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BH3 Profiling Identifies Three Distinct Classes of Apoptotic Blocks to Predict Response to ABT-737 and Conventional Chemotherapeutic Agents

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SUMMARY

Cancer cells exhibit many abnormal phenotypes that induce apoptotic signaling via the intrinsic, or mitochondrial, pathway. That cancer cells nonetheless survive implies that they select for blocks in apoptosis. Identifying cancer-specific apoptotic blocks is necessary to rationally target them. Using a panel of 18 lymphoma cell lines, we show that a strategy we have developed, BH3 profiling, can identify apoptotic defects in cancer cells and separate them into three main classes based on position in the apoptotic pathway. BH3 profiling identifies cells that require BCL-2 for survival and predicts sensitivity to the BCL-2 antagonist ABT-737. BCL-2 dependence correlates with high levels of proapoptotic BIM sequestered by BCL-2. Strikingly, BH3 profiling can also predict sensitivity to conventional chemotherapeutic agents like etoposide, vincristine, and adriamycin.

INTRODUCTION

Apoptosis, a form of programmed cell death (PCD), can be triggered by numerous types of cellular damage and derangement. Genomic instability, oncogene activation, cell cycle checkpoint violation and loss of prosurvival signaling all have been shown to induce apoptosis via the intrinsic apoptotic pathway. Cancer cells often exhibit some or all of these "deadly" phenotypes. To survive, therefore, it is necessary that cancer cells select for a block in apoptotic signaling at the mitochondrion. Such blocks likely involve alterations in the function and control of the BCL-2 family of proteins. Understanding these blocks is essential for selective therapeutic targeting of apoptotic pathways in cancer cells.

Proteins of the BCL-2 family are key mediators of programmed cell death at the mitochondria (Figure 1A) (Danial and Korsmeyer, 2004; Green and Kroemer, 2004; Wang, 2001). They may be conveniently broken into three main groups based on sequence homology and function (Cory and Adams, 2002; Gross et al., 1999). BAX and BAK, also known as multidomain proapoptotic proteins or "effectors," share homology in the BCL-2 homology (BH) 1, 2, and 3 regions. The antiapoptotic proteins, including BCL-2, MCL-1, BCL-XL, BCL-w, and BFL-1/A1, share homology in the BH1, BH2, BH3, and BH4 domains. The proapoptotic BH3-only family members are so named because they share homology only in the BH3 domain, which is essential for prodeath function. In response to damage and derangement signals, BH3-only proteins are activated, either by stabilization of protein, increased transcription, or posttranslational modification (Huang and Strasser, 2000; Kelekar and Thompson, 1998; Wei et al., 2000). Certain of the BH3-only proteins, including BID and BIM, are called "activators," and induce activation by causing an allosteric change in BAX and BAK (Cartron

SIGNIFICANCE

The key to effective rational therapies is the identification of targets that are required for cancer cell survival but dispensable for normal cell survival. Due to death signaling generated by their phenotypic aberrancies, cancer cells require blocks in cell death pathways not required by normal cells. Here we present a method, BH3 profiling, for identifying apoptotic blocks in cancer cells and separating them into three distinct classes. We identify cancer cells that depend upon the block conferred by BCL-2 expression. These cells can be killed by a compound, ABT-737, that antagonizes BCL-2 function. Furthermore, BH3 profiling can identify cancer cells already heavily primed with death signals. Significantly, these cells are more sensitive to conventional agents like etoposide, vincristine, and adriamycin.

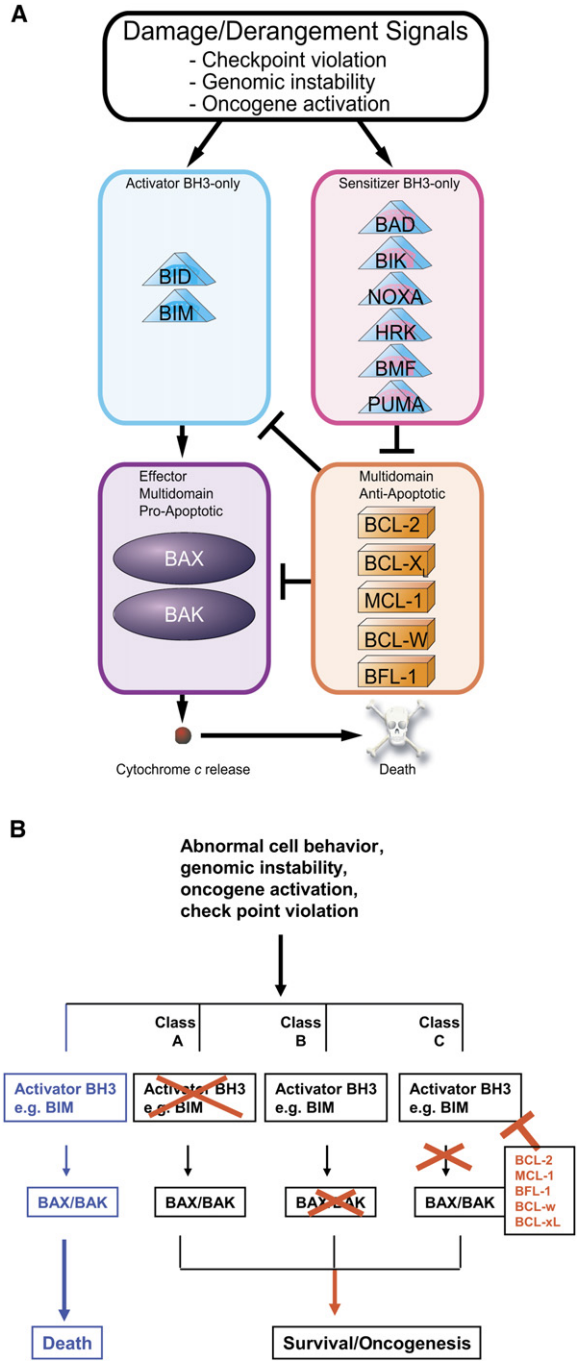


Figure 1. Three Distinct Classes of Apoptotic Blocks in Diffuse Large B Cell Lymphoma Cells

(A) Model of BCL-2 family control over mitochondrial apoptosis. In response to damage or derangement, activators activate effectors, causing mitochondrial permeabilization and commitment to death. Antiapoptotic proteins sequester activators to prevent their contacting effectors, and sensitizers act as selective antagonists of antiapoptotic proteins. Adapted from Certo et al. (2006).

(B) Three possible apoptotic blocks that cancer cells might select to escape apoptosis.

et al., 2004; Certo et al., 2006; Desagher et al., 1999; Kuwana et al., 2002; Letai et al., 2002; Luo et al., 1998; Marani et al., 2002; Wei et al., 2000). Recent evidence has been presented that as an intact protein PUMA may also function as an activator (Kim et al., 2006). BAX and BAK oligomerization follows, with subsequent mitochondrial outer membrane permeabilization (MOMP). BAX and BAK activation and MOMP may be considered the critical steps at which point the cell is irretrievably committed to PCD. BAX and BAK are absolutely essential for the proapoptotic function of BH3-only proteins, and deficiency of BAX and BAK affords broad protection from apoptosis against numerous insults (Wei et al., 2001; Zong et al., 2001). Multiple proapoptotic factors are released following MOMP, including cytochrome *c*, AIF, and SMAC (Wang, 2001). Resulting widespread proteolysis by the family of cysteine proteases, known as caspases, induces cellular dysfunction and the tagging of the cell with signals that trigger the engulfment of the apoptotic cell by phagocytic cells.

Antiapoptotic proteins like BCL-2 oppose PCD primarily by binding and sequestering activator BH3-only proteins, preventing their activation of BAX and BAK (Certo et al., 2006; Cheng et al., 1996, 2001; Kuwana et al., 2005; Letai et al., 2002). The hydrophobic face of the BH3 domains' amphipathic α helix binds into a hydrophobic groove formed by the BH1, BH2, and BH3 domains of the antiapoptotic proteins (Cheng et al., 1996; Kelekar et al., 1997; Kelekar and Thompson, 1998; Muchmore et al., 1996; Sattler et al., 1997). Antiapoptotic proteins also bind BAX and BAK, particularly in their activated monomeric forms (Hsu et al., 1997; Hsu and Youle, 1997). When a cell or mitochondrion contains antiapoptotic proteins that are largely occupied by activator BH3-only proteins due to ongoing death signaling, we describe that cell type as "primed for death" (Certo et al., 2006). Cells that are primed for death are uniquely dependent on the function of antiapoptotic proteins of the BCL-2 family for survival. We have thus far found that being "primed for death" is more common among malignant than among nonmalignant cells.

