

Mitochondria primed by death signals determine cellular addiction to antiapoptotic BCL-2 family members

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Summary

We show that the antiapoptotic proteins BCL-2, BCL-XL, MCL-1, BFL-1, and BCL-w each bear a unique pattern of interaction with a panel of peptides derived from BH3 domains of BH3-only proteins. Cellular dependence on an antiapoptotic protein for survival can be decoded based on the pattern of mitochondrial sensitivity to this peptide panel, a strategy that we call BH3 profiling. Dependence on antiapoptotic proteins correlates with sequestration of activator BH3-only proteins like BID or BIM by antiapoptotic proteins. Sensitivity to the cell-permeable BCL-2 antagonist ABT-737 is also related to priming of BCL-2 by activator BH3-only molecules. Our data allow us to distinguish a cellular state we call "primed for death," which can be determined by BH3 profiling and which correlates with dependence on antiapoptotic family members for survival.

Introduction

The BCL-2 family of proteins contains key regulators of the mitochondrial (also called "intrinsic") pathway of apoptosis (Danial and Korsmeyer, 2004). The family may be subdivided into three main groups based on regions of BCL-2 homology (BH domains) and function: multidomain antiapoptotic (BCL-2, BCL-XL, BCL-w, MCL-1, BFL-1/A1), multidomain proapoptotic (BAX, BAK), and BH3-only proapoptotic (BID, BIM, BAD, BIK, NOXA, PUMA, BMF, HRK). Proapoptotic function of BH3-only proteins requires BAX or BAK (Cheng et al., 2001; Wei et al., 2001; Zong et al., 2001) and an intact BH3 domain (Chittenden et al., 1995; O'Connor et al., 1998; Wang et al., 1996; Zha et al., 1997), the only BCL-2 homology (BH) region this class of protein possesses. The BH3 domain is an amphipathic α helix that interacts with multidomain family members via the hydrophobic cleft formed by their BH1, BH2, and BH3 domains (Cheng et al., 1996; Kelekar et al., 1997; Kelekar and Thompson, 1998; Muchmore et al., 1996; Sattler et al., 1997). BH3-only family members serve as sentinels for cellular derangement primarily by modulating function of the multidomain proteins (Huang and Strasser, 2000; Kelekar and Thompson, 1998; Wei et al., 2000). In response to a wide variety of damage signals, including DNA damage, growth factor withdrawal, or oncogene

activation, BH3-only family members are activated by transcription or posttranslational modification (Nakano and Voutsden, 2001; Oda et al., 2000; Puthalakath et al., 1999; Zha et al., 1996). Certain of these proteins (which we named activators, including BID and BIM [Letai et al., 2002]) induce the oligomerization of BAX and/or BAK resulting in mitochondrial outer membrane permeabilization (MOMP) (Cartron et al., 2004; Desagher et al., 1999; Kuwana et al., 2005; Kuwana et al., 2002; Letai et al., 2002; Luo et al., 1998; Marani et al., 2002; Wei et al., 2000), allowing the release of proapoptotic factors including SMAC, AIF, and cytochrome c into the cytoplasm (Wang, 2001). Cytochrome c forms a complex with APAF-1 and caspase-9 known as the apoptosome; this holoenzyme then cleaves and activates caspase-3, resulting in widespread proteolysis and cell death.

Antiapoptotic family members prevent death by interrupting signaling upstream of BAX/BAK oligomerization, largely by binding and sequestering activator BH3 domains and preventing their interaction with BAX/BAK (Cheng et al., 1996, 2001; Kuwana et al., 2005; Letai et al., 2002). Another class of BH3-only proteins, which we term sensitizers (Letai et al., 2002), induce BAX/BAK oligomerization indirectly, by binding antiapoptotic proteins and displacing activator BH3-only proteins. Another perspective suggests that interactions among BH3-only proteins and BAX/BAK, if they occur at all, are of little

SIGNIFICANCE

With the advent of effective antagonists of antiapoptotic protein BCL-2, it is vital to understand the mechanism underlying cellular "addiction" to antiapoptotic proteins in the BCL-2 family. Using a panel of peptides that selectively antagonize the individual BCL-2 family members BCL-2, BCL-XL, BCL-w, MCL-1, and BFL-1, we show that cellular "addiction" to individual antiapoptotic proteins may be diagnosed based on mitochondrial response to these peptides. We show that not all cells are sensitive to antagonism of antiapoptotic proteins. Sensitive cells are "primed for death" with death signals carried by a select subset of proapoptotic proteins of the BCL-2 family. Some cancer cells may be tonically primed for death, and thus selectively susceptible to agents that provoke or mimic sensitizer BH3-only domains.

A

BID	EDIIRNIARHLAQVGD SMDR
BIM	MRPEIWIAQELRRIGDEFNA
BIDmut	EDIIRNIARHAAQVGASMDR
BAD	LWAAQRYGRELRMSDEFEGSFKGL
BIK	MEGSDALALRLACIGDEMDV
NOXA A	AELPPEFAAQLRKIGDKVYC
NOXA B	PADLKDECAQLRRIGDKVNL
HRK	SSAAQLTAARLKALGDELHQ
BNIP	VVEGEKEVEALKKSADWVSD
PUMA	EQWAREIGAQLRRMADDLNA
BMF	HQAEVQIARKLQLIADQFHR

B

	BID	BIM	BIDmut		BAD	BIK	NOXA A	NOXA B	HRK	BNIP	PUMA	BMF
BCL-2	66 (6)	<10	-		11 (3)	151 (2)	-	-	-	-	18 (1)	24 (1)
BCL-XL	12 (9)	<10	-		<10	10 (2)	-	-	92 (11)	-	<10	<10
BCL-w	<10	38 (7)	-		60 (19)	17 (12)	-	-	-	-	25 (12)	11 (3)
MCL-1	<10	<10	-		-	109 (33)	19 (2)	28 (3)	-	-	<10	23 (2)
BFL-1	53 (3)	73 (3)	-		-	-	-	-	-	-	59 (11)	-

Figure 1. Binding pattern for BH3 peptide interaction with antiapoptotic BCL-2 family proteins

A: BH3 peptides used in these studies. BIDmut differs from BID by two alanine substitutions.

B: Selective binding between antiapoptotic and BH3-only family members. Dissociation constants for interactions between antiapoptotic BCL-2 family proteins (left) and BH3 domains from BH3-only proteins (top) are shown in nM. Standard deviations of at least three independent measurements are in parentheses. Yellow blocks signify high-affinity binding; blue blocks and minus sign signify no observed binding ($K_d > 2500$ nM). Activators are in purple, and sensitizers in green. Note that, for BCL-2 binding, K_d values are lower than those previously reported (Letai et al., 2002) due to improved purity of GST-BCL-2 protein in this assay.

significance. Rather, apoptosis is triggered by the neutralization of antiapoptotic proteins by BH3-only proteins. In this model, the link between neutralization of antiapoptotic proteins and MOMP remains obscure (Chen et al., 2005; Willis et al., 2005).

It has been postulated that inhibition of apoptosis is a requirement of oncogenesis (Green and Evan, 2002; Hanahan and Weinberg, 2000). In what may be an attempt to meet this requirement, many types of cancer overexpress antiapoptotic BCL-2 family members. Understanding how these proteins function is therefore critical to understanding how cancer cells maintain survival. Here, we systematically investigate how antiapoptotic BCL-2 family members interact with BH3-only family members to control MOMP and commitment to apoptosis. We demonstrate that antiapoptotic proteins show selective affinity for binding BH3 peptides derived from BH3-only proteins. We further show that antagonism of antiapoptotic family members results in MOMP only when the antiapoptotic proteins are “primed” with activator BH3 proteins, validating the critical role of activator BH3 domains in activating BAX/BAK. In cell culture models, we show that activator “priming” can be observed following experimentally induced death signaling, and that such priming confers dependence on antiapoptotic family members. Remarkably, we show that dependence on antiapoptotic BCL-2 family members can be captured functionally by the pattern of mitochondrial sensitivity to sensitizer BH3 domains. Finally, in a previously credentialed model of BCL-2-dependent leukemia, we show that these cancer cells are tonically “primed” with activator BH3 molecules, conferring sensitivity to sensitizers. Hence, we show that oncogene addiction in this model has its correlate in sensitivity to BH3 peptides that bind BCL-2.

Heretofore, two states had been identified with respect to programmed cell death: alive and dead. We distinguish a third state, which we call “primed for death.” Cells in this state require tonic antiapoptotic function for survival. This state can be characterized based on sensitivity to sensitizer BH3 domains and BCL-2 antagonists like ABT-737.

Results

Antiapoptotic proteins demonstrate distinct profiles of binding sensitizer BH3 peptides

To determine selectivity in interactions among antiapoptotic BCL-2 family members and BH3 domains of BH3-only proteins, we used fluorescence polarization binding assays (FPA). Antiapoptotic proteins BCL-2, BCL-XL, MCL-1, BCL-w, and BFL-1 were purified from transfected bacteria as GST fusion proteins. BH3 domains were synthesized as 20–25-mers as shown in Figure 1A. Oligopeptides used for FPA were tagged with an N-terminal FITC moiety. Figure 1B quantitates binding by dissociation constants.

It is immediately notable that the antiapoptotic family members may be distinguished from each other based on affinity for individual BH3 domains. For instance, BCL-XL may be distinguished from BCL-2 and BCL-w by its much greater affinity for HRK BH3. Otherwise, though there are quantitative distinctions among binding patterns of BCL-2, BCL-XL and BCL-w, the qualitative binding patterns are quite similar, suggesting similarity in the hydrophobic binding pockets of these three molecules.

