might also be individually targeted pharmacologically. Twenty years after the cloning of Bcl-2, we are starting to reap the translational harvest of much fundamental research into the molecular mechanisms controlling apoptosis.

Acknowledgments

A. Letai is supported by NIH grant K08 CA10254, the Claudia Adams Barr Foundation, and the Richard and Susan Smith Family Foundation (Chestnut Hill, Massachusetts, USA).

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