A second class of proapoptotic BH3-only proteins, called "sensitizers," are unable to induce activation of BAX and BAK directly. Rather, they exert their proapoptotic function by competing for the BH3 domain-binding cleft in antiapoptotic proteins, displacing or preventing the binding of activators (Certo et al., 2006; Kuwana et al., 2005; Letai, 2003; Letai et al., 2002). Sensitizers may thus be considered inhibitors of the inhibitors of apoptosis. BH3 domains that behave as sensitizers include BAD, BIK, NOXA, BMF, Harakiri (HRK), and PUMA (Certo et al., 2006).

An important recent observation is that interactions between members of the sensitizer BH3-only class and the antiapoptotic class are selective (Certo et al., 2006; Chen et al., 2005; Kim et al., 2006; Kuwana et al., 2005; Opferman et al., 2003). For instance, while BCL-2 is bound and antagonized by the BAD BH3 domain, NOXA BH3 binds BCL-2 very poorly. The exactly opposite pattern is

observed for MCL-1. Since peptides derived from the BH3 domains of the sensitizers exhibit selective inhibitory interactions with antiapoptotic proteins, we realized that they could be exploited as probes of antiapoptotic protein function. Therefore, using a strategy we call "BH3 profiling," we can determine (1) if the cell is "primed for death" and (2) if the cell is primed, which antiapoptotic protein(s) are primarily responsible for maintaining survival (Certo et al., 2006).

BH3 profiling is a potentially powerful tool in determining how cancer cells evade apoptosis. To perform BH3 profiling, mitochondria are isolated from the cell of interest and then exposed to a series of peptides derived from the BH3 domains of BH3-only proteins. The ability of these peptides to induce mitochondrial permeabilization is measured by cytochrome *c* release. In model systems, the ability of activator BH3 domains to induce cytochrome *c* release indicates the presence of either BAX or BAK. The ability of sensitizer BH3 domains to induce cytochrome *c* release implies the presence of BAX and BAK, but also the presence of antiapoptotic protein(s) primed with a protein capable of activating BAX or BAK, as sensitizer BH3 peptides lack this function. By examining the pattern of sensitizer BH3 peptides that induce cytochrome *c* release, the identity of the antiapoptotic protein necessary for maintaining survival can be identified by comparison to a table summarizing the interaction pattern between the antiapoptotic proteins and the range of BH3 domains (Certo et al., 2006).

We have previously used BH3 profiling for the interrogation of the intrinsic, or mitochondrial, apoptotic pathway in chronic lymphocytic leukemia (CLL) (Del Gaizo Moore et al., 2007). We found that BH3 profiling was very effective in demonstrating the uniform BCL-2 expression and BCL-2 dependence of the many CLL samples tested. This dependence was reflected in a uniform sensitivity of CLL cells from chemotherapy-naive patients to the BCL-2 antagonist ABT-737. While this study was instructive regarding CLL biology, the homogeneity of apoptotic blocks represented in our CLL samples did not allow us to investigate the wider range of apoptotic blocks employed by cancer cells. In order to study cancers that escape apoptosis by a wider variety of mechanisms we turned to a more heterogeneous disease, diffuse large B cell lymphoma (DLBCL). In DLBCL, roughly 15% of cases express high levels of BCL-2 due to the t(14;18) translocation, which places the BCL-2 gene under the control of the immunoglobulin heavy chain gene transcriptional elements. In another subset of DLBCL, BCL-2 is overexpressed as a result of gene amplification or gain of an extra copy of chromosome 18, whereas others show high BCL-2 expression without identifiable genetic abnormality. Remaining cases, which may represent at least half of al DLBCL, show little expression of BCL-2 and thus must rely on a different apoptotic block (Gascoyne et al., 1997; Iqbal et al., 2006; Kramer et al., 1996). We turned to a large panel of 18 DLBCL cell lines as a model system to study classes of apoptotic escape exploited by cancer cells.

Many chemotherapeutic agents kill cancer cells via apoptosis, and there is considerable interest and investment in the direct targeting of abnormal apoptotic pathways in cancer (Letai, 2005). It is therefore of crucial importance to investigate methods that can rapidly and convincingly decipher the abnormalities in the cell death pathways in cancer cells to better understand the critical phenomena of therapeutic resistance and sensitivity. In order to address this question, we needed a range of cancer cells that evaded apoptosis in heterogeneous ways but that were otherwise biologically similar. For this reason, we turned to the study of apoptotic abnormalities in the most common lymphoid malignancy, diffuse large B cell lymphoma.

In this study, we analyzed a panel of 18 diffuse large B cell lymphoma cell lines with tools that included BH3 profiling. We found that we were able to assign cells to one of three classes based on the type of apoptotic block they possessed. Membership in these classes was predictive of response to a BCL-2 antagonist as well as to conventional chemotherapy. These findings show that identification of the class of apoptotic block in cancer cells is potentially of great clinical importance and utility.

RESULTS

BH3 Profiling Detects Three Classes of Apoptotic Blocks in Lymphoma Cells

A priori, one can consider three distinct ways that cancer cells might select to block intrinsic apoptotic signaling generated by their abnormal phenotypes (Figure 1B). First, upstream activation of BH3-only proteins might be inhibited (class A block). Second, the effector arm of the apoptotic pathway might be blunted by loss of BAX and BAK (class B block). Finally, activation of BAX and BAK might be prevented via expression of antiapoptotic proteins like BCL-2 and MCL-1 (class C block). Note that cells with a class C block are those we describe as being "primed for death" and would be detectable by mitochondrial sensitivity to sensitizer BH3 domains in BH3 profiling. It should also be noted that presence of any one block does not rule out the presence of another, though one might expect selection pressure for additional blocks to be attenuated after selection for an effective initial block.

We initiated our studies of lymphoma in four lymphoma cell lines: SU-DHL4, SU-DHL6, SU-DHL8, and SU-DHL10 cell lines. These were chosen because two contained the t(14;18) (SU-DHL4, SU-DHL6), while the other two did not. To perform BH3 profiling, we tested the ability of a panel of sensitizer peptides to induce mitochondrial outer membrane permeabilization (MOMP) in mitochondria isolated from the lymphoma cells. For easy reference, Figure 2A shows the interaction pattern between the BH3 peptides and antiapoptotic proteins. MOMP was measured by quantifying cytochrome *c* release by ELISA.

BH3 profiling proved able to distinguish these three classes of blocks in our sample of four lymphoma lines (Figure 2). SU-DHL4 and SU-DHL6 demonstrated class C blocks. They showed a "primed" phenotype, based

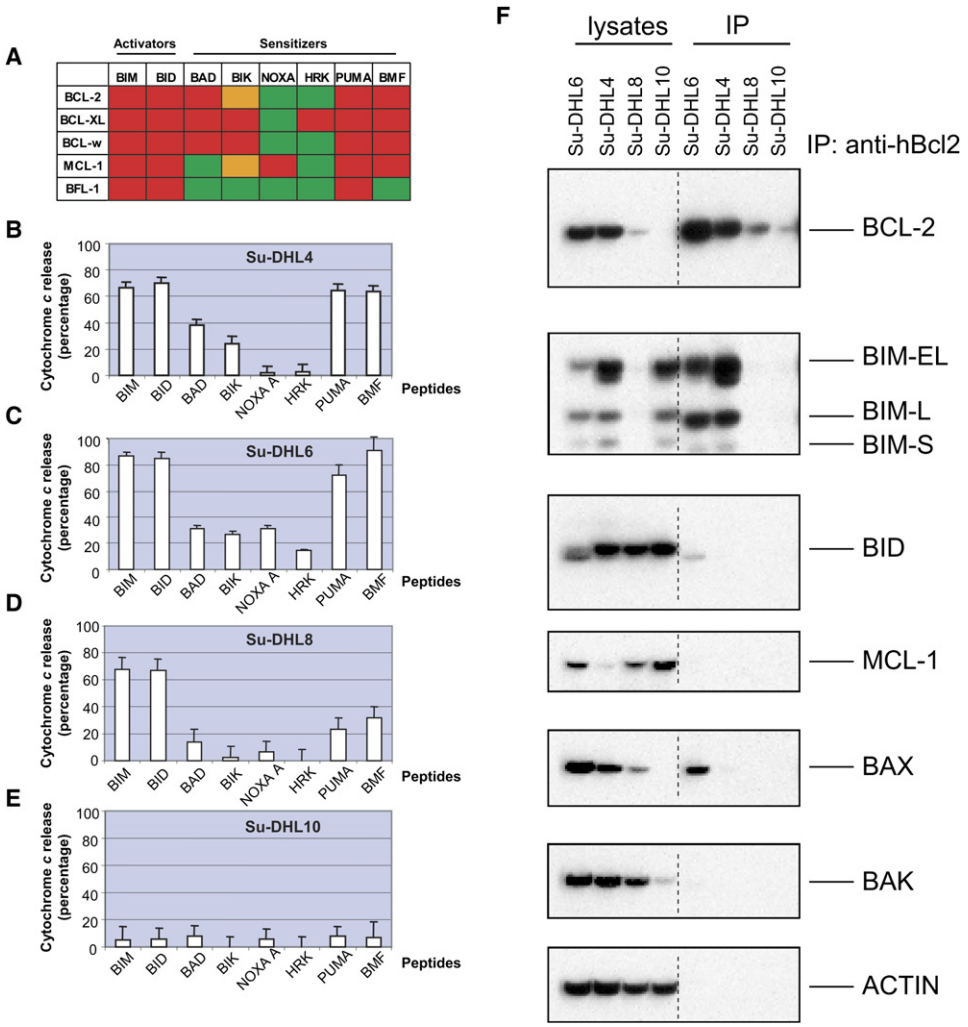


Figure 2. BH3 Profiling of SU-DHL4, -6, -8, and -10 Cells Discriminates Three Distinct Classes of Apoptotic Blocks

