Bcl-2, Bcl-x<sub>L</sub>, and Bcl-w are not equivalent targets of ABT-737 and navitoclax (ABT-263) in lymphoid and leukemic cells

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The BH3-mimetic ABT-737 and an orally bioavailable compound of the same class, navitoclax (ABT-263), have shown promising antitumor efficacy in preclinical and early clinical studies. Although both drugs avidly bind Bcl-2, Bcl-x<sub>L</sub>, and Bcl-w in vitro, we find that Bcl-2 is the critical target in vivo, suggesting that patients with tumors overexpressing Bcl-2 will probably benefit. In human non-Hodgkin lymphomas, high expression of Bcl-2 but not Bcl-x<sub>L</sub> predicted sensitivity to ABT-263. Moreover, we show that increasing Bcl-2 sensitized normal and transformed lymphoid cells to ABT-737 by elevating proapoptotic Bim. In striking contrast, increasing Bcl-x<sub>L</sub> or Bcl-w conferred robust resistance to ABT-737, despite also increasing Bim. Cell-based protein redistribution assays unexpectedly revealed that ABT-737 disrupts Bcl-2/Bim complexes more readily than Bcl-x<sub>L</sub>/Bim or Bcl-w/Bim complexes. These results have profound implications for how BH3-mimetics induce apoptosis and how the use of these compounds can be optimized for treating lymphoid malignancies. (Blood. 2012;119(24):5807-5816)

**Introduction**

Defects in the mitochondrial apoptotic pathway regulated by the Bcl-2 family of proteins play a major role in cancer development and in conferring chemoresistance. Within the family, Bax and Bak are essential for mitochondrial membrane permeabilization and cell death. Prosurvival proteins (Bcl-2, Bcl-x<sub>L</sub>, Bcl-w, Mcl-1, A1) oppose Bax and Bak and ensure mitochondrial integrity and cell survival. These prosurvival proteins also interact with distant relatives that share only 1 BH3 homology region, BH3, that is critical for their proapoptotic function. The BH3-only proteins such as Bim, Bad, Puma, and Noxa act as stress sensors and relieve the inhibition of Bax and Bak by the prosurvival proteins.

The clinical efficacy of most anticancer therapeutics primarily reflects their ability to induce apoptosis. Resistance to conventional anticancer therapeutics (eg, etoposide) is often because of a failure to activate BH3-only proteins, for example because of mutation of the tumor suppressor p53, which is critical for transcriptional induction of *Puma* and *Noxa* after DNA damage. Overexpression of prosurvival Bcl-2 proteins, or silencing of BH3-only protein expression, are also associated with inferior therapeutic outcomes. BH3-mimetic drugs, such as ABT-737, were developed to directly counter such apoptotic blocks. ABT-737 binds avidly to Bcl-2, Bcl-x<sub>L</sub>, and Bcl-w, but not Mcl-1 or A1. In preclinical studies, it demonstrated single agent efficacy against tumors with low Mcl-1 or A1 levels, such as follicular lymphoma (FL), chronic lymphocytic leukemia (CLL), and small cell lung carcinoma (SCLC). ABT-737 shows limited toxicity toward normal cells, although there is transient reduction of platelets and lymphocytes. ABT-263 (navitoclax), an orally bioavailable compound in the same class with similar target specificity, also exhibited efficacy in various cancer-derived cell lines both in vitro and in vivo, and is undergoing phase I/II clinical trials with encouraging results.

Overexpression of Bcl-2 or Bcl-x<sub>L</sub> has been implicated in the pathogenesis of lymphoid malignancies, and is frequently observed in CLL, acute lymphoblastic leukemia (ALL), FL, and diffuse large B-cell lymphoma (DLBCL). In some settings, elevated Bcl-2 or Bcl-x<sub>L</sub> expression directly correlates with poor responses to conventional therapies.