In contrast with this group, MCL-1 does not bind BAD BH3, in agreement with data generated by pull-down (Opferman et al.,

2003), yeast two-hybrid (Leo et al., 1999), and surface plasmon resonance (Chen et al., 2005) assays. Murine NOXA is unique among the known BH3-only proteins in that it possesses two putative BH3 domains (Oda et al., 2000). It is significant that, while the other four proteins interact with neither of the NOXA BH3 domains tested, MCL-1 interacts with both. This suggests that the interaction between NOXA and MCL-1 is indeed biologically significant, congruent with prior findings (Chen et al., 2005). The ability to bind both BH3 domains suggests the possibility of multimeric interactions between MCL-1 and murine NOXA, or alternatively, differential control over exposure of the two BH3 domains in NOXA.

Also distinct is BFL-1. While it binds BID and BIM, it binds only PUMA among the sensitizers tested. It is also notable that the activators BID and BIM BH3 are bound by all of the antiapoptotics tested, distinguishing them from the sensitizers, which, except PUMA, show a more selective pattern of binding. Of additional note is that the BH3 domain obtained from BNIP-3 α binds to none of the proteins tested and does not activate BAX or BAK. While we cannot exclude the possibility that BNIP BH3 interacts with an untested multidomain pro- or antiapoptotic BCL-2 family member, it is also possible that BNIP-3 α does not function as a BH3 family member at all (Ray et al., 2000).

Dependence on individual antiapoptotic proteins may be deduced by pattern of sensitivity to sensitizer BH3 peptides; Inhibition of antiapoptotic protein is insufficient for MOMP unless activator tBID is present

We have previously shown that the BH3 domains of BID and BIM possess the ability to induce BAX and BAK oligomerization and cytochrome c release in a purified mitochondrial system (Letai et al., 2002). We termed this class of BH3 domain “activators.” BH3 domains from BAD and BIK (which we termed “sensitizers”) were unable to induce cytochrome c release on their own. However, when an activator was bound and sequestered by BCL-2, preventing interaction of the activator with BAX or BAK, sensitizers could provoke mitochondrial apoptosis by competitively inhibiting BCL-2’s binding of the activator, freeing the activator to oligomerize BAX or BAK and induce cytochrome c release. Thus, the two sensitizer BH3 domains were shown to be antagonists of BCL-2 antiapoptotic function. The ability to antagonize BCL-2 function correlated with high-affinity binding to BCL-2.

In Figure 1B, the expanded range of BH3 domains tested in the present study demonstrate distinct patterns of binding to antiapoptotic proteins. To test if selective binding corresponded to ability of individual BH3 domains to selectively antagonize antiapoptotic function, we constructed a purified mitochondrial system in which we reconstituted the critical apoptosis decision-making molecular machinery. For the activator function, we used caspase-8 cleaved BID protein, tBID. tBID is an archetypical activator protein, capable of inducing BAX/BAK oligomerization and cytochrome c release in purified mitochondria (Wei et al., 2000) and synthetic liposomes (Kuwana et al., 2002, 2005). tBID’s induction of cytochrome c release and apoptosis requires BAX or BAK (Cheng et al., 2001; Wei et al., 2001). The multidomain proapoptotic function was provided by the BAK that resides in mouse liver mitochondria; mouse liver mitochondria contain no detectable BAX protein (Letai et al., 2002). The dominant antiapoptotic function was provided by

one of the five different recombinant antiapoptotic proteins used in the binding assays. BH3 peptides provided the sensitizer function.

As cytochrome c release is the readout for our system, it was important to test whether our peptides by themselves release cytochrome c in mouse liver mitochondria like activators BID or BIM BH3 (Letai et al., 2002). In Figure 2A, we confirm that none of the sensitizer BH3 peptides by themselves can induce cytochrome c release significantly above background, even at concentrations 10-fold higher than those used in Figures 2B–2F. While we had previously shown this for BAD, BIK, NOXA A, and NOXA B BH3s, this is to our knowledge the first time this finding has been described for the HRK, BNIP, PUMA, and BMF BH3 domains.

In each of the subsequent panels, cytochrome c release by tBID is demonstrated, followed by inhibition of cytochrome c release by addition of either BCL-2 (Figure 2B), BCL-X_L (Figure 2C), BCL-w (Figure 2D), MCL-1 (Figure 2E), or BFL-1 (Figure 2F). We then tested the ability of our panel of BH3 domains to antagonize antiapoptotic protection as measured by cytochrome c release. Remarkably, in each case, the ability to antagonize antiapoptotic function maps to the binding specificities in Figure 1B. This is important confirmation that the binding pattern elucidated in Figure 1B corresponds to biological function.

It is important to emphasize that treatment with sensitizer peptides alone, even those that bind and antagonize all the antiapoptotics tested, such as PUMA BH3, or the combination of NOXA and BAD BH3 is insufficient to cause cytochrome c release (Figure 2A). Furthermore, when the panel of sensitizer BH3 peptides was tested in the presence of the antiapoptotic protein BCL-X_L, there was still no cytochrome c release, formally ruling out the possibility that the BH3 peptides were somehow directly converting antiapoptotic proteins to a proapoptotic function (Figure S1 in the Supplemental Data available with this article online). To induce MOMP and cytochrome c release, there appears to be an absolute requirement for an activator function, here provided by the tBID protein.

These data critically demonstrate that our panel of peptides can determine whether a mitochondrion depends on an antiapoptotic protein to maintain integrity. Furthermore, the identity of the critical antiapoptotic protein can be deduced based on the pattern of sensitivity to our panel of sensitizer BH3 peptides. We call this strategy BH3 profiling.

Sensitizers displace activators from antiapoptotic proteins

Since sensitizer BH3 peptides cannot induce cytochrome c release on their own but can induce cytochrome c release when activator and antiapoptotic proteins are present, in a pattern that mirrors their binding to antiapoptotic proteins, we hypothesized that the sensitizers are displacing activators from the antiapoptotic proteins. As one test of this hypothesis, we tested the ability of sensitizer peptides to displace tBID from antiapoptotic protein Bcl-w. In Figure 3A, tBID is displaced from BCL-w by sensitizer BH3 peptides in a pattern that replicates the pattern in Figure 2D. As an additional test, we examined displacement of the activator BIM BH3 peptide from BCL-2 and MCL-1 by BAD and NOXA BH3 peptides. In Figure 3B, consistent with Figure 1B, BAD BH3 efficiently displaces BIM BH3 from BCL-2, but not MCL-1, whereas NOXA A BH3 efficiently displaces BIM from MCL-1, but not BCL-2. These experiments support the ability of

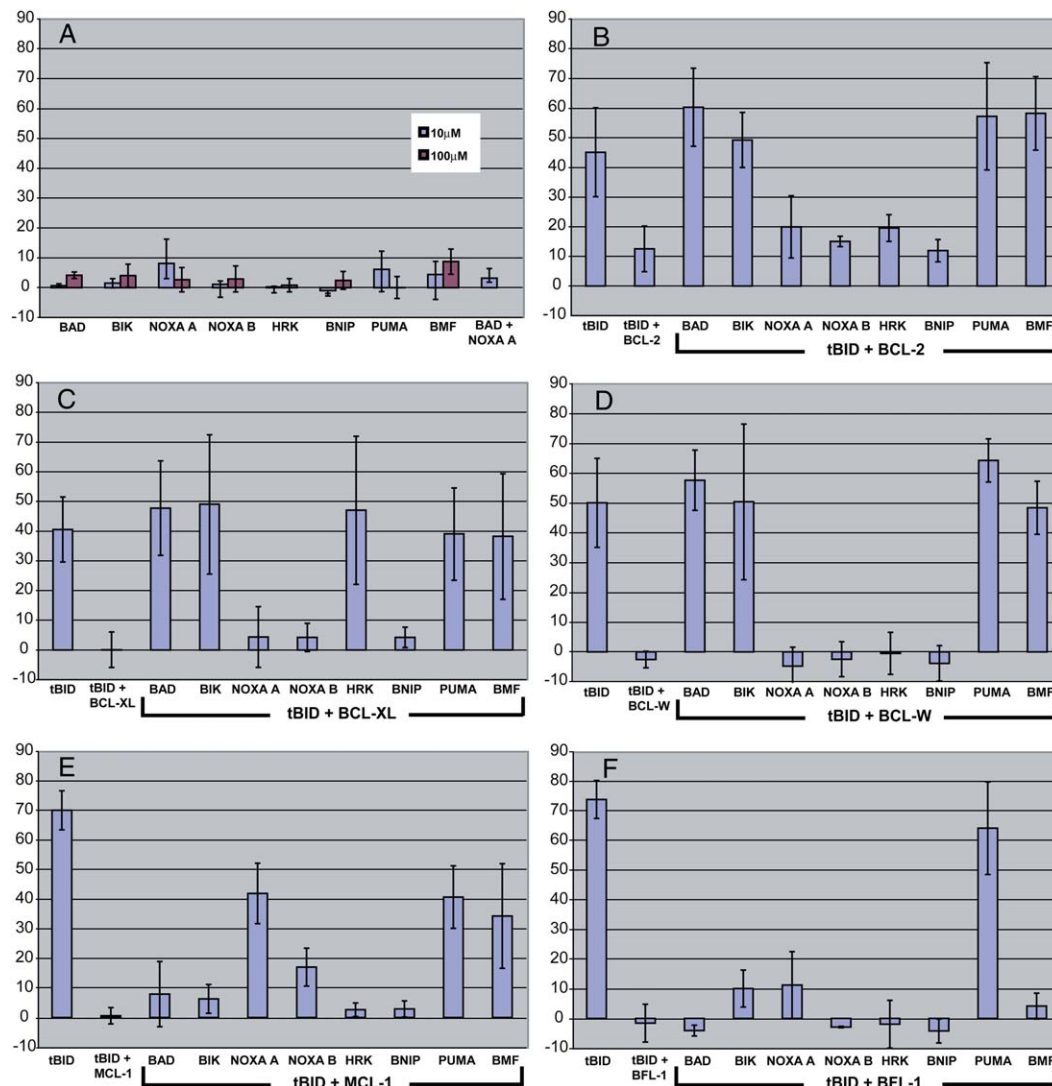


Figure 2. Pattern of antiapoptotic functional antagonism matches binding chart

Mouse liver mitochondria were treated as indicated, and cytochrome c release was measured. Peptide concentrations were 10 μ M unless otherwise indicated; tBID concentration was 13 nM.