(A) Interaction pattern between BH3 peptides and antiapoptotic proteins (Certo et al., 2006). Red indicates high-affinity binding, orange indicates low-affinity binding, and green indicates undetectable binding. Mitochondria were isolated from SU-DHL4 (B), SU-DHL6 (C), SU-DHL8 (D), and SU-DHL10 (E) cells, and incubated with a panel of BH3 peptides (100 μM). Release of cytochrome *c* was determined by a comparison of cytochrome *c* in the pellet and supernatant quantitated by ELISA. Results were shown as percentage minus solvent control DMSO values, and mean of triplicates with error bars for standard deviation. (F) Protein lysates were prepared and subjected to immunoprecipitation with an antibody specific for human BCL-2 (6C8, Phamingen). Both lysates (left) and immunoprecipitates (right) were separated by denaturing electrophoresis. Membranes were blotted with antibodies against BCL-2 family proteins as indicated. Actin was a loading control.

on the sensitivity to sensitizer BH3 peptides. Note that a strong response to the PUMA BH3 peptide, which interacts with all of the antiapoptotic proteins, provides a useful gauge of whether the mitochondria are primed. The pattern of sensitivity (PUMA, BMF, BAD, ± BIK) indicated a dependence on BCL-2 for SU-DHL4. SU-DHL6 also was primed, as shown by a strong PUMA BH3 and BMF BH3 signal. The weaker, but definite, response to both of the more selective BH3 peptides BAD BH3 and NOXA A BH3 implicate combined dependence on BCL-2 and MCL-1. SU-DHL8 appeared to be poorly primed, given the limited response to PUMA BH3 and other sensitizers, but nonetheless demonstrated an intact effector arm by responding strongly to activators BIM BH3 and BID BH3. This suggested a class A block for SU-DHL8. SU-DHL10 responded poorly to both sensitizer and activator peptides, indicating the loss of the effector arm, suggesting a class B block.

BH3 profiling predicted that SU-DHL4 and SU-DHL6 would exhibit priming by an activator BH3-only protein, that SU-DHL8 would be poorly primed, and that SU-DHL10 would lack BAX and BAK. To test these predictions, we examined the abundance of BCL-2 family proteins and complexes of BCL-2 with other members of the BCL-2 family (Figure 2F). BIM was present in high levels in SU-DHL4 and SU-DHL6, and it was likewise sequestered in a complex with BCL-2. Notably, SU-DHL4, which showed singular dependence on BCL-2,

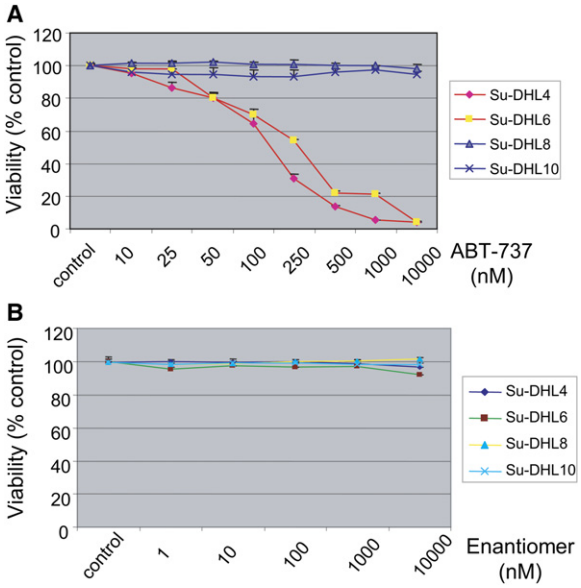


Figure 3. BH3 Profiling Predicts Sensitivity to BCL-2 Antagonism by ABT-737

SU-DHL4, -6, -8, and -10 cells were treated with increasing doses of ABT-737 (A) or its negative control enantiomer (enant, B) for 48 hr and stained with Annexin V-FITC for flow cytometry analysis. Viability was shown as a percentage of control (DMSO-treated) cells with standard deviation of triplicates shown by error bars.

had very low MCL-1 expression, whereas SU-DHL6, which showed dependence on both BCL-2 and MCL-1, showed higher levels of MCL-1 than SU-DHL4. BIM expression was nearly undetectable in SU-DHL8, consistent with the class A block shown by BH3 profiling. Whereas uncleaved BID was present in roughly equal amounts in all four cell lines, the active cleaved form of BID was not detected, and there was very little BID seen in complex with BCL-2 in any of the cells. Strikingly, the class B block shown by BH3 profiling had also predicted the nearly undetectable levels of BAX and BAK in SU-DHL10, in stark contrast to the expression levels in the other three cell lines. Thus, BH3 profiling accurately assessed the mechanisms of apoptotic block present in all four cell lines. It furthermore accurately predicted the abundance of key proteins and protein complexes.

BH3 Profiling Predicts Response to ABT-737

Next, we investigated whether this single test also correctly predicted sensitivity to BCL-2 antagonism by ABT-737. BAD BH3 response and a BCL-2-dependent pattern from BH3 profiling have previously been strong predictors of cellular response to ABT-737 in other models (Certo et al., 2006; Del Gaizo Moore et al., 2007). Therefore, BH3 profiling predicts in this case a response of SU-DHL4 and SU-DHL6, but not SU-DHL8 or SU-DHL10, to ABT-737. We tested this prediction by generating a dose-response curve to ABT-737 for each of the cell lines (Figure 3A). Clearly, the cell lines demonstrate sensitivity and resistance exactly as predicted by BH3 profiling (EC_{50} 140 nM and 250 nM for SU-DHL4 and SU-DHL6, respectively, >>10,000 nM for SU-DHL8 and SU-DHL10). Supporting action by the intended mechanism of BCL-2 antagonism, all four cell lines showed very limited toxicity from treatment with an enantiomer of similar physiochemical properties, but much lower affinity to BCL-2 (Oltersdorf et al., 2005) (Figure 3B).

BCL-2 and BIM:BCL-2 Complex Levels Quantitatively Predict Sensitivity to ABT-737

Above we showed that relatively high abundance of the BCL-2:BIM complex predicted sensitivity to ABT-737. To test how generalizable this predictor is, we examined a larger panel of lymphoma cell lines. First, we prepared dose-response curves for each of the 18 cell lines, which demonstrated a wide range of EC_{50} (Figure 4A). To validate the use of BH3 profiling in this additional cohort of lymphoma cell lines, we tested the ability of BH3 profiling to distinguish apoptotic blocks in two cell lines that both bore the t(14;18), but which had very different responses to ABT-737 (TOLEDO, EC_{50} = 74 nM; PFEIFFER, EC_{50} = 5619 nM) (Figures 4B and 4C). Both profiles demonstrate a class C block. In the case of TOLEDO, the pattern of response to BH3 peptides suggests that BCL-2 is primarily responsible for the class C block, consistent with sensitivity to ABT-737. For PFEIFFER, however, the pattern suggests protection by a protein other than BCL-2, consistent with relative resistance to ABT-737. The pattern, in fact, is more suggestive of protection by the antiapoptotic protein BFL-1, which is not antagonized by ABT-737 (see Figure 2A) (Certo et al., 2006; Oltersdorf et al., 2005). In support of the selective importance of BFL-1 in the PFEIFFER cell line, we found that levels of BFL-1 mRNA expression are much higher in PFEIFFER than in any of the other cell lines studied by BH3 profiling (Figure 4D). Note that of these seven cell lines, only PFEIFFER revealed a pattern suggestive of BFL-1 dependence. Thus, results of BH3 profiling here again accurately predict response to ABT-737 and expression of BCL-2 family proteins.

Next, we again examined the abundance of BCL-2 family proteins and complexes by immunoblots of cell lysates (Figure 4E) and immunoprecipitates of BCL-2 (Figure 4F). We quantified the intensity of the bands by gel densitometry, as presented in Table 1. Note that absolute levels of other targets of ABT-737, BCL-w, and BCL-XL were much lower than those of BCL-2 as established by comparison to recombinant standards (Figure S2 in the Supplemental Data available with this article online). Thus, no further studies of BCL-w and BCL-XL were pursued. Note also that BID was found to be uncleaved and not in complex with BCL-2 throughout the cell lines, and therefore unlikely to be playing a role in BCL-2 dependence or sensitivity to ABT-737 (Figures 4E and 4F).