Hence, direct targeting of Bcl-2 and Bcl-x<sub>L</sub> constitutes a promising approach for treating such malignancies. In this study, we exploited a substantial panel of gene-targeted mouse strains to address the mechanism of cell killing by ABT-737 and ABT-263 in normal and transformed lymphoid cells. Given both ABT-737 and ABT-263 target Bcl-x<sub>L</sub>, Bcl-2, and Bcl-w, we investigated whether these drugs kill by inhibiting each of these, or by inhibiting 1 or 2 preferentially. Our study reveals that Bcl-2 is the key target in lymphoid cells. Indeed, whereas overexpression of Bcl-2 sensitizes cells to killing by ABT-737, elevated expression of Bcl-x<sub>L</sub> or Bcl-w confers resistance instead.


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Methods

Mice

Generation of varP-Bcl-2,18 varP-Mcl-1,20 Mcl-IRES,8 Ep-Myc,20 Bin88, Bin88 and Bim88,21 Bim88,22 Puma88, Noxa88, Bmf88, Bax88 (The Jackson Laboratory), Bak88, and Bax88/Bak88 mice were previously described. All animal experiments were performed in accordance with the guidelines of the Walter and Eliza Hall Institute (WEHI) Animal Ethics Committee.

Microarray and predictive model

IC50 values are the average of 3 independent experiments; curves were generated with a 4 parameter fit using XLFit. The mRNA expression levels of 10 Bcl-2 family members have been assessed by microarray, and correlated to resistance of 39 non-Hodgkin lymphoma (NHL) cells to ABT-263. The coefficient of correlation of each gene is represented in the linear predictor, ABT-263 Dx, with the positive sign indicating a resistant marker and the negative sign indicating a sensitive marker. The size of the coefficient of each gene correlates to its importance in the predictor. The linear predictor was generated with penalized lasso regression (cv.glmnet function in glmnet R package) based on 5-fold cross validation using the standardized expression intensities of the Bcl2 family members (Z scores). The ABT-263 Dx can be specified as: ABT-263 Dx = Z_{mBim} - 0.6 Z_{Bak} + 0.14 Z_{Noxa} - 0.05 Z_{Bax}. The publicly available DLBCL patient dataset was downloaded from GEO database (GSE10846, HGU133plus2). The linear regression predictor was then applied to the Z-score standardized patient microarray data to predict the ABT-263-Dx score of each patient. The survival analysis was conducted using Kaplan-Meier curves, which plots the 3 evenly divided patient groups based on their ABT-263-Dx score, and Cox proportional hazard, which fits the Dx score as a continuous variable (survival, R package).

Both microarray datasets were normalized using Robust Multi-array Average.

Cell lines, FACS analysis, and cell survival assays

Murine DO11.10 T-hybridoma cells expressing murine Bcl-2 or Bcl-xL subcloned into the mammalian expression vector pEF-FLAG PGK puro25 were cultured in DMEM containing 10% FCS, Methyl-10%/Eμ-Myc lymphoma cells were isolated from 2 independent tumors and cultured in Iscove modified Dulbecco medium (IMDM) containing 20% FCS, 2mM L-glutamine, 100 mg/mL murine stem cell factor, 10 mg/mL IL-3 (Peprotech). These cells were transduced with pMSCV-ires greens reporter protein (GFP)–based expression vectors as described,7 treated with 4-OHT (R-CHOP) or without rituximab (CHOP). In CHOP-treated cells were injected into lethally irradiated (2 × 550 rads) recipient mice, and the hematopoietic system of reconstituted mice was analyzed 8 weeks later.

Immunoprecipitations and Western blotting

Coimmunoprecipitations were performed as previously described21 and used a rat mAb for Bim (3C5, Enzo Biosystems) or a hamster monoclonal anti-human Bcl-2 (3F11), Anti-FLAG (clone 9H11). Anti-Bim (14A8 or 3C5; Alexis), anti-Bcl-2 (BD Pharmingen), anti–Bcl-xL (BD Bioscience), anti–Mcl-1 (Rockland), anti–Bcl-w (Stressgen), or anti-actin (Sigma-Aldrich) antibodies were used for Western blotting.