A: Panel of sensitizer BH3 peptides induces no cytochrome c release, even at 100 μ M.

B: tBID treatment induces cytochrome c release (first bar), which is inhibited by BCL-2 (1.2 μ M, second bar). The panel of sensitizer BH3 peptides antagonize BCL-2 protection as indicated by restoration of cytochrome c release. Note that pattern of release mirrors pattern of BCL-2 binding in Figure 1B.

C–F: Remaining panels are analogous except that the following antiapoptotic proteins are used: 0.25 μ M BCL-XL (**C**), 6.3 μ M BCL-w (note tBID 43 nM this experiment) (**D**), 1.1 μ M MCL-1 (**E**), 2.4 μ M BFL-1 (**F**). DMSO (1%) is included as a solvent control. Note that, in each case, restoration of cytochrome c release corresponds to a high-affinity interaction in Figure 1B. Average and standard deviation from at least three independent assays performed for each antiapoptotic protein are shown.

sensitizer BH3 peptides to displace activators from the antiapoptotic binding cleft.

A cellular requirement for BCL-2 corresponds to a “BCL-2 pattern” of mitochondrial sensitivity to the sensitizer BH3 panel

In order to test whether mitochondrial dependence on individual antiapoptotic protein function can be correlated with cellular behavior, we investigated cellular models of defined antiapoptotic dependence. We first tested whether a cellular requirement for BCL-2 for survival correlates with the BCL-2 signature of mitochondrial sensitivity to sensitizer BH3 domains found in Figure 2B. The polymorphocytic murine FL5.12 cell line requires

IL-3 to maintain survival. Apoptosis induced by IL-3 withdrawal is inhibited by overexpression of BCL-2 (Figure 4A). Therefore, BCL-2-overexpressing FL5.12 (FL5.12-BCL-2) cells deprived of IL-3 are a model of BCL-2-dependent survival. FL5.12-BCL-2 cells grown in the presence of IL-3 are examples of BCL-2-independent cells.

While the dependence on BCL-2 of IL-3-deprived FL5.12 cells is demonstrated genetically in Figure 4A, we confirmed the dependence using a cell-permeable BCL-2 antagonist. ABT-737 has been shown to antagonize BCL-2 (and BCL-XL and BCL-w) (Oltersdorf et al., 2005). In agreement with the prior report, ABT-737 induced cell death in the IL-3-starved, but not the IL-3-replete BCL-2-protected cells (Figure 4B). Moreover,

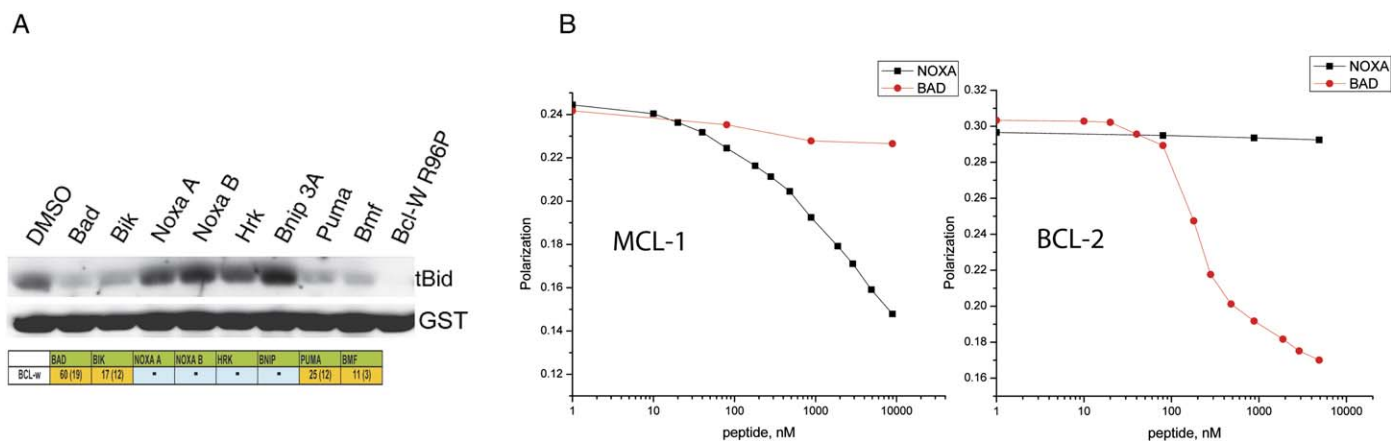


Figure 3. Sensitizer BH3 peptides displace activator BH3 proteins from antiapoptotic proteins

A: GST pull-down assay. GST-BCL-w (or point mutant R96P) was combined with tBid protein and the indicated peptides (10 μ M). The BH1 domain R96P mutant of BCL-w lacks the ability to bind BH3 domains. Proteins bound to glutathione-agarose beads were eluted with glutathione and analyzed by Western blot. For convenience, BCL-w binding pattern from Figure 1B is excerpted below.

B: Displacement of a fluorescein-tagged BIM BH3 peptide from BCL-2 and MCL-1 proteins by BAD and NOXA BH3 peptides by fluorescence depolarization. Shown are representative plots from three independent experiments for each combination.

ABT-737 was nontoxic to the unstressed IL-3-replete wild-type (wt) FL5.12 cells. This cell death was caspase dependent, demonstrating that death occurred using the apoptotic pathway (Figure 4C).

Having credentialed a BCL-2-dependent cellular system, we wanted to test whether this BCL-2 dependence could be isolated at the level of mitochondria. We hypothesized that removal of IL-3 would “load” the BCL-2 on the mitochondria with activator BH3 proteins. We further hypothesized that mitochondria bearing “loaded” BCL-2 would release cytochrome c when treated with sensitizer BH3 peptides that compete for the BCL-2 binding cleft. The interpretation that the IL-3-starved FL5.12-BCL-2 cells were “primed” for death is supported by the rapidity of their death following ABT-737 treatment (Figure S2).

We isolated mitochondria from wt FL5.12 cells and FL5.12-BCL-2 cells in the presence of IL-3, and from FL5.12-BCL-2 cells following 24 hr of IL-3 deprivation. Due to advanced apoptosis, mitochondria could not be isolated in sufficient quantities from wt FL5.12 cells after IL-3 deprivation. In Figure 5A, we show that, while activators BID and BIM potently induce cytochrome c release from mitochondria isolated from wt FL5.12 cells, the remaining sensitizer peptides do not (blue bars). Thus, inhibition of antiapoptotic family members is by itself not sufficient to induce MOMP. Next, BCL-2 overexpression inhibits release induced by 10 μ M BID BH3, but not 10 μ M BIM BH3, in accordance with dose-response curves previously demonstrated (red bars) (Letai et al., 2002). When mitochondria from FL5.12-BCL-2 cells deprived of IL-3 are tested, however, certain sensitizer peptides now demonstrate the ability to induce cytochrome c release (tan bars), and sensitivity to 10 μ M BID BH3 is restored. It is most notable that only those sensitizer peptides with high affinity for BCL-2 cause MOMP. BIK BH3 induces cytochrome c release only at 30 μ M in this setting, consistent with its approximately 10-fold lower affinity than BAD, PUMA, or BMF BH3 for BCL-2. It can be seen, therefore, that cellular BCL-2 dependence can be “diagnosed” from the pattern of mitochondrial sensitivity to our panel of sensitizer BH3 peptides. This dependence can be “diagnosed” whether the activator involved is

recombinant protein, as in Figure 2, or a more complex mix involving more than one molecule, as is likely the case following IL-3 withdrawal. Note that inhibition of BCL-2 alone is not sufficient to induce cytochrome c release, as seen by the failure of all of the sensitizer peptides to induce release in the IL-3-replete FL5.12-BCL-2 mitochondria (Figure 5A). In fact, even the combination of the peptides BAD and NOXA BH3, which provide a broad spectrum of antiapoptotic protein binding, cannot induce cytochrome c release in the absence of an activator molecule (Figure 5B). To induce MOMP, the BCL-2 must first be “primed” by molecules communicating a death signal, generated by IL-3 withdrawal. We found that BCL-2 blocks apoptosis upstream of BAX oligomerization and that BAD BH3 and ABT-737 inhibition of BCL-2 on IL-3-starved mitochondria results in BAX oligomerization (Figure 5C). Therefore, we hypothesized that this death signal might be an activator BH3 protein.

BIM has previously been shown to play a role in death following IL-3 withdrawal in FL5.12 cells (Harada et al., 2004). In Figure 5D, we show that total cellular BIM levels, as well as levels of BIM complexed to BCL-2, dramatically increase following IL-3 withdrawal. It is notable that levels of BCL-2, BAX, and BAK stay roughly constant during the same time period. These results suggest that the activator BIM (and perhaps PUMA) is a dynamic mediator of the death response following IL-3 withdrawal in FL5.12 cells and that it is sequestered to prevent apoptosis. Cells and mitochondria bearing “loaded” BCL-2 are then “addicted” to BCL-2 and die when BCL-2 function is antagonized. Furthermore, cellular BCL-2 addiction can be diagnosed by the pattern of mitochondrial sensitivity to sensitizer BH3 domains.