We examined the ability of the different protein and complex levels to dictate response to ABT-737 by a linear regression analysis of protein or complex abundance and log EC_{50}. We found that levels of BCL-2 (Figure 5A) and BCL-2:BIM complex (Figure 5B) quantitatively predicted

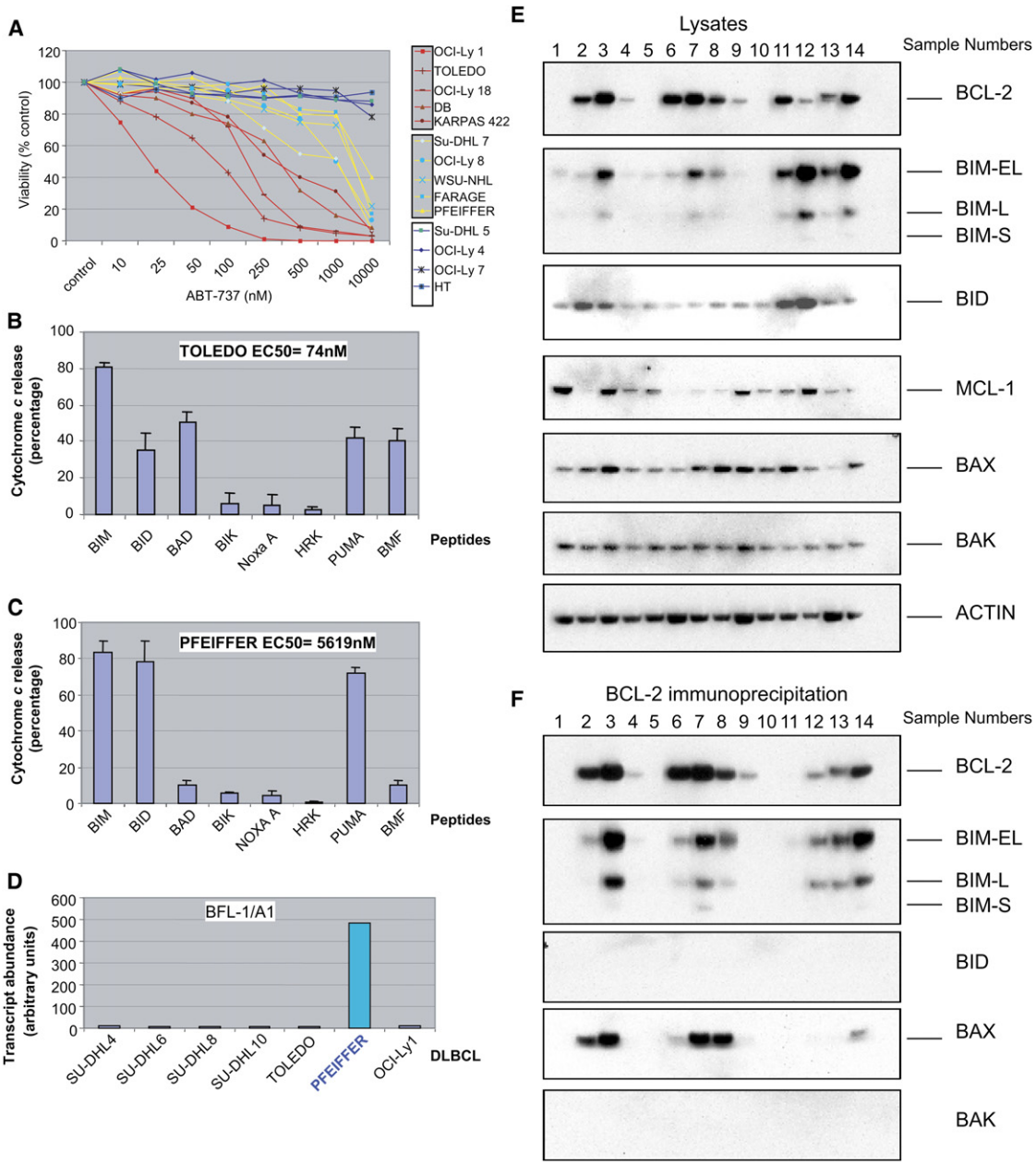


Figure 4. Sensitivity to ABT-737 Falls across a Wide Range in a Panel of Lymphoma Cell Lines

(A) A panel of 14 DLBCL cell lines (not including the SU-DHL4, -6, -8, and -10 cells shown in Figure 3) was examined for their sensitivity to ABT-737. Cells were treated with increasing doses of ABT-737 for 48 hr and stained with Annexin V-FITC for flow cytometry analysis as in Figure 3. Shown are the means of experiments performed in triplicate. Dose-response curves indicating highest sensitivity are red, those indicating intermediate sensitivity are yellow, and those indicating low sensitivity are blue.

(B and C) (B) and (C) were BH3 profiling results for DLBCL cell lines TOLEDO and PFEIFFER, respectively. The assays were performed with 100 μM BH3-only peptides as in Figure 2. Shown is the mean of three experiments; error bars show standard deviation.

(D) Relative mRNA levels of BFL-1 found in seven lymphoma cell lines.

(E) Immunoblot analysis of BCL-2 family proteins in lysates prepared from the 14 DLBCL cell lines with indicated antibodies.

(F) Immunoblot analysis of immunoprecipitates prepared from the same lysates in (E) using an antibody against human BCL-2 (6C8) for immunoprecipitation. Sample numbers correspond to sample names as shown in Table 1.

sensitivity to ABT-737 better than any of the other proteins or complexes. Levels of BCL-2:BAX complex (Figure 5C) and BAX (Figure 5D) also correlated with response to ABT-737, though the correlation was weaker than for BCL-2 or BCL-2:BIM. Correlation of MCL-1 and log EC_{50} did not reach statistical significance ($p = 0.081$, $r^2 = 0.18$), but there is a weak trend for higher MCL-1 levels to reduce sensitivity to ABT-737. Notably, BIM and BAK

Table 1. Summary of Densitometric Analysis of Immunoblots, EC_{50} of ABT-737, and t(14: 18) Status in 18 DLBCL Cells

Sample Name	Sample No.	BCL-2	MCL-1	BIM	BAX	BAK	BIM (IP)	BAX (IP)	EC_{50} (nM)	t(14:18)
SU-DHL5	1	2	196	8	32	122	0	0	20,000	negative
SU-DHL7	2	177	29	11	70	138	22	129	807	negative
OCI-Ly1	3	228	127	51	97	97	139	273	21	positive
OCI-Ly4	4	26	128	3	60	120	10	8	20,000	negative
OCI-Ly7	5	0	101	5	32	99	6	4	20,000	negative
OCI-Ly8	6	140	8	7	13	52	36	30	1216	positive
OCI-Ly18	7	224	28	39	57	86	100	274	159	positive
DB	8	185	46	35	131	108	60	264	279	positive
FARAGE	9	15	70	2	55	66	7	10	2769	negative
HT	10	2	74	0	58	103	9	5	20,000	negative
KARPAS 422	11	220	105	53	129	87	11	2	350.6	positive
PFEIFFER	12	69	312	275	57	219	45	7	5619	positive
WSU-NHL	13	127	43	28	0	59	62	4	2405	positive
TOLEDO	14	158	99	183	93	172	108	52	74	positive
SU-DHL6	15	124	128	16	92	109	38	349	250	positive
SU-DHL4	16	97	116	37	76	100	28	0	140	positive
SU-DHL8	17	36	256	12	51	145	5	13	20,000	negative
SU-DHL10	18	32	268	41	17	8	8	30	20,000	negative

Immunoblots, shown in Figure 4D (lysates), Figure 4E (immunoprecipitates [IP]), and Figure S1 (where SU-DHL4, -6, -8, and -10 samples were normalized to cell lines OCI-Ly1, DB, and FARAGE in Figures 4D and 4E), were subjected to densitometric analysis. Arbitrary units for protein lysates were normalized to actin levels. EC_{50} of ABT-737 of DLBCL cells were calculated from dose-response curve experiments shown in Figures 3 and 4A. t(14:18) translocation status determined by standard cytogenetic analysis.

levels by themselves offered no ability to predict response to ABT-737 (Figures 5F and 5G). While using a nonparametric comparison, it is notable that the presence of the t(14;18) was also strongly correlated to sensitivity to ABT-737 ($p < 0.0001$, Mann-Whitney rank sum test, see Table 1), supporting its mechanism of killing via interaction with BCL-2. All of these findings support our model that BIM only predisposes to BCL-2 dependence and response to ABT-737 when it is sequestered by BCL-2, priming the cell for a death that is prevented by BCL-2.