Affinity measurements

The affinity of recombinant mouse Bim, mouse NoxaA, Bin88, or human Bax for mouse Bcl-2 or mouse Mcl-1 was determined by solution competition assay using a Biacore 3000 instrument, as previously described.26 Peptides were purchased from Mimotopes Pty. Peptide sequences were: mBim DLRRIEIQRLIGDEMNLYTR; mBax BAXAFAALPKRLGKDYCTWTRR; mNoxaA RAELPPEFAAQLRKGIDKVKYCTWSAP; hNoxaA PAELEVCAATQLRFRGDKLNFQKLL.

Cellular protein redistribution assay

Genes encoding fluorescently tagged proteins were generated and cloned into the pFIT vector, and stable Flp-In T-REx 293 cell lines were generated as described.27 Cell culture, imaging, and protein redistribution were carried out as described.28

Statistics

Values used in heat maps are presented in supplemental Tables 1 and 2 (available on the Blood Web site; see the Supplemental Materials link at the top of the online article). They represent the mean of the EC50 collected from at least 3 independent experiments done in triplicate (except for Bax88/Bak88 transgenic mice for which only 1 experiment could be done in triplicate). Statistical tests were performed on the Log-EC50 values rather than he EC50 values to uphold the assumption of normality. Two sample t tests comparing each transgenic to WT were performed where Log-EC50 values were available. P values were adjusted for multiple testing within a cell type using the Holm method.

Results

High Bcl-2 expression correlates with sensitivity to ABT-263

To clarify further which Bcl-2 family members determine sensitivity of lymphoma cells to ABT-263, we analyzed the sensitivity of 39 human NHL cell lines in relation to the expression of 10 Bcl-2 family members, as determined by microarray analysis (Figure 1A). High expression of BCL-2 and NOXA mRNAs proved the strongest predictor of ABT-263 sensitivity (Figure 1B), as previously reported.28 In contrast, high MCL-1 expression was a strong indicator of resistance to ABT-263, in agreement with studies with diverse cell lines. Expression of the other genes tested (BID, PUMA, BAX, BAK, BIM, BAD, BCL-X, BCL-W, or A1) did not significantly correlate with ABT-263 sensitivity.

Based on these observations, we developed a linear predictor, denoted ABT-263-DX (see “Microarray and predictive model” for formula), to model correlations between gene expression and sensitivity to ABT-263, which we validated using a publicly available dataset (GSE10846)29 for human DLBCL. Patients in this cohort had received a standard combination chemotherapy of cyclophosphamide, doxorubicin, vincristine, and prednisone, with (R-CHOP) or without rituximab (CHOP).33 In CHOP-treated
DLBCL patients, low ABT-263-DX values (predicting high sensitivity to ABT-263) were strongly associated with inferior outcomes (Figure 1C, HR \( < 0.61 \), \( P < 0.00025 \)). Notably, this association was largely lost (\( P < 0.04 \)) when the data were adjusted for lymphoma subtype (germinal center B cell–like: GCB versus activated B cell–like: ABC), suggesting a probable correlation between ABT-263-DX and GCB/ABC subtype. A similar, albeit smaller, trend was also evident in R-CHOP–treated DLBCL patients (Figure 1C, HR \( < 0.82 \), \( P = 0.026 \)).

Taken together, these analyses suggest that the DLBCL patients most probable to respond to ABT-263 are those who fare poorly with conventional standard-of-care regimens.

High Bcl-2 levels also sensitize normal cells to ABT-263 and ABT-737

As elevated BCL-2 expression correlated with sensitivity to ABT-263 in lymphoma cells (Figure 1), we investigated whether
increased Bcl-2 also sensitizes their nontransformed counterparts. We focused on vavP–Bcl-2 transgenic mice, which overexpress human BCL-2 in all nucleated hematopoietic cells.18 As expected,18 Bcl-2 overexpression rendered vavP–Bcl-2 lymphocytes highly resistant to lymphotoxic agents such as dexamethasone (Figure 2A) and etoposide (Figure 2B).