Our model predicts that BCL-2 acts upstream of BAX activation by intercepting activator BH3 molecules. To test this prediction, in Figure 5E, we performed immunoprecipitation with an antibody that recognizes only the activated form of BAX, which exposes an N terminus epitope (Desagher et al., 1999; Hsu and Youle, 1997). We found that IL-3 withdrawal induced BAX activation in wt FL5.12 cells, while total BAX levels remained constant. However, when BCL-2 protected against death from IL-3 withdrawal, it also prevented BAX conformational change,

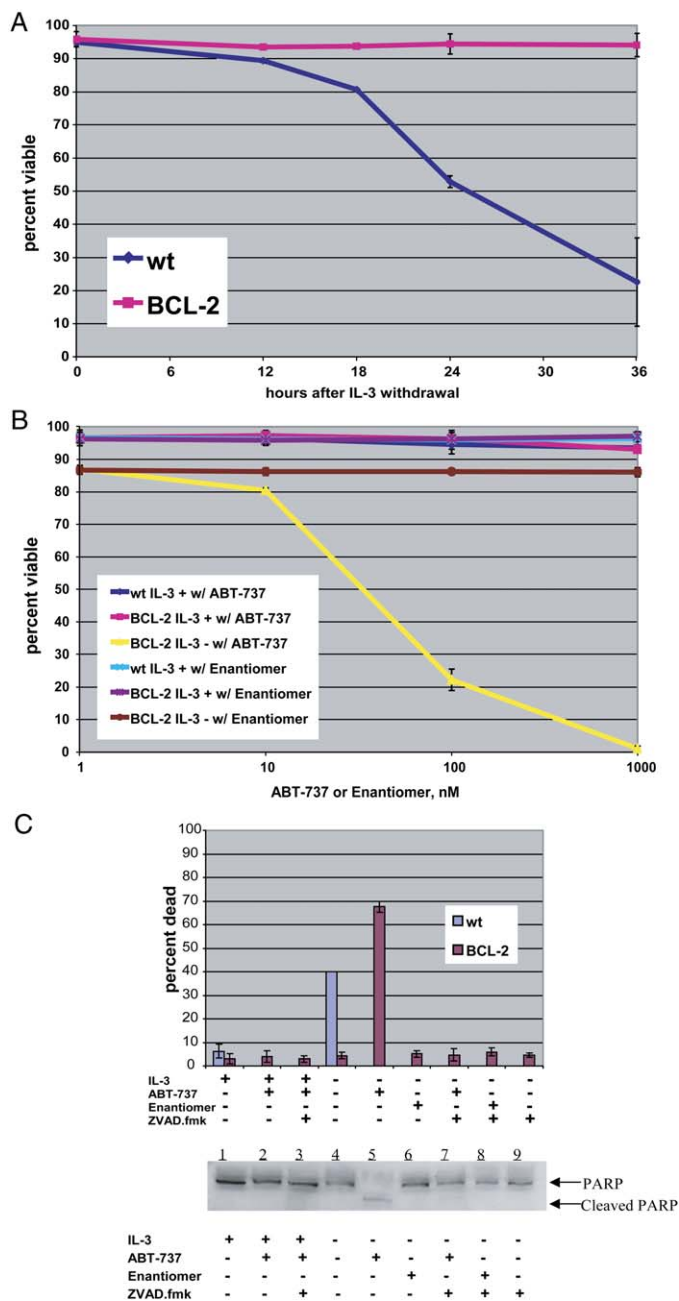


Figure 4. IL-3 withdrawal in BCL-2-protected FL5.12 cells provides a model of BCL-2 dependence

A: Time course of survival following IL-3 withdrawal for wtFL5.12 and FL5.12-BCL-2 cells. Survival was imputed for cells not staining with Annexin V by FACS analysis. Shown are average and standard deviation of three independent experiments.

B: Sensitivity to ABT-737, a BCL-2 antagonist. IL-3- cells were grown in the absence of IL-3 for 24 hr prior to initiation of treatment with compound. All cells were treated with compounds for 24 hr prior to harvest. Viability assayed by absence of Annexin V staining. Shown are average and standard deviation of three independent experiments.

C: Death caused by ABT-737 is caspase dependent. (Top) Death of FL5.12 cells treated as indicated. Shown are average and standard deviation of three independent experiments. (Bottom) PARP cleavage correlates with cell death.

consistent with BCL-2's sequestering activators like BIM prior to their interaction with BAX (compare fourth and eighth lanes). Furthermore, treatment with ABT-737 restored cytochrome c release and BAX activation, consistent with ABT-737 functioning by displacing activators from BCL-2.

BH3 profiling can discriminate MCL-1 cellular dependence from BCL-2 cellular dependence

To test if our model of antiapoptotic "priming" could be extended beyond BCL-2 to other antiapoptotic proteins, we compared the behavior of cells protected by BCL-2 with those protected by MCL-1. The murine hybridoma 2B4 cell line is sensitive to dexamethasone treatment. Overexpression of FLAG-tagged MCL-1 or BCL-2 confers resistance to dexamethasone-induced apoptosis (Figure 6A). Therefore, dexamethasone-treated, FLAG-MCL-1-expressing cells are a model of cellular MCL-1 dependence, while dexamethasone-treated, BCL-2-expressing cells are a model of cellular BCL-2 dependence. Treatment of the MCL-1-protected dexamethasone-treated cells with ABT-737 has no effect, showing that the cells are not dependent on BCL-2 for survival. In stark contrast, 2B4 cells protected from dexamethasone-induced apoptosis by BCL-2 are very sensitive to ABT-737 (Figure 6B).

The cellular data provoke the prediction that mitochondria isolated from 2B4-MCL-1 cells treated with dexamethasone would be sensitive to NOXA and insensitive to BAD BH3, the opposite of the pattern observed with IL-3-starved FL5.12-BCL-2 cells. We isolated mitochondria from dexamethasone-treated and untreated vector-transfected and FLAG-MCL-1-transfected 2B4 cells. Apoptosis was too advanced to permit isolation of mitochondria from dexamethasone-treated vector-transfected 2B4 cells. As can be seen in Figure 6C, only mitochondria isolated from the MCL-1-dependent cells recapitulate an "MCL-1 pattern" of sensitivity to sensitizer BH3 peptides. As with the FL5.12 cells, since sensitizer BH3 peptides cause little cytochrome c release in untreated cells, it is clear that sensitizer BH3 peptide inhibition of MCL-1 (and other antiapoptotic proteins that might be present) is not by itself sufficient to induce apoptosis. An additional death signal (initiated by dexamethasone treatment in this case) is needed to "prime" MCL-1 so that MCL-1 antagonism by sensitizers results in mitochondrial permeabilization. To demonstrate the robustness of this strategy, we also performed BH3 profiling on 2B4 cells treated with dexamethasone, but this time protected with BCL-2. Consistent with our priming model, a BCL-2 pattern is revealed (Figure 6D). Thus, MCL-1 dependence, like BCL-2 dependence, also can be "diagnosed" by mitochondrial sensitivity to the sensitizer BH3 panel.

As with the FL5.12 cells, we investigated whether dexamethasone treatment resulted in increased sequestration of an activator BH3 protein by MCL-1 and BCL-2. In Figure 6E, we show that FLAG-MCL-1 sequesters increased amounts of BIM following the death signaling induced by dexamethasone treatment, as does BCL-2 (Figure 6F). Note that levels of BAX and BAK stay constant during the treatment. Also note that it appears that the small amount of BAX bound to cells before treatment with dexamethasone decreases after treatment. One interpretation is that the BAX is displaced by increased levels of BIM binding to BCL-2. This is significant because it suggests that displacement of BAX from MCL-1 is insufficient to induce MOMP and death.

To further demonstrate that the mitochondrial assays reflect true cellular dependence, we transfected peptides via electroporation into FLAG-MCL-1-transfected 2B4 cells that had been treated with dexamethasone, putatively priming MCL-1 with death signals, carried at least in part by BIM. Supporting the cellular relevance of our mitochondrial BH3 profiling assays, an MCL-1 pattern of response to sensitizer peptides was observed (Figure 6G; compare with Figures 1B, 2E, and 6C).

Dependence on BCL-2 in a leukemia corresponds to mitochondrial sensitivity to sensitizers in a “BCL-2 pattern” and sequestration of BIM

Dependence on antiapoptotic proteins is perhaps of greatest importance in the context of cancer, in which antiapoptotic BCL-2 family proteins are subjects of intense investigation as therapeutic targets. While the concept of oncogene addiction has received attention recently (Jonkers and Berns, 2004; Weinstein, 2002), the molecular details of the addiction to specific oncogenes is poorly understood. We therefore turned to a validated model of oncogene addiction, a BCL-2-dependent murine leukemia, to examine the molecular basis for BCL-2 “addiction.”

We have previously described a mouse acute lymphocytic leukemia model in which *c-myc* is constitutively expressed and human BCL-2 is repressibly expressed. In this model, when BCL-2 transgene expression is eliminated by administration of doxycycline, the leukemic cells undergo apoptosis, resulting in rapid resolution of the leukemia (Letai et al., 2004). This provides us with an ideal in vivo model of a BCL-2-dependent cancer. We wondered if the dependence on BCL-2 was due to a similar mechanism to that of the IL-3-deprived FL5.12-BCL-2 cells—that is, a death signal was being initiated and carried by an activator BH3 molecule, but BCL-2 was binding it and preventing its interaction with multidomain proapoptotic proteins.