BCL-2 Overexpression Does Not Cause Sensitivity, but BIM Knockdown Reduces Sensitivity to ABT-737

While BIM:BCL-2 levels correlate well with ABT-737 sensitivity, so do BCL-2 levels. Our hypothesis was that the level of the BIM:BCL-2 complex is mechanistically critical, and the correlation of sensitivity to ABT-737 with BCL-2 simply reflects the requirement for sufficient BCL-2 to sequester the amount of BIM generated by death signaling. However, it remained a formal possibility that BCL-2 levels alone dictated sensitivity by an obscure mechanism. To test this possibility, we expressed BCL-2 in SU-DHL8 and HT cells that were relatively resistant to ABT-737 and had expressed low levels of BCL-2 (Figure 6A). By transfection with a BCL-2 cDNA, we obtained BCL-2 expression levels comparable to those from the ABT-737-sensitive cell line SU-DHL6. Low BIM expression levels remained unaltered. Congruent with our model and with prior results (Certo et al., 2006; Oltersdorf et al., 2005), overexpression of BCL-2 alone did not confer sensitivity (Figure 6B).

If the presence of BIM sequestered by BCL-2 dictates sensitivity, then we would predict a knockdown of BIM to reduce sensitivity. We used shRNA against BIM to reduce BIM levels in the cell line most sensitive to ABT-737, OCI-Ly1 (EC_{50} = 21 nM; Figure 6C). Reduction of BIM levels caused a reduced sensitivity to ABT-737 (Figure 6D). Furthermore, we examined the effect of BIM reduction on BCL-2 dependence at the mitochondrial level using BH3 profiling. Parental OCI-Ly1 mitochondria revealed a BCL-2-dependent pattern upon BH3 profile, and a mitochondrial sensitivity to the compound, consistent with the cell line's sensitivity to ABT-737 (Figure 6E). Key to this analysis is the response to the sensitizer BAD BH3 peptide, which maps tightly with ABT-737 sensitivity (Certo et al., 2006). In an abbreviated BH3 profile, we confirmed that reduction in BIM levels had no effect on sensitivity to the activator BH3 peptide BIM BH3 (Figure 6F). However, decrease in BIM levels caused reduction in mitochondrial response to both BAD BH3 and ABT-737. Note that mitochondrial response to ABT-737 correlated with cellular response in other cell lines as well, supporting mitochondrial BCL-2 as the target for ABT-737 action (Figure S3). This finding implicates

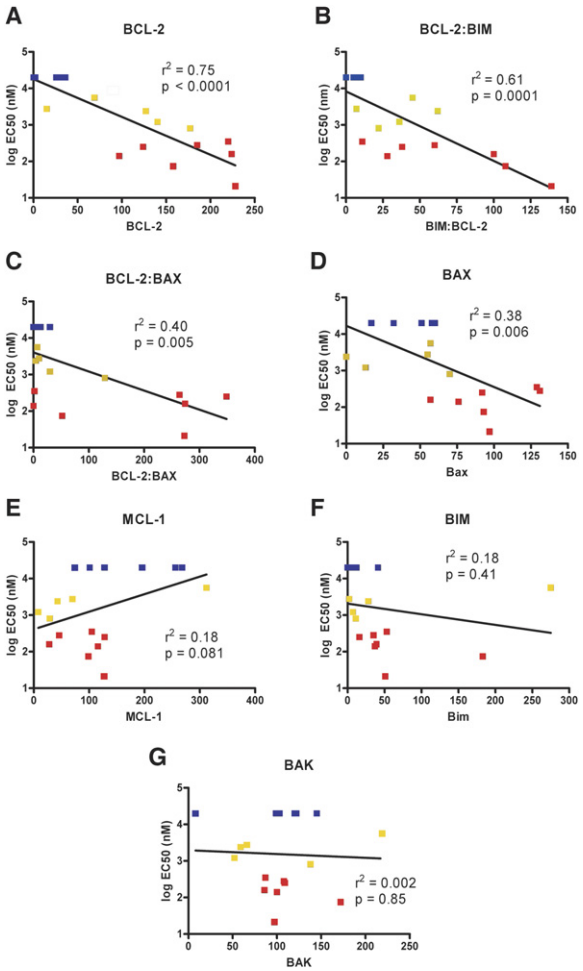


Figure 5. Levels of BCL-2 and BCL-2:BIM Complex Are Best Predictors of ABT-737 Sensitivity in DLBCL Cells

(A–G) BCL-2 family proteins analyzed by immunoblotting in DLBCL cells and EC_{50} of ABT-737 of those cells (both listed in Table 1) were subjected to statistical analysis via a linear regression model. y axis is log EC 50 of ABT-737 (nM); x axis represents the arbitrary units of different BCL-2 family proteins or immunoprecipitates as indicated, obtained by densitometric scanning of immunoblots. Colors of filled squares corresponds to the color code used in Figure 4A: most sensitive to ABT-737 in red, intermediate sensitivity in yellow, and low sensitivity in blue.

displacement of BIM from a complex with BCL-2 as a key event in the mitochondrial and cellular toxicity resulting from antagonism of BCL-2 by ABT-737 in lymphoma.

Figure 7A shows a summary of our classification of cells into three classes based on type of apoptotic block. It occurred to us that many types of conventional chemotherapy also kill via the mitochondrial apoptotic pathway. If our classification is accurate, we would predict that agents that rely on the mitochondrial pathway for killing would be more active against the "primed" subset of class C compared to the "unprimed" subset of class A and B. To test this hypothesis, we treated seven cell lines that had been already classified by BH3 profiling (summarized in Figure 7B) with agents that have been characterized as inducing apoptosis through the mitochondrial pathway (Decaudin et al., 1997; Kojima et al., 1998; Wei et al., 2001; Zong et al., 2004). Strikingly, in each case we found that the primed subset was more sensitive than the unprimed subset (Figures 7C–7E). The case of the putatively BFL-1-dependent PFEIFFER cell line is particularly instructive, as it shows that it is the state of being "primed" rather than specifically being sensitive to ABT-737 that dictates sensitivity to the chemotherapy drugs. It has been shown previously that alkylating agents like N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) can use the intrinsic apoptotic pathway but can also induce death via an alternative PARP-dependent necrotic pathway independent of BCL-2 family proteins (Zong et al., 2004). Specifically, MEFs from mice lacking BAX and BAK were highly resistant to etoposide, but not to MNNG. Therefore, we would predict that lymphoma lines would respond to MNNG independent of their classification by BH3 profiling. That is precisely what we found (Figure 7F), providing confirmation in a human cancer setting of what was previously proposed in genetically modified murine cells. Therefore, just as BH3 profiling can identify cancer cells (class C) that are likely to respond to agents using the intrinsic apoptotic pathway like vincristine, etoposide, and adriamycin, it may also be able to identify those cells (class A or B) that will selectively respond to alkylating agents. BH3 profiling therefore can provide fundamental parameters of a cell's phenotype.

DISCUSSION

Many, perhaps even all, cancer cells require blocks in death signaling for oncogenesis and maintenance of the malignant phenotype (Green and Evan, 2002; Hahn and Weinberg, 2002). Since the most common abnormalities of cancer behavior, like genomic instability and oncogene activation, signal death via the mitochondrial apoptotic pathway, it seems necessary that cancer cells select means for blocking apoptosis at the mitochondrion. Using diffuse large B cell lymphoma as a model, we show that blocks in the intrinsic pathway of apoptosis may be broken into three main classes, and that BH3 profiling can assign a cancer cell to one of these three classes (Figure 7). Due to the prior lack of availability of a technology to make such distinctions, such a classification has previously been lacking. We hope its introduction will facilitate systematic study of cancer cells and how to kill them.

A class A block occurs when normal generation of proapoptotic activators by aberrant behavior is inhibited. Mitochondrial outer membrane permeabilization requires activation and oligomerization of BAX and BAK. Activation of BAX and BAK requires the action of activator BH3-only proteins. These proteins include BID and BIM, but other proteins may possibly operate as activators, including PUMA and p53 (Chipuk et al., 2004; Kim et al., 2006; Letai et al., 2002). Given the difficulty in identifying BH3 domains from primary sequence, there may well be several undiscovered important activator proteins, and even molecules that activate BAX and BAK without a discernable

BH3 domain. The mechanisms by which aberrant behavior such as genomic instability and oncogene activation generate death signals via BH3-only proteins is as yet poorly understood, and therefore how this upstream "pre-BH3-only" signaling is attenuated is also poorly understood. Since transcriptional activation certainly can induce upregulation of activators, loss of transcriptional proteins like p53 may certainly play a role. Posttranslational modifications, including phosphorylation, also are important in regulating function of BH3-only proteins, so we can speculate that the modification of phosphorylation cascades that occurs in cancer may play a role in establishing a class A block. This is an area of ongoing study.