In striking contrast, multiple lymphoid subpopulations isolated from the thymi or BM of vavP–Bcl-2 transgenic mice were markedly more, not less, sensitive to ABT-263 or ABT-737 (Figure 2C). This was most evident for the lymphoid populations normally resistant to these BH3-mimetics, such as BM transitional B cells (IgMhiIgDmed) or immature B cells (IgM hiIgDlo). This sensitization is not a general consequence of prosurvival protein overexpression because similar cells isolated from vavP–Mcl-1 transgenic mice19 were highly resistant to ABT-737 (Figure 2D). Treatment with ABT-737 did not induce a detectable change in the expression of Bcl-2, Bim, Mcl-1, or Bcl-xL in the cells analyzed (supplemental Figure 1).

Loss of Bim, or combined absence of Bax and Bak, renders lymphoid cells resistant to ABT-737

We next studied the impact on ABT-737–induced cell death when Bim, Noxa, Puma, or Bmf are deleted because the corresponding BH3-only proteins are key regulators of lymphoid apoptosis.22 Whereas loss of Noxa, Puma or Bmf had minimal impact on sensitivity to ABT-737, loss of Bim rendered all lymphoid subpopulations examined highly resistant to ABT-737 (Figure 2D). Treatment with ABT-737 did not induce a detectable change in the expression of Bcl-2, Bim, Mcl-1, or Bcl-xL in the cells analyzed (supplemental Figure 1).

Overexpression of Bcl-2 causes Bim accumulation

These studies demonstrate the critical role of Bim in ABT-737–induced apoptosis. Because Bim mRNA expression did not predict sensitivity to ABT-263 (Figure 1), we next examined the expression of Bim protein in 14 of the NHL cell lines classified as sensitive (IC50 < 1μM) or resistant (IC50 ≥ 5μM) to ABT-263. Notably, all ABT-263–sensitive cell lines exhibited higher levels of Bim and Bcl-2 than the resistant lines (Figure 4A), consistent with earlier observations.15 Bim protein levels were also markedly higher in vavP–Bcl-2 lymphocytes than their WT counterparts (Figure 4B). Moreover, the half-life of Bim was significantly extended in vavP–Bcl-2 compared with WT cells (supplemental Figure 2A). These observations suggest that binding to Bcl-2 protects Bim from degradation.

To test this further, we crossed vavP–Bcl-2 mice with Bim-BH3 “knock-in” mice (BimBW, BimN, or BimP) that express mutant Bim proteins that have had their native BH3 domain replaced with that of mouse Bad, mouse NoxaA, or mouse Puma (BimBad, BimN, and BimP) and exhibit their binding specificities.21 Consistent with the notion that binding to Bcl-2 is necessary to drive Bim accumulation in lymphoid cells expressing the vavP–Bcl-2 transgene, BimBad and BimP were as elevated as WT Bim (Figure 4B). BimN levels were elevated to a lesser degree, reflecting the lower affinity of native NoxaA for Bcl-2.19 The strong association of Bcl-2 with WT Bim, BimBad and BimP was confirmed by coimmunoprecipitation (Figure 4C). It is
formation with prosurvival proteins significantly prolongs the half-life from control mice. Taken together, these results indicate that complex expressed. By contrast, WT Bim and BimNoxa were elevated in the Table 1). In contrast, ABT-737, regardless of Bcl-2 expression (Figure 4E, supplemental heat map. Blue represents sensitivity, and red, resistance. (B) The hematopoietic system of lethally irradiated mice was reconstituted with both BimNoxa and BimPuma can bind Mcl-1, but BimBad cannot. Notably, the level of Bcl-2 bound to Bim or the Bim mutants consistently decreased after ABT-737 treatment, whereas the level of Mcl-1 complexed with Bim or its mutants increased, except as anticipated in BimBad-expressing cells (Figure 4F).

Collectively, these findings demonstrate that for ABT-737 to trigger killing of Bcl-2–overexpressing lymphoid cells, Bim must be available to neutralize Mcl-1, which is not directly targeted by ABT-737.

Overexpression of Bcl-xL, or Bcl-w, does not confer sensitivity to ABT-737

Because ABT-737 binds purified recombinant Bcl-2 and Bcl-xL proteins with similar affinities, it was surprising that the level of BCL-XL expression did not correlate with the sensitivity of NHL cells to ABT-263 (Figure 1). To investigate this further, we reconstituted the hematopoietic system of lethally irradiated mice with WT fetal liver cells infected with retroviruses encoding FLAG-tagged Bcl-2, Bcl-xL, Bcl-w, or Mcl-1. We confirmed that all 4 proteins were functionally overexpressed, clearly protecting against etoposide and dexamethasone (supplemental Figure 3A).