We isolated mitochondria from leukemia cells and exposed them to sensitizer BH3 peptides and measured release of cytochrome c. As an internal control, mitochondria were isolated from liver from the leukemic mice in parallel (Figure 7A). The sensitizer BH3 peptides were unable to induce cytochrome c release from nonmalignant hepatocyte mitochondria from the leukemic mice, just as they were unable to induce cytochrome c release from nonmalignant liver mitochondria from normal mice (Figure 2A) or from nonmalignant FL5.12 (Figure 5A) or 2B4 mitochondria (Figure 6C). Intriguingly, certain sensitizer BH3 peptides were capable of inducing near total cytochrome c release from the leukemic mitochondria (Figure 7B). Significantly, the pattern of peptides that induced release corresponded exactly to those peptides that bind with high affinity to BCL-2 (Figure 1B), namely BAD, BIK, PUMA, and BMF. Note that, consistent with its approximately 10-fold lower affinity than BAD BH3 for BCL-2, BIK BH3 requires a 10-fold higher concentration to demonstrate cytochrome c release. A 10-fold increase in NOXA A peptide concentration has no effect, consistent with the extremely low affinity NOXA A has for BCL-2.

These results suggest that, in this leukemia model, death signals are being continually initiated, and BCL-2 is required to sequester the activator BH3 molecule to prevent apoptosis. In contrast to the nonmalignant systems tested above, leukemic cell BCL-2 behaves as if already “primed” with activator protein(s) without any further intervention, such as growth factor

withdrawal or dexamethasone treatment. In Figure 7C, we show that BIM is expressed in the leukemia cells, and it is bound by BCL-2. Supporting the signal importance of BIM in transmitting death signals in this model, BID is also present in the lysate but is not bound by BCL-2. Note that PUMA is also found to be bound by BCL-2, consistent with a report showing that PUMA deficiency could accelerate myc-induced lymphomagenesis (Hemann et al., 2004). Since in our hands the PUMA BH3 lacks the ability to directly activate BAX or BAK, we hypothesize that PUMA is acting as a sensitizer in this context, in effect decreasing the amount of BCL-2 available to bind BIM and possibly BAX or BAK.

If BCL-2 maintains survival of this leukemia cell primarily by sequestering BIM, then one would predict that BIM loss of function could substitute for BCL-2 overexpression to cooperate with *c-myc* in leukemogenesis. In fact, this experiment has already been performed. It was found that BIM deficiency can indeed cooperate with *c-myc* to produce a pre-B lymphocytic leukemia like the one produced here by the cooperation of BCL-2 overexpression with *c-myc* (Egle et al., 2004). These results support a model in which BCL-2 is necessary for survival of our leukemia largely because it is required to sequester BIM, preventing activation of BAX/BAK and subsequent MOMP. The leukemia cells are therefore neither normal and healthy, nor dead, but rather primed for death.

BH3 profiling predicts sensitivity to ABT-737

As another test of the ability of BH3 profiling to detect in vivo BCL-2 dependence, we turned to two small cell lung cancer (SCLC) cell lines that were sensitive to treatment with ABT-737 in vitro and in an in vivo murine xenograft model (Oltersdorf et al., 2005). Both H146 and H1963 demonstrate a pattern of sensitivity diagnostic of BCL-2 sensitivity (Figure 7D). This provides further support, in addition to the results of Figures 4, 5, and 6, that mitochondrial BH3 profiling is a powerful predictor of what cells are sensitive to BH3 mimetic drugs in vitro and in vivo.

Discussion

Life on the edge: “Primed for death”

Conventionally, study of the apoptotic machinery has been able to discriminate cells into two states: alive or dead. Here, we show that certain cells live in a state that can be distinguished by dependence on antiapoptotic proteins for survival. We describe these cells as being “primed for death,” as death signaling has caused their antiapoptotic family members to sequester significant quantities of proapoptotic BH3 proteins. Inhibition of the antiapoptotic proteins in these cells, but not unprimed cells, results in BAX/BAK oligomerization and MOMP. We suggest, therefore, that there are three functionally distinguishable states with respect to programmed cell death: unprimed, primed for death, and dead. We suggest that the state of being primed for death is a continuum, as the magnitude of BH3 proteins priming the mitochondrion can, of course, vary continuously until the antiapoptotic reserve is overwhelmed and the cell commits to programmed cell death. A summary of this model with reference to the data in this paper can be found in Figure 8.

We probed mitochondria to determine a cell’s state using our panel of sensitizer BH3 peptides, selective antagonists of antiapoptotic BCL-2 family members. Mitochondria that are primed

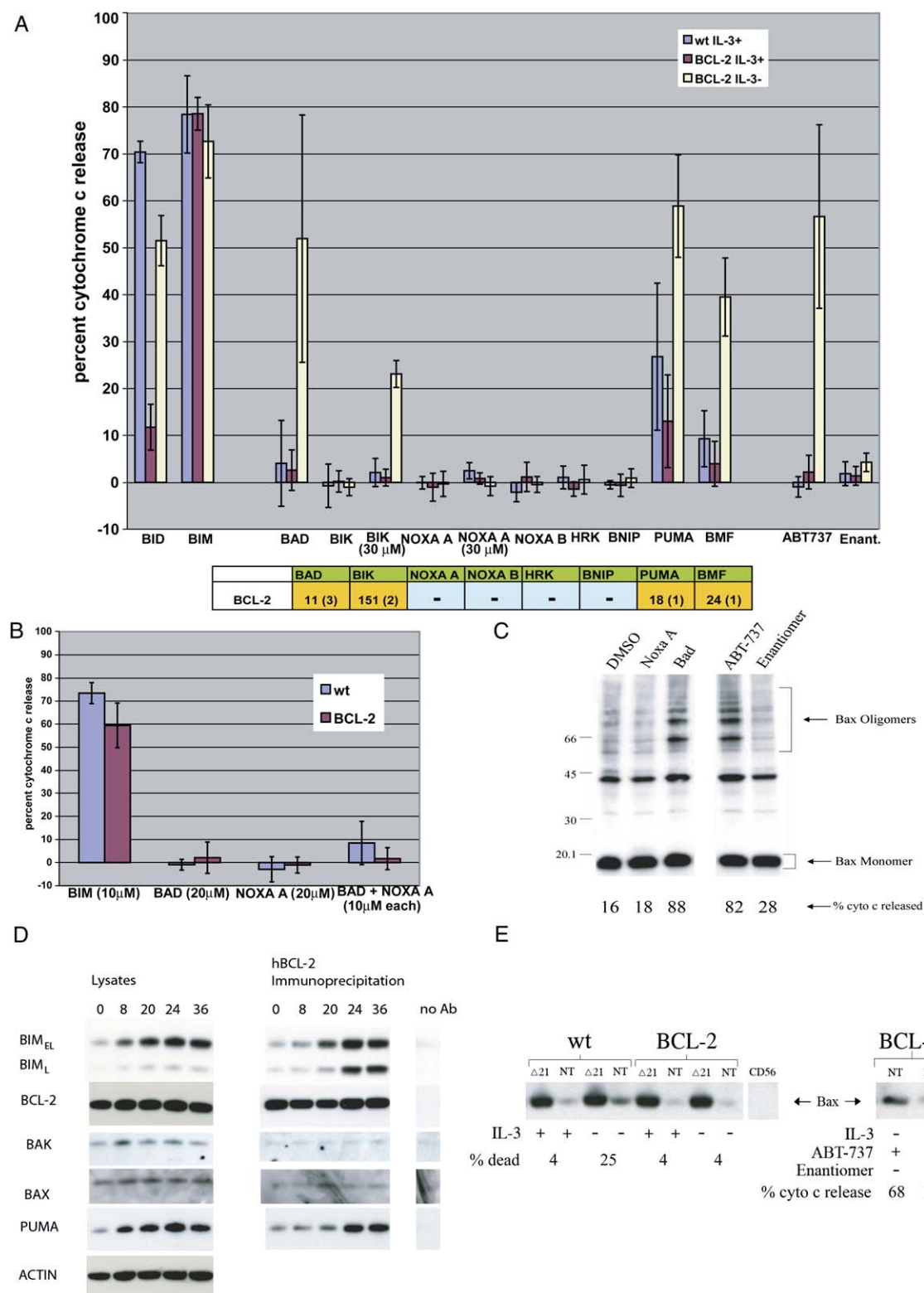


Figure 5. BCL-2 dependence is revealed by mitochondrial sensitivity to BCL-2 binding BH3 peptides

A: Cytochrome c release induced by BH3 peptides (10 μ M) from mitochondria isolated from wtFL5.12 cells grown in the presence of IL-3 (blue bars); FL5.12-BCL-2 cells grown in the presence (red bars) or absence (tan bars) of IL-3 for 24 hr. Note that BH3 sensitizer peptides, which tightly bind BCL-2 in Figure 1, are effective antagonists of BCL-2 protection. Shown are average and standard deviation of three independent experiments. For convenience, BCL-2 binding pattern from Figure 1 is excerpted below.

B: Combination of NOXA and BAD BH3 induces no cytochrome c release from mitochondria isolated from wt and BCL-2 FL5.12 cells grown in the presence of IL-3. Shown are average and standard deviation of three independent experiments.

for death are dependent on antiapoptotic protein function to prevent MOMP, so that they release cytochrome c when exposed to sensitizer BH3 peptides (Figures 2B–2F, 5A, 6C, 6D, 7B, and 7D). In contrast, unprimed cells do not release cytochrome c when exposed to sensitizer BH3 peptides. In theory, any cell from which mitochondria can be isolated can therefore be so tested and categorized as being primed or unprimed. Testing of mitochondria directly has the advantage of eliminating any contribution of transcription, translation, or posttranslational modification events that might be triggered by transfection of peptide, protein, or expression vector into a living cell. A “snapshot” of the apoptotic state at a given time may be taken with minimal perturbation of the extant apoptotic machinery. In summary, we were able to capture information about a fundamental aspect of cellular physiology in an assay that can be performed using primary or cultured cells in a single day.

Importantly, we link mitochondrial behavior to whole-cell behavior in several models. Mitochondria were primed when cells were enduring a physiologic challenge, and BH3 profiling revealed a dependence on antiapoptotic proteins only when a cellular dependence was also demonstrated. In the case of FL5.12 cells, cells and mitochondria became primed for death only after IL-3 withdrawal. For 2B4 cells, cells and mitochondria were primed for death only after dexamethasone treatment. For the primary BCL-2-dependent leukemia cells, the genomic instability, myc oncogene activation, and checkpoint violation inherent to the cancer phenotype were sufficient to induce mitochondrial priming without further external intervention. The SCLC cell lines H164 and H1963 that revealed a BCL-2 pattern of sensitivity to BH3 profiling likewise are sensitive to the BCL-2 antagonist ABT-737. In each case, mitochondrial studies correctly diagnosed the cellular dependence on an antiapoptotic BCL-2 family member. Furthermore, the identity of the individual family member could be decoded based on the pattern of mitochondrial sensitivity to our peptide panel.

An implication of our results is that in some cells, like IL-3-replete FL5.12-BCL-2 cells, BCL-2 overexpression provides extra antiapoptotic reserve. In others, like the murine leukemias, high levels of BCL-2 are present, but the BCL-2 is so highly occupied by activator BH3 proteins that the cell has very poor antiapoptotic reserve and is actually primed for death.

The binding code

An important property of our panel of sensitizer BH3 peptides is its ability to distinguish among the antiapoptotic proteins based on binding specificity. Others have recently demonstrated similar selectivity in interaction among BH3 peptides and antiapoptotic proteins using a different technique, surface plasmon resonance (SPR) (Chen et al., 2005). While dissociation constants were not explicitly determined, the overall pattern of binding was similar, with some notable differences. The binding of HRK BH3 peptide, restricted to BCL-XL in our work, was

promiscuous throughout the antiapoptotic proteins tested by SPR. BFL1/A1, which bound only PUMA among the sensitizer BH3 peptides in our work, bound BIK, HRK, and NOXA as well in the work by Chen et al. There are several possible explanations for these differences. First, the peptides are not identical. Their peptides were 26 amino acids in length, while those we use are shorter (Figure 1A). In addition, they do not test direct binding of the peptides to the antiapoptotic proteins, as we did by fluorescence polarization, but rather test the ability of each of the peptides to displace a BIM BH3 peptide from the antiapoptotic protein by SPR. We find our peptide panel particularly useful, as it allows us to distinguish BCL-XL protection from the rest due to its selective binding of HRK BH3. Furthermore, our demonstration that MCL-1 selectively binds both murine NOXA BH3 peptides suggests biological relevance to the binding pattern observed.

Do these interactions between BH3 peptides and antiapoptotic proteins mirror protein-protein interactions in actual cellular systems? Consistent with our binding chart, we have shown that MCL-1 preferentially binds BIM but not BAD, governing murine lymphocyte dependence on MCL-1 for survival (Opferman et al., 2003). A yeast two-hybrid interaction study supports the specificity we found for BAD's binding of antiapoptotic family members in Figure 1B (Bae et al., 2001). Others (Chen et al., 2005) showed that interactions of proteins overexpressed in cells showed fidelity with the patterns found in both our systems. Since BH3 domains are the critical ligands for the binding pockets of antiapoptotic proteins, we expect our BH3 peptide binding patterns to be consistent with *in vivo* protein-protein interaction specificity. Since length of peptide might affect function, a longer, 26 amino acid HRK peptide was tested. While lengthening the peptide increased slightly its ability to antagonize BCL-2 and MCL-1 in cytochrome c release assays, it still did not demonstrate activator function (data not shown). It is nonetheless possible that different conformations or posttranslational modifications play a role *in vivo* that was not evaluated in our binding assays. Regardless, our peptide panel captures binding differences that allow it to function as a diagnostic of antiapoptotic protein dependence. BCL-2 and BCL-w are most similar, with BCL-XL distinguished by its binding to HRK BH3. MCL-1 is quite different, binding both NOXA peptides but not BAD, and BFL-1 binds none of the sensitizers except PUMA. These binding specificities suggest, moreover, that it is possible for cells to adjust sensitivity to distinct apoptotic insults by adjusting levels of individual BCL-2 family member expression.

The basic apoptotic paradigm

Our results shed light on a controversial issue—how interactions among BCL-2 family members control MOMP. Our results consistently show that ligation of antiapoptotic family members alone is insufficient to induce MOMP. Rather, antiapoptotic

C: Mitochondria isolated from FL5.12-BCL-2 cells grown in the absence of IL-3 for 24 hr were treated with NOXA A or BAD peptides (30 μ M) or ABT-737 or control enantiomer at 10 μ M for 35 min. Mitochondrial pellets were subjected to chemical crosslinking as previously described (Letai et al., 2002).

D: Immunoblots of FL5.12-BCL-2 whole-cell lysates (left) and samples immunoprecipitated by an antibody directed against the human BCL-2 transgene product (right). Numbers at top refer to hours after IL-3 withdrawal. Control lane performed without anti-human BCL-2 antibody in pull-down at right. Negative control immunoprecipitation using anti-human BCL-2 antibody on lysates from IL-3-starved FL5.12-BCL-XL cells yielded no bands (data not shown).

E: BCL-2 blocks apoptosis upstream of BAX activation. wt or BCL-2-expressing FL5.12 cells were exposed to IL-3 withdrawal as indicated. BAX was immunoprecipitated using an antibody recognizing all BAX conformations (Δ 21) or only the activated conformation with N-terminal exposure (NT). Death induced in the cells is indicated below. At right, mitochondria isolated from IL-3-starved cells were treated with ABT-737 or control enantiomer, and immunoprecipitation with NT performed as indicated. CD56 indicates control immunoprecipitation by an irrelevant antibody recognizing CD56.

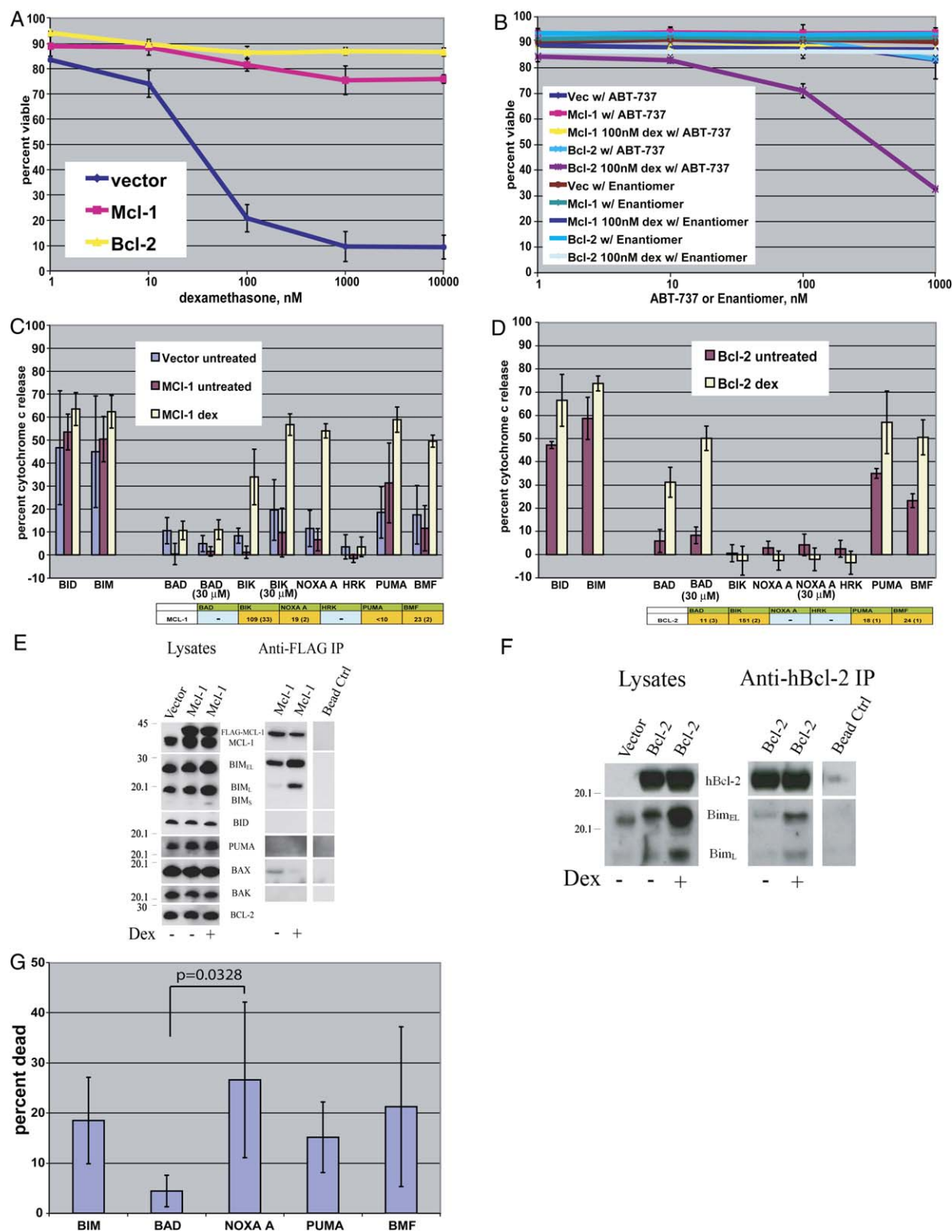


Figure 6. BH3 profiling discriminates BCL-2 and MCL-1 dependence

A: MCL-1 and BCL-2 inhibit cell death induced by dexamethasone in 2B4 cells. 2B4 cells transfected with Flag-MCL-1, BCL-2, or empty vector constructs were cultured for 24 hr in the presence of the indicated concentration of dexamethasone. Viability determined by absence of Annexin V staining by FACS. Shown are average and standard deviation of three independent experiments.

B: BCL-2 antagonist ABT-737 has no effect on wt or MCL-1-dependent 2B4 cells but kills BCL-2-dependent cells. 2B4 cells were incubated for 24 hr with dexamethasone and either ABT-737 or enantiomer for 24 hr. Shown are average and standard deviation of three independent experiments.

C: Mitochondria isolated from 2B4 cells treated as indicated were exposed to BH3 peptides at the concentrations indicated. Cytochrome c release is induced by MCL-1 binding sensitizer BH3 peptides only when mitochondria are derived from MCL-1-dependent cells. Shown are average and standard deviation of three independent experiments. For convenience, MCL-1 binding pattern from Figure 1 is excerpted below.

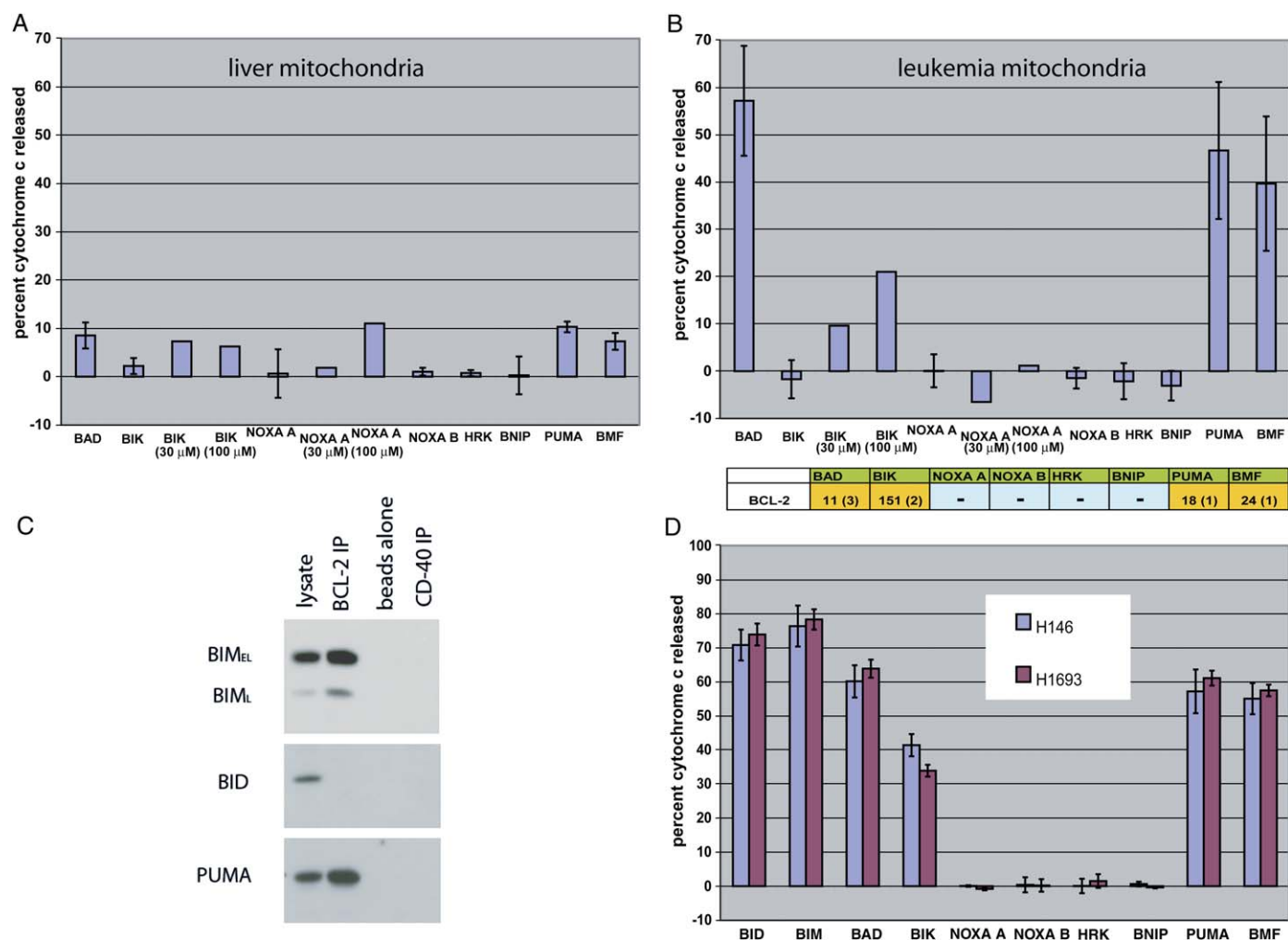


Figure 7. Leukemia mitochondria are uniquely sensitive to BCL-2 antagonism by sensitizer BH3 peptides

A and B: Cytochrome c release by BH3 peptides (10 μ M, unless otherwise noted) using mitochondria isolated from liver (**A**) and BCL-2-dependent leukemia (**B**) from the same animal as **A**. For convenience, BCL-2 binding pattern from Figure 1 is excerpted below. Shown are average and standard deviation of three independent experiments, except 30 and 100 μ M treatments, which were performed once.

C: Immunoblot of samples from a BCL-2-dependent leukemia. First lane shows a whole-cell lysate, 25 μ g loaded; second lane shows products of an immunoprecipitation using an antibody against the human BCL-2 transgene product; third lane shows a control with Protein A beads alone; fourth lane shows control immunoprecipitation using an irrelevant hamster monoclonal antibody recognizing murine CD-40.

D: BH3 profiling predicts ABT-737 sensitivity of SCLC cell lines. Mitochondria were isolated from two SCLC cell lines, H146 and H1693, and exposed to panel of BH3 peptides. Cytochrome c release quantitated by ELISA. $n = 3$ for each and the error bars represent the standard deviation.

inhibition causes MOMP only when an activator is present that can be freed from the antiapoptotic protein to activate BAX or BAK. Based on these results, we propose a model of BCL-2 member interaction summarized in Figure S3. Our studies provide strong evidence that our earlier model (Letai et al., 2002) of a sensitizer/activator dichotomy governing interactions of BH3-only family members with BCL-2 and BAX/BAK can be applied across a wide range of antiapoptotic and BH3-only family members.

Strong support for this interpretation can be found in another recent report (Kuwana et al., 2005). In a biochemically completely defined system using BAX and liposomes, it was shown that only those BH3 domains that we have designated activators (BID or BIM) are capable of directly activating BAX to oligomerize and permeabilize liposomes. Those BH3 domains, which we here categorize as sensitizers, were found to induce permeabilization only when an activator like cleaved BID protein was available to be displaced from an antiapoptotic protein.

D: As in **C**, but using BCL-2-expressing 2B4 cells and 1000 nM dexamethasone.

E: Vector- or FLAG-MCL-1-transfected 2B4 cells were treated with 0 or 100 nM dexamethasone and lysed. FLAG antibody linked to agarose beads immunoprecipitated proteins complexing with FLAG-MCL-1. Increased BIM sequestration by MCL-1 correlates with MCL-1 dependence.

F: As in **E**, but using BCL-2-expressing 2B4 cells and 1000 nM dexamethasone.

G: Primed FLAG-MCL-1 2B4 cells transfected with BH3 peptides illustrate an MCL-1 pattern. Dexamethasone-treated FLAG-MCL-1 2B4 cells were electroporated with 100 μ M of the indicated peptides and percent killing ascertained by Annexin-V staining. Note that killing by transfection with NOXA A is significantly greater than that with BAD, recapitulating the same MCL-1 pattern observed in isolated mitochondria from this same cell line (as in **C**). $n = 3$ and the error bars represent the standard deviation.

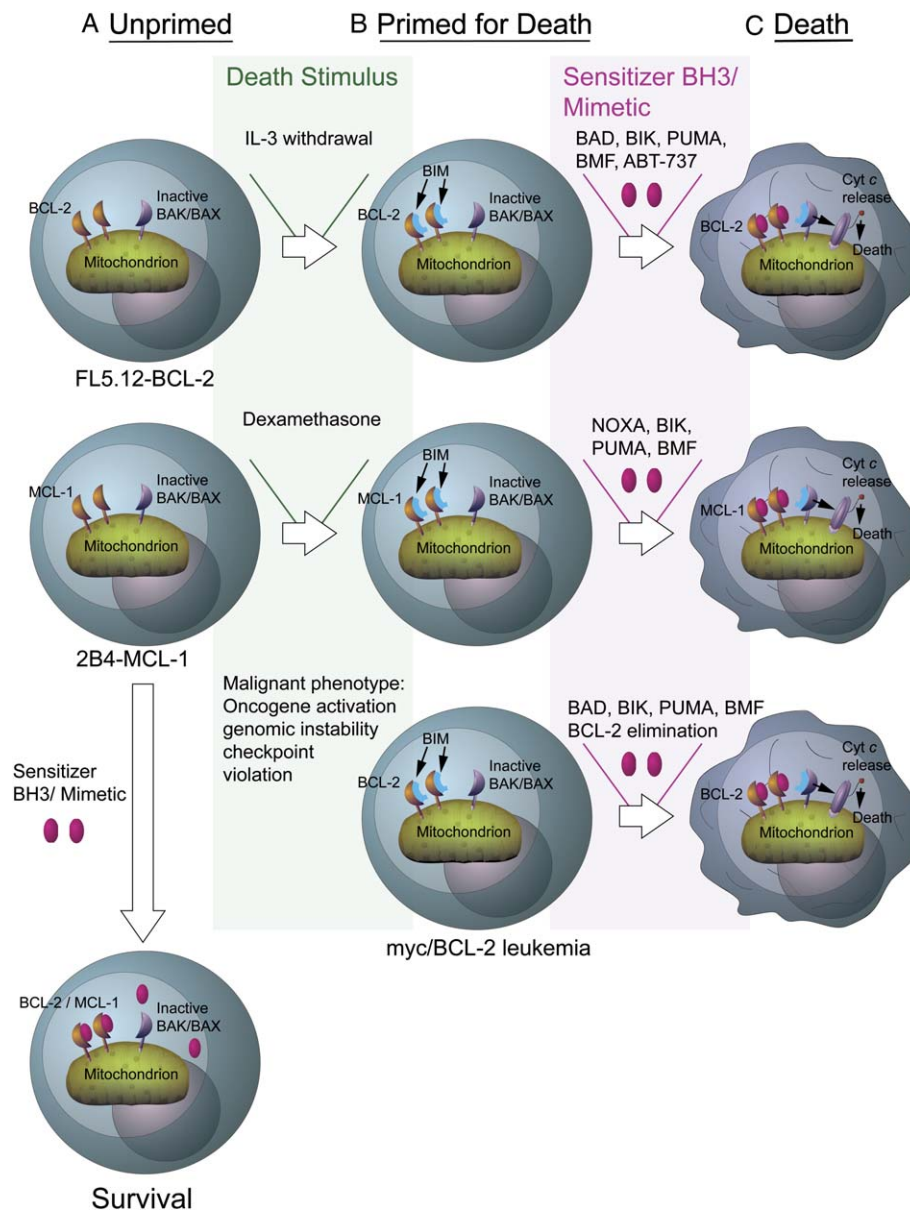


Figure 8. Model of selective cancer sensitivity to sensitizer BH3 mimetic treatment

Living unprimed cells (**A**) are primed for death following death stimuli (**B**) (IL-3 withdrawal for FL5.12-BCL-2 cells, dexamethasone for 2B4-MCL-1 cells). The leukemia cell is tonically primed for death due to cancer phenotype, without external intervention (**B**). Cells in the primed state undergo apoptosis in response to antiapoptotic antagonists (**C**)—those in the unprimed state do not.

Furthermore, this displacement from either BCL-X_L or MCL-1 took place in accordance to a binding code consistent with Figure 1B.

It is worth noting how our model contrasts with other models recently proposed. Chen et al. present a model for control of apoptosis in which BH3-only family members provoke apoptosis exclusively by inhibition of antiapoptotic BCL-2 family members, without interacting with multidomain proapoptotic BAX or BAK (Chen et al., 2005). In contrast, our results clearly demonstrate that mere inhibition of antiapoptotic proteins is insufficient for apoptosis. Instead, activation of BAX and/or BAK by activator BH3-only proteins like BIM or BID is required. The same authors also suggested, based on transfection and overexpression in cellular systems, that the combination of NOXA and BAD is sufficient to trigger apoptosis, as together they bind to a broad range of antiapoptotic proteins. We tested this directly in both MLM and FL5.12 mitochondria, systems in which translation and transcription were unavailable to alter levels of activator

proteins. The combination of NOXA BH3 and BAD BH3 peptides by themselves could not induce MOMP (Figures 2A and 5B). However, if an activator protein (BID or BIM) was present and sequestered by an antiapoptotic protein to which NOXA or BAD BH3 could bind, either NOXA BH3 or BAD BH3 by itself was effective (Figures 2B, 2E, 5A, 6C, and 6D).

It has recently been suggested that, on mitochondria containing BAK, survival is maintained when BAK is sequestered by select antiapoptotic proteins. BAK can be sequestered by MCL-1 and BCL-X_L, but not BCL-2. In this model, apoptosis is initiated when BAK is displaced from MCL-1 or BCL-X_L by competition of BH3-only proteins (Willis et al., 2005). A prediction of this model, therefore, is that BCL-2 should be unable to prevent apoptosis on mitochondria bearing only BAK, but no BAX. This model is inconsistent with the data we present here. The murine liver mitochondria in Figure 2 contain BAK, but no BAX (Letai et al., 2002). Figure 2B, however, demonstrates that BCL-2 can prevent tBID-induced apoptosis in this system. In addition,

in Figure S1, inhibition of antiapoptotic function of BCL-X_L by sensitizer BH3 domains is unable to induce apoptosis; activator tBID is required. Our data are more consistent with a model in which BCL-2 and other antiapoptotic BCL-2 family members inhibit BAK-dependent apoptosis by sequestering activators like tBID and preventing their activation of BAK. Our data do not exclude, however, the possibility that in some systems tonic sequestration of BAK by MCL-1 or BCL-X_L might play a role in preventing apoptosis. Further studies will be necessary to determine the relative relevance of each of these models across a range of biological systems.

We have here classified PUMA BH3 as a sensitizer. While PUMA BH3 sometimes induced low levels of cytochrome c release in the absence of exogenous death stimuli, the levels were always significantly lower than those found for BID and BIM BH3. Additionally, in these cases it is possible that the low levels of BIM already present in these cells provided the key activator function, as can be seen in Figure 6. Some studies have provided evidence that PUMA can indeed interact directly with BAX (Cartron et al., 2004; Liu et al., 2003), while others suggest that PUMA lacks this property (Kuwana et al., 2005). Left untested by these prior studies is the ability of PUMA to interact with BAK. It is notable that PUMA shares with activators BID and BIM the ability to interact with all five antiapoptotics tested. However, we showed that, under conditions where PUMA BH3 could not induce cytochrome c release by itself, it could nonetheless indirectly provoke cytochrome c release by antagonism of antiapoptotic proteins occupied by an activator. While we cannot rule out that PUMA may have significant activator function in other conditions, under the conditions tested here its BH3 domain did not, and we were able to evaluate the spectrum of its sensitizer function in isolation.

An important consequence of our results is that interpretation of experiments in apoptosis in vivo, on cells, and mitochondria must consider whether the extent of priming of the system is affecting results. This consideration may clarify some discrepancies present in the field of apoptosis research.

Antiapoptotic oncogene addiction in cancer

It is noteworthy that, while our nonmalignant models like mouse liver (Figure 2) and FL5.12 (Figures 4 and 5) require exogenous death signaling to acquire a requirement for antiapoptotic proteins, the cancer cells tested in these studies (like the murine leukemia and SCLC cell lines in Figure 7) are apparently already enduring death signaling that renders them dependent on BCL-2.

In our murine leukemia model, BCL-2 sequesters BIM (Figure 7C), the likely explanation for BCL-2's requirement for leukemia maintenance in vivo (Letai et al., 2004). When these mitochondria were treated with sensitizer antagonists of BCL-2, therefore, they required no additional activator function, as one was already present and bound to BCL-2. Cancer cells violate many normal rules, including oncogene activation (*c-myc* in this case), cell cycle checkpoint violation, genomic instability, etc. These and other abnormal properties of cancer cells provoke death signals, likely producing a requirement for apoptotic deficiency in cancer cells (Fanidi et al., 1992; Green and Evan, 2002; Hanahan and Weinberg, 2000; Schmitt, 2003). Relevant to this model, *c-myc* has been shown to induce BIM expression (Egle et al., 2004; Hemann et al., 2005). Our experiments lay a biochemical framework for studying the widely discussed

notion that cancer cells may teeter on the brink between survival and death.

These experiments suggest an intriguing dichotomy with potentially profound therapeutic significance in cancer. It will be interesting to explore the extent to which tonic activator BH3 priming of antiapoptotic proteins contributes to the preferential sensitivity of many cancer cells to standard cytotoxic chemotherapy. This model suggests that there may be a natural therapeutic window provided between unprimed normal cells and BH3-only protein-primed cancer cells. This is of therapeutic significance, since our data suggest that primed cells would likely be selectively sensitive both to sensitizer BH3 mimetics like ABT-737 as well as to agents that provoke sensitizer BH3-only protein expression. Since conventional chemotherapeutic agents do activate or induce sensitizer BH3-only proteins, it may be that the priming by death signals we identify may underlie many instances of cancer sensitivity to cytotoxic chemotherapy.

Sensitizer BH3 mimetic small molecule inhibitors of antiapoptotic proteins are currently in preclinical and clinical development (Oltersdorf et al., 2005). Our results shed light on how such agents might selectively cause death in "primed" cells, a state that may be preferentially occupied by cancer cells. Furthermore, the binding code presented here suggests that the binding pockets of the antiapoptotic proteins are structurally and functionally distinct. Antiapoptotic proteins may therefore be susceptible to individual targeting by drugs developed as selective mimetics of sensitizer BH3 domains.

Experimental procedures

Reagents

ABT-737 and its negative control enantiomer, which has lower affinity for BCL-2 family members, were obtained from Abbott Laboratories (Oltersdorf et al., 2005).

GST pull-down

Ten micrograms GST-BCL-w (or BH3 binding-defective R96P point mutant) were incubated with glutathione-agarose beads for 1 hr at 4°C in binding buffer (140 mM NaCl, 10 mM Tris [pH 7.4]). Beads were rinsed and incubated with approximately 0.2 µg tBID for 1 hr at 4°C. Beads were washed again and incubated with peptides for 1 hr at 4°C. tBID protein was eluted from beads with 50 mM glutathione and loaded on a denaturing NuPAGE gel.

Cytochrome c release

Mitochondria were purified from liver and FL5.12 cells as previously described (Letai et al., 2002). Mitochondria were purified from leukemia cells and 2B4 cells as previously described for FL5.12 cells. Mitochondria were incubated with treatments for 45 (mouse liver mitochondria) or 35 min (FL5.12, 2B4, and leukemic mitochondria). Release of cytochrome c was determined by a comparison of cytochrome c in the pellet and supernatant following treatment, quantitated by ELISA (R&D systems). When results of multiple experiments were averaged, results from solvent-only (DMSO) treatments values were subtracted from each, so that 0 release reflects that observed in solvent-only treatments.

Other procedures are in the Supplemental Data.

Supplemental data

The Supplemental Data include Supplemental Experimental Procedures and three supplemental figures and can be found with this article online at <http://www.cancerres.org/cgi/content/full/65/5/351/DC1/>.

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