A class B block occurs when there is a significant loss of BAX and BAK. It has been shown that the presence of either BAX or BAK is necessary for apoptotic signaling through the mitochondrial pathway (Wei et al., 2001; Zong et al., 2001). Again, it is not clear how this might occur, whether by genetic or epigenetic means, but we observed its spontaneous occurrence in the case of SU-DHL10. Given how profound the block in apoptosis is that results from loss of BAX and BAK, it is reasonable to ask why such a loss does not occur more often in cancer. The answer well may lie in other nonapoptotic roles played by BAX and BAK, including control of mitochondrial fusion and calcium homeostasis (Karbowski et al., 2006; Scorrano et al., 2003). Loss of these roles may be too costly to allow for frequent selection in natural oncogenesis.

One can readily identify examples of the genetic basis of the class C block in certain human cancers. The t(14;18) found in follicular lymphoma and some DLBCL drives overexpression of the antiapoptotic BCL-2 protein (Bakhshi et al., 1985; Cleary and Sklar, 1985; Tsujimoto et al., 1985). In CLL, there is evidence that chromosomal deletions causing loss of micro-RNAs that downregulate BCL-2 expression are responsible for BCL-2 expression in a large proportion of cases of CLL (Cimmino et al., 2005). It is notable that, in this study, nine of the ten cell lines that bear the t(14;18) are dependent on BCL-2 as shown by sensitivity to ABT-737 and BH3 profiling (Table 1). This supports the model that high levels of expression of antiapoptotic proteins in cancer cells license oncogenic phenotypes, which generate death signals carried by BH3-only proteins. These phenotypes, which would be intolerable without antiapoptotic protection, include genomic instability, hyperproliferation, and oncogene activation. Thus, when selected for during oncogenesis, the BCL-2 resulting from the t(14;18) may not provide any additional resistance to cytotoxic agents, as it is already occupied by BH3-only death signals generated by cancer phenotypes.

Superficially, this model might seem to contradict the traditional view of BCL-2 expression as uniformly providing protection from cytotoxic insults like chemotherapy. Yet it is important to understand the context under which this view arose. BCL-2 was initially studied via overexpression in stable cell lines that had already reached an equilibrium with their in vitro environment. In these circumstances, additional BCL-2 provided extra antiapoptotic reserve which produced resistance to a variety of insults, including genotoxic agents, growth factor withdrawal, and broad spectrum kinase inhibition. However, cancers cannot select for antiapoptotic protein expression in the expectation that they will some day be subject to apoptotic signaling from chemotherapy. Rather, the only selection pressure available during oncogenesis is that generated by the cancer's own aberrant behavior. While the magnitude of death signaling may vary with an individual cancer cell's local environment or position in cell cycle, it seems likely that much of the antiapoptotic reserve that might otherwise be provided by the selected BCL-2 expression will be eliminated by ongoing prodeath signaling. To put it simply, in cell culture overexpression models, BCL-2 is largely "empty," whereas BCL-2 is largely already "full" when it is selected for in cancer. This may offer an explanation of the high sensitivity of certain cancer cells to chemotherapy and radiation, a sensitivity that often lacks adequate molecular explanation. Strong support for this view can be found in the clinically obvious, but perhaps scientifically underappreciated, observation that BCL-2-expressing follicular lymphoma and CLL are among the most chemosensitive cancers known. An explanation for this seeming paradox may be found in the observation that, in CLL cells, the BCL-2 is primed with abundant amounts of BIM, rendering CLL "primed for death" (Del Gaizo Moore et al., 2007).

A particularly significant finding here is that BH3 profiling can be used to predict sensitivity to conventional chemotherapy agents. As predicted, cells that are "primed" with prodeath signaling proteins, class C cells, respond better to chemotherapy agents using the intrinsic apoptotic pathways like vincristine, etoposide, or adriamycin. Perhaps just as important, we can identify cells, class A or B, that may be unlikely to respond to those agents, but rather more likely to benefit from alkylating agents. This paradigm certainly suggests itself to clinical application, but clearly more work on a wider range of cell types and wider range of agents is necessary before clinical use. This approach is especially attractive as BH3 profiling can be performed on tumor mitochondria without the need for ex vivo cell culture (Del Gaizo Moore et al., 2007; Letai et al., 2004), thus overcoming one of the major hurdles that has plagued determination of chemosensitivity in vitro. While needing confirmation, this model is thus far supported by the observation that previously untreated CLL, which we found to be uniformly a class C disease (Del Gaizo Moore et al., 2007), is also quite uniformly sensitive to a wide range of chemotherapeutic agents.

BH3 profiling proves to be a very useful tool in detecting each of the three classes of block, and furthermore, detecting which antiapoptotic protein is most critical for survival in the case of a class C block. Consequently, BH3 profiling proves to be an excellent predictor of cellular response to ABT-737. Cells that show a class C block and BCL-2 dependence on BH3 profiling are predicted to be sensitive to ABT-737, and those with other profiles as relatively resistant. On this basis,

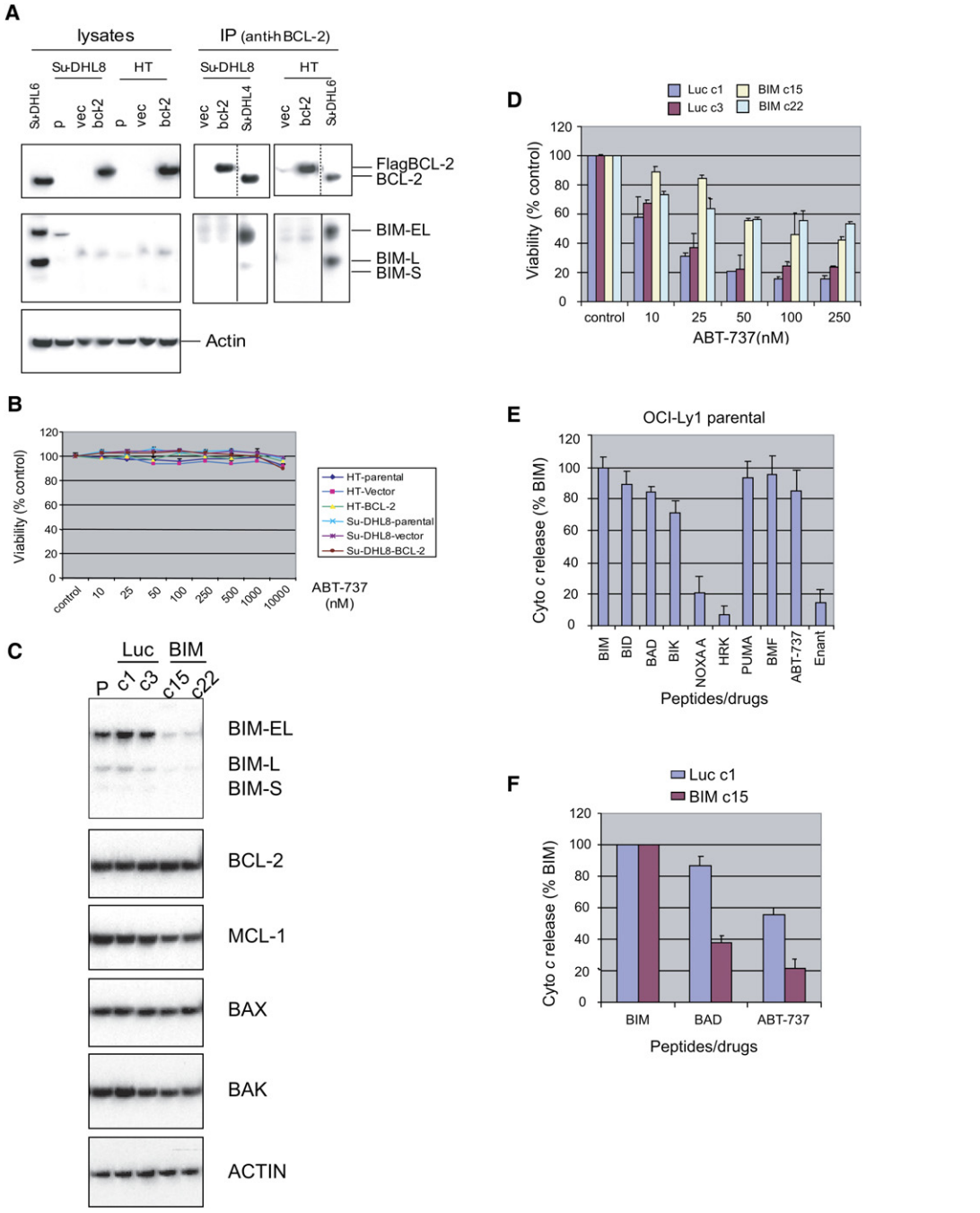


Figure 6. Overexpression of BCL-2 Alone Does Not Confer Cell Sensitivity to ABT-737-Resistant Cells, but BIM Loss Confers Resistance to Sensitive Cells

(A) Immunoblot of BCL-2 and BIM levels in whole cell lysates (left) and anti-BCL-2 immunoprecipitates (right) in HT and SU-DHL8 stable clones. The endogenous BCL-2 in SU-DHL6 cells is shown for comparison. P, parental cells; vec, vector-transfected cells; bcl-2, FlagBCL-2-transfected cells.

(B) Dose-response curve experiments were carried out in parental and vector- and Flag-BCL-2-transfected HT and SU-DHL8 cells as in Figure 4. Shown is the mean of three experiments; error bars show standard deviation.

(C) Immunoblots of OCI-Ly1 parental (p), Luc ShRNA, and BIM ShRNA knockdown stable cells. c1, c3 and c15, c22 are two representative clones for OCI-Ly1 Luc ShRNA and BIM ShRNA cells, respectively. Various antibodies against BIM, BCL-2, MCL-1, BAX, BAK, and loading control ACTIN were used for immunoblot analysis.

(D) Dose response to ABT-737 experiments in OCI-Ly1 Luc ShRNA and BIM ShRNA clones. Luc (c1, c3) and BIM (c15, c22) cells were treated with increased doses of ABT-737 for 4 hr and stained with Annexin-V-FITC for flow cytometry analysis as in Figure 4.

(E) BH3 profiling for OCI-Ly1 parental cells. Mitochondria were isolated from OCI-Ly1 parental cells and incubated with a panel of BH3-only peptides (100 μM), ABT-737, and enantiomer (both were 1 μM). Results were shown as percentage minus DMSO values and normalized to BIM-treated sample.

SU-DHL4, SU-DHL6, TOLEDO, and OCI-Ly1 were all correctly predicted to be sensitive to ABT-737, while SU-DHL8, SU-DHL10, and PFEIFFER were all correctly predicted to be resistant. While we had previously shown in CLL that a BCL-2 BH3 profile corresponded to sensitivity to ABT-737, the surprising homogeneity of the leukemia samples studied (all were exquisitely sensitive to ABT-737) did not allow for the testing of the ability of ABT-737 to discriminate between sensitive and resistant cell lines in a heterogeneous group of samples (Del Gaizo Moore et al., 2007).

This same heterogeneity allowed for the testing of protein correlates to ABT-737 sensitivity. We found that sensitivity to ABT-737 correlated most tightly with abundance of BCL-2 and BCL-2:BIM complex (Figures 5A and 5B). This finding is consistent with our model in which priming of BCL-2 with activators is the primary determinant of BCL-2 dependence (Figure 7). To demonstrate that BCL-2 expression in the absence of priming was insufficient to cause ABT-737 sensitivity, we showed that overexpression of BCL-2 failed to convert a resistant cell line to a sensitive cell line (Figures 6A and 6B). These results were congruent with results we obtained in model systems, which showed that BCL-2 expression in the absence of priming was insufficient to cause sensitivity to ABT-737 (Certo et al., 2006). To demonstrate that BIM's priming of BCL-2 was critical for the dependence on BCL-2, we showed that knockdown of BIM expression decreased sensitivity of both cells and mitochondria to BCL-2 antagonism (Figures 6C–6F). A weaker correlation was found linking sensitivity to BAX and BCL-2:BAX levels (Figure 5D). This may suggest that BCL-2 can be primed with BAX in certain lymphoma cells. If so, this is likely to reflect sequestration of BAX that has already been activated, as BCL-2 binds poorly to BAX before it undergoes the allosteric changes that accompany activation.

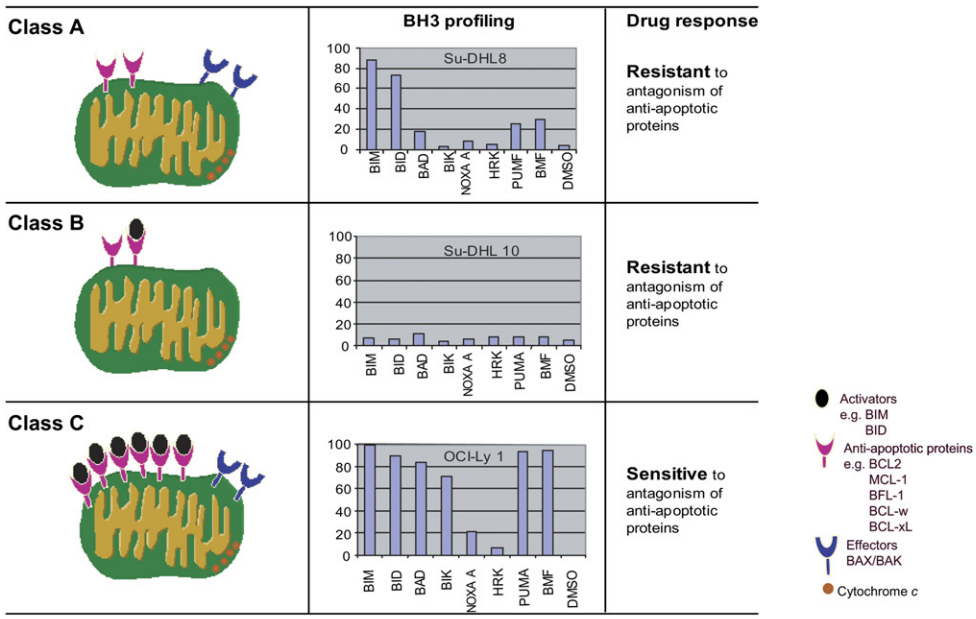
Recent work in model systems and acute myelogenous leukemia has suggested that expression of MCL-1 is a key determinant of resistance to ABT-737 (Konopleva et al., 2006; Lin et al., 2006; van Delft et al., 2006). It is indeed very plausible that high levels of MCL-1 expression are likely to render a cell resistant to ABT-737 treatment. Our results indicate, however, that while MCL-1 levels may well influence resistance, lack of MCL-1 is not the sole determinant of sensitivity, nor likely the most critical, at least in lymphoma. For instance, a simple inspection of Table 1 shows that OCI-Ly1 and OCI-Ly4 have nearly identical levels of MCL-1, but EC_{50} values that differ by more than 100-fold. Other similar examples are seen in Table 1. A more comprehensive assessment is found in the linear regression modeling, which reveals a suggestive, but fairly weak correlation between MCL-1 and log EC_{50} (Figure 5E). It remains to be seen whether other cell types show a stronger correlation between MCL-1 and EC_{50} of ABT-737. Mechanistically, we believe it is likely that MCL-1 overexpression would protect cells from ABT-737, as the drug cannot target MCL-1. However, while high levels of expression of MCL-1 may be expected to induce resistance, results here and elsewhere (Certo et al., 2006; Oltersdorf et al., 2005) confirm that even total absence of MCL-1 is unlikely to cause sensitivity to BCL-2 antagonism unless BCL-2 is present and primed with prodeath activator BH3-only proteins.

Recently, a report suggested that there are circumstances in which activation of BAX or BAK by BID or BIM is dispensable to the MOMP required for apoptosis in the intrinsic pathway (Willis et al., 2007). However, our results here further support the important role of BIM in activating BAX and BAK. Using a combination of BH3 profiling and ABT-737 treatment, we are able to determine which lymphoma lines are most dependent on BCL-2 function for ongoing survival. Remarkably, dependence on BCL-2 correlates very tightly, even quantitatively, with amount of BIM:BCL-2 complex (Figure 5B). Reduction of BIM reduces this dependence (Figures 6C, 6D, and 6F). Furthermore, overexpression of BCL-2 in the absence of BIM alone does not induce BCL-2 dependence (Figures 6A and 6B). BCL-2 dependence instead requires significant amounts of BIM to be sequestered by BCL-2. It is difficult to understand how BIM:BCL-2 complex levels would so quantitatively confer dependence on BCL-2 in a model in which BIM plays no role in activating BAX or BAK. These results are therefore less consistent with the "indirect activation" model in which the exclusive antiapoptotic function of BCL-2 is to bind unspecified subsets of BAX or BAX (Willis et al., 2007). They are instead more consistent with a model in which BCL-2 sequesters activators like BIM from activating BAX or BAK, resulting in a "primed" cell that is dependent upon BCL-2 function. It is worth noting that we cannot, and do not, claim that BIM or BID are the only activators. Indeed, evidence already exists that activation of BAX and BAK can occur via PUMA or p53, and there is no reason to believe that all activators have been discovered (Chipuk et al., 2004; Kim et al., 2006).

In this study, we focused on a single disease, diffuse large B cell lymphoma. We found that, in a group of cells that were superficially quite similar, vital molecular details could be revealed by BH3 profiling and by protein analysis driven by BH3 profiling results. These details explained an important therapeutic phenomenon, sensitivity to BCL-2 antagonism by ABT-737. BH3 profiling of lymphoma cells provides a valuable and unique insight into the diverse mechanisms maintaining their survival, immediately suggesting targets for therapeutic intervention. We suggest that, by identifying the class of apoptotic block, BH3 profiling can provide valuable information about a fundamental biological parameter of any cell.

(F) BH3 profiling for OCI-Ly1 Luc shRNA c1 and BIM shRNA c15 stable clones. Mitochondria were isolated from the respective clones and subjected to incubation with peptides BIM BH3 (10 μM), BAD BH3 (3 μM), or ABT-737 (30 nM).
(D)–(F) show the mean of three experiments; error bars show standard deviation.

A



B

Cell line	Apoptotic block	primed vs. unprimed	sensitivity to ABT-737
Su-DHL8	Class A	unprimed	resistant
Su-DHL10	Class B	unprimed	resistant
Su-DHL4	Class C - BCL-2	primed	sensitive
Su-DHL6	Class C - BCL-2/MCL-1	primed	sensitive
TOLEDO	Class C - BCL-2	primed	sensitive
OCI-Ly1	Class C - BCL-2	primed	sensitive
PFEIFFER	Class C - BFL-1	primed	resistant

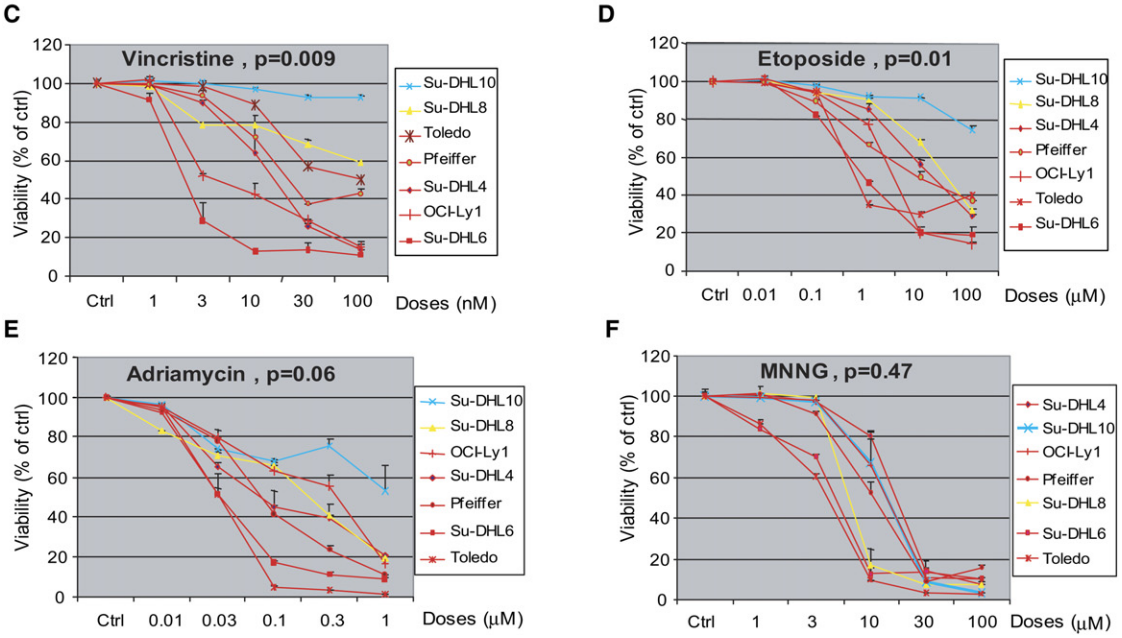


Figure 7. Three Classes of Apoptotic Escape for Cancer Cells Predict Sensitivity to Conventional Chemotherapy Agents

(A) Class A cells survive due to suppression of activator upregulation. Class B cells survive due to loss of BAX and BAK, the effector BCL-2 family proteins. Class C cells survive due to high expression of antiapoptotic proteins like BCL-2. In the middle are shown representative actual BH3 profiles, and at right are expected responses to antagonists of antiapoptotic proteins like ABT-737.

EXPERIMENTAL PROCEDURES

Cell Lines

DLBCL cells were cultured in suspension with RPMI 1640 medium or Iscove's modified Dulbecco's medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Sigma, F-6178), L-glutamine, and penicillin/streptomycin (Invitrogen).

Stable HT and SU-DHL8 cell lines carrying pCI-Neo.FlagBcl2 or control vector pCI-Neo.Flag were obtained by electroporation of plasmids into lymphoma cells (Gene Pulser II, Bio-Rad) followed by selection with G418 (Sigma, 1 mg/ml).

BIM ShRNA (pLKO.1puro-Bim) or Luc ShRNA (pLKO.1puro-luciferase) was obtained from the RNAi Consortium (Cambridge, MA). Knockdown cells were prepared by infecting lymphoma cells with retroviral supernatants produced by cotransfection of 293T cells with pCMVΔR8.91, pMD.G, and either pLKO.1puro-Bim or pLKO.1puro-luciferase (Del Gaizo Moore et al., 2007; Ernst et al., 2004). Stable clones were selected with puromycin (Sigma, 250 ng/ml) and maintained by changing cell culture media daily with fresh L-glutamine (2 mM).

BH3 Profiling

Mitochondria were purified from DLBCL cells by mechanical disruption followed by differential centrifugation, as previously described (Letai et al., 2002). Mitochondrial suspensions were made at 0.1 mg protein/ml in experimental buffer and exposed to BH3 domain peptides at 100 μM for 40 min at room temperature. Peptides used in this assay were synthesized by Tufts University Core Facility and purified by HPLC. Identity was confirmed by mass spectrometry. Stock solutions were made in dimethyl sulfoxide (DMSO). Peptide sequences are as previously reported (Certo et al., 2006). ABT-737 and its negative control enantiomer (Enant) were kindly provided by Abbott Laboratories (Abbott Park, IL). Release of cytochrome *c* was determined by a comparison of cytochrome *c* in the pellet and supernatant quantitated by ELISA (R&D Systems).

Cell Viability Assay

DLBCL cells were treated with ABT-737 or enantiomer as described in the figure legends. DMSO was used as a solvent-only negative control. After treatments, cells were stained with fluorescent conjugates of Annexin-V (BioVision) and analyzed on a FACSCalibur machine (Becton Dickinson). Viable cells are Annexin V-FITC negative. Vincristine, etoposide, and adriamycin were obtained from Sigma. MNNG was obtained from Fisher.

Immunoprecipitation and Immunoblotting

Protein lysates were obtained by cell lysis in Triton X-100 buffer (50 mM Tris-HCL [pH 7.4], 150 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 10% Glycerol, 1% Triton X-100 [Sigma]). Immunoprecipitation was performed in 600 μl lysates containing 400 μg proteins, which was precleared by centrifugation followed by exposure to 12 μl (50% slurry) protein A beads (Santa Cruz) at 4°C for 1 hr. Cleared extracts were incubated overnight with protein A beads pre-exposed for 1 hr to anti-BCL-2 antibody (6C8, PharMingen). Immunoprecipitates were then washed three times with Triton X-100 buffer and boiled in loading buffer (Invitrogen). Protein samples were electrophoretically separated on NuPAGE 10% Bis-Tris polyacrylamide gels (Invitrogen). Antibodies were used to detect the following proteins on membrane: BCL-2 (Epitomics, 1017-1); BCL-xL (kind gift from Larry Boise); BCL-w (Oncogene, 75-1); MCL-1 (Santa Cruz, S-19); BIM (Calbiochem, 22-40); BID (Santa Cruz, FL-195); BAX (Santa Cruz, N20); BAK (Upstate, NT), Actin (Chemicon, MAB1501).

Western Blot Protein Quantification

Densitometry of protein bands were acquired using an AlphaImager EC gel documentation system (Alpha Innotec Germany), and bands were analyzed with the spot densitometry analysis tool (Alpha Ease FC software, version, 4.1.0).

mRNA Comparison

Quantities of BFL-1 mRNA were measured according to the manufacturer's specification on an Affymetrix chip using the 205681_at probe.

Statistical Analysis

GraphPad Prism 4 software was used to determine EC_{50} values by nonlinear dose-response curve fitting. Correlation of EC_{50} with BCL-2 family proteins was obtained by a linear regression model.

Supplemental Data

The Supplemental Data include three supplemental figures and can be found with this article online at http://www.cancercell.org/cgi/content/full/12/2/171/DC1/.

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(B) Summary of BH3 profiling results on seven cell lines. Class C lines are further subdivided based on dependence on individual antiapoptotic proteins. For convenience, ABT-737 sensitivity also shown.
(C–F) Dose-response curves for the seven cell lines treated with vincristine (C), etoposide (D), adriamycin (E), and MNNG (F). Shown is the mean of three experiments; error bars show standard deviation. p values obtained from single-tailed Student's t test comparing primed (red lines) and unprimed (blue or yellow lines) cell lines.

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