In the experiments described above (Figure 2), overexpression of Bcl-2 sensitized cells to ABT-737 treatment, whereas overexpression of Mcl-1 conferred resistance (Figure 5A-B). In surprising contrast to the effect of Bcl-2 excess, overexpressed Bcl-xL and Bcl-w induced dramatic ABT-737 resistance in all BM and thymic lymphoid subsets (Figure 5A, supplemental Figure 3B, and supplemental Table 2 for values). As with Bcl-2 and Mcl-1, overexpression of Bcl-xL or Bcl-w elevated levels of Bim (Figure 5B), which was sequestered in complexes with these prosurvival proteins (supplemental Figure 4A). High Bcl-2, Bcl-xL, or Bcl-w levels protected Bim from degradation (supplemental Figure 4B) but did not increase Bim mRNA levels (supplemental Figure 4C). These results were confirmed in the T-cell hybridoma line DO11.10. Overexpression of Bcl-2 sensitized DO11.10 cells to ABT-737, whereas overexpression of Bcl-xL protected them against this compound (Figure 5C), even though each augmented the levels of Bim comparably (Figure 5D).

To determine whether the opposing effects of Bcl-2 and Bcl-xL on ABT-737–induced apoptosis hold in a tumor model, we used the Eμ-Myc transgenic mice. Eμ-Myc–driven lymphomas exhibit a high degree of resistance to ABT-737, because of high levels of Mcl-1. We generated lymphomas in Eμ-Myc/Mcl-1flox/flox/...
RosaCreER mice, so that Mcl-1 could be deleted at will by 4-hydroxytamoxifen (4-OHT) treatment. Overexpression of Bcl-2 in Eμ-Myc/Mcl-1<sup>Flox/Flox</sup>/RosaCreER cells sensitized these lymphoma cells to in vitro treatment with ABT-737, especially when endogenous Mcl-1 was removed (Figure 5E-F). In contrast, Bcl-xL or Mcl-1–overexpressing cells were resistant to ABT-737, even in the absence of endogenous Mcl-1 (Figure 5E-F). These data are consistent with our findings in human NHL cells. Overall, our conclusion is that ABT-737 is much more efficient at killing cells that overexpress Bcl-2 than it is at killing cells that overexpress Bcl-xL or Bcl-w (Figure 1B).

**Figure 4. Sensitization of vav-Bcl-2 lymphocytes to ABT-737 requires the binding of Bim to Mcl-1.** (A) Western blot analysis of NHL cell lines having higher or lower levels of Bcl-2 RNA by microarray analysis (Figure 1). Those expressing higher levels of Bcl-2 protein are sensitive to ABT-263 and express higher levels of Bim. (B) Expression of Bcl-2 and Bim in vavP–Bcl-2/Bim<sup>+/+</sup>, vavP–Bcl-2/Bim<sup>RR</sup>, vavP–Bcl-2/Bim<sup>NN</sup>, and vavP–Bcl-2/Bim<sup>RR</sup> thymocytes was determined by Western blotting. (C) Thymocyte lysates were prepared and the protein complexes were analyzed by immunoprecipitation and Western blotting. (D) Affinities of Bim and Noxa BH3 peptides for mouse Bcl-2 and mouse Mcl-1 were determined by solution competition assay using a Biacore optical biosensor. Numbers in brackets represent standard deviations for n = 2 to 4 experiments. (E) The sensitivity of BM-derived B cells and thymocytes of the indicated genotypes to ABT-737 was measured and data are represented in a heat map as described in Figure 2. Results represent the mean of at least 3 independent experiments per genotype (detailed in supplemental Table 1). Restricting the binding specificity of Bim to that of Bad renders all cell types resistant to ABT-737, regardless of Bcl-2 overexpression. As a control, the BH3 mutations in Bim did not affect the resistance of vavP–Mcl-1–transgenic cells to ABT-737–induced cell death (vavP–Mcl-1/Bim<sup>NN</sup> and vavP–Mcl-1/Bim<sup>RR</sup>). (F) Thymocytes from vavP–Bcl-2/Bim<sup>+/+</sup> mice were treated with ABT-737 (1 μM) for 5 hours before Bim immunoprecipitation. The composition of the protein complexes was analyzed by Western blotting.

ABT-737 releases Bim from sequestration by Bcl-2 more readily than Bim bound to Bcl-xL or Bcl-w

To fully investigate why overexpressing Bcl-2, but not Bcl-xL or Bcl-w, sensitizes cells to ABT-737 even though it binds all of them in vitro,6,7 we tested the susceptibility of their cellular complexes to ABT-737 treatment. In this cellular redistribution assay,26 mCherry-tagged Bim<sub>ΔC</sub> (a mutant lacking the C-terminal membrane targeting domain) was sequestered to the mitochondria by binding to eGFP-tagged full-length Bcl-2, Bcl-xL, or Bcl-w (Figure 6). On addition of ABT-737, mCherry Bim<sub>ΔC</sub> was displaced from the
mitochondria in a time-dependent manner (Figure 6). Strikingly, ABT-737 was 5-fold more potent at displacing Bim from Bcl-2 than from Bcl-xL or Bcl-w. The 6-fold higher affinity of Bim for Bcl-xL than for Bcl-2 that we previously reported probably contributes to this difference. Thus, the differential impact of ABT-737 on cells with elevated Bcl-2, Bcl-xL, or Bcl-w can be accounted for by its differential ability to disrupt cellular complexes of Bim with these prosurvival proteins (supplemental Figure 6).

Discussion

The recently completed phase 1 trials have established the safety and optimal dosing of navitoclax (ABT-263). Significant objective responses were achieved in 30%-50% of patients with CLL, but not in patients with other lymphoid neoplasms or SCLC, despite encouraging preclinical activity of ABT-263 in all these tumors. To better understand determinants of ABT263/ABT-737 activity, we conducted detailed mechanism-of-action studies in cell lines as well as primary mouse lymphocytes isolated from a large panel of mouse strains that harbor mutations in one or more Bcl-2 family member. Our focus here is on lymphoid compartment because lymphoid malignancies are the most promising indications for ABT-263 therapy.

Consistent with recent studies demonstrating that Bcl-2 levels correlate with sensitivity to ABT-737, we found that high BCL-2 was the best predictor of sensitivity to ABT-263 in a panel of lymphoid cells.
Figure 6. ABT-737 exhibits greater potency at displacing BimS&LC from Bcl-2 than from Bcl-xL or Bcl-w. (A) Displacement of mCherry-BimS&LC from the mitochondria was measured at 350 minutes after compound addition and normalized to DMSO controls at 1.0. (B) EC₅₀ values were estimated by fitting mitochondrial displacement of mCherry-BimS&LC in response to ABT-737 to sigmoid curves and averaged for 2 to 4 biologic replicates. *P* values comparing Bcl-2 with Bcl-xL and Bcl-w were .0009 and .028, respectively. Error bars represent SE, and *P* values were calculated using a Student *t* test.

39 NHL cell lines. Our data thus provide support of why ABT263 may have such benefit in CLL as these tumors have minimal to no BCL-xL. In accord with this, our initial studies revealed that the lymphoid subpopulations known to express low levels of Bcl-2, such as immature B cells, are insensitive to ABT-737. Notably, the resistant subpopulations were markedly sensitized to ABT-737 by overexpression of Bcl-2, which in contrast provided dramatic protection against other chemotherapeutics.

Why are Bcl-2–overexpressing cells more sensitive to ABT-737? At first glance, this is counterintuitive because in most cases, increasing the amount of the drug target should raise the barrier to cytotoxicity. Our studies confirm that ABT-737 triggered Bax/ Bak-dependent apoptosis and establish definitively that increased Bim is the key driver of ABT-737 lymphotoxicity, in general agreement with previous studies in cell lines and the central role of Bim in lymphoid homeostasis. Overexpression of any prosurvival Bcl-2 family protein caused the accumulation of Bim in nontransformed as well as malignant lymphoid cells, confirming that the formation of complexes with prosurvival proteins is an important mechanism regulating the steady state level of Bim.

We show that Bcl-2/Bim complexes are highly susceptible to disruption by ABT-737, and that the released Bim then binds to Mcl-1. It is also conceivable that some of the Bim released from Bcl-2 also directly activates Bax or Bak. Our studies with Bim mutants indeed revealed that the 3 forms of Bim able to bind Mcl-1 with high affinity (WT Bim, BimPuma or BimNoo) were able to kill lymphoid cells on ABT-737 treatment, whereas the one form (BimBud) that binds Mcl-1 poorly was associated with complete resistance, regardless of whether Bcl-2 is overexpressed. The lack of a good antibody against A1 prevented us from considering it further. However, we do not exclude that A1 may play a similar role as Mcl-1 in certain subpopulations, because it was shown to be up-regulated, together with Bcl-xL, in response to cell-cell interactions within the lymph node microenvironment and be the reason of acquired resistance to ABT-737.

Perhaps the most important and unexpected outcome from our study, confirmed in different lymphoid cell types, is that increasing Bcl-2, but not Bcl-xL or Bcl-w, sensitizes cells to ABT-737 even though it binds tightly to all 3 in cell-free assays. Of note, protection of lymphoid cells from ABT-737 by Bcl-w was previously reported, although this study also reported that both Bcl-2 and Bcl-xL overexpressing Eµ-Myc lymphoma cells were sensitive to ABT-737. Interestingly, CD4⁺8⁺ thymocytes, which are highly sensitive to many apoptotic stimuli (eg, DNA damage, glucocorticoids) were the cell type most resistant to ABT-737 treatment in WT mice. We can now account for this observation, as CD4⁺8⁺ thymocytes depend on Bcl-xL for their survival and ABT-737 appears to be relatively inefficient at disrupting Bcl-xL/Bim complexes. Consistent with this notion, we recently observed that exposure of Mcl-1–deficient fibroblasts to increasing doses of ABT-737 selected for the emergence of resistant clones that had markedly up-regulated Bcl-xL, but not for cells increased Bcl-2 (our unpublished observations).

Interestingly, the primary dose-limiting toxicity of ABT-263 (recapitulated by ABT-737 in mice) is thrombocytopenia because of Bcl-xL antagonism. It thus appears that although these BH3-mimetics target Bcl-xL with sufficient avidity to kill platelets, they are not efficient antagonists of Bcl-xL in lymphoid or leukemic cells. This probably reflects the importance of Bad and Bak, compared with Bim, for platelet survival; indeed, ABT-737 is more efficient at disrupting Bad/Bcl-xL complexes than Bim/Bcl-xL complexes in cells (supplemental Figure 5). Sensitivity or resistance to ABT-737 thus depends on the composition of the key complex formed between the predominant prosurvival and proapoptotic proteins in a given cell.

The differential impact of compounds like ABT-737 on Bcl-2 and Bcl-xL is highly relevant for cancers because elevation of Bcl-xL is closely linked to chemoresistance including to ABT-737. Our studies presage the requirement to identify the critical complex that needs to be targeted by BH3-mimetics for optimal cell killing. Whereas elevated levels of Bim/Bcl-2 complexes indicate sensitivity to ABT-737 in lymphoid cells (this study) and certain breast tumors, high levels of Bim/Bcl-xL complexes are associated with resistance. Our results thus highlight the importance of determining the ability of BH3-mimetics at disrupting complexes between pro and antiapoptotic proteins in vivo, which might not reflect their affinity for prosurvival proteins in vitro. The strategy to achieve high kill rates will then depend on choosing the optimal BH3-mimetic to target the key complex in each tumor. Although these key complexes have not been established for most tumors, our studies lay the groundwork and principles necessary for the optimal preclinical development and the choice of malignancy probably to be targeted by specific BH3-mimetics currently under development.
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Authorship

Contribution: D.M. and S.L.K. conducted most of the experiments; S.P.G. generated the Mcl-1i30/Ep-Myc lymphoma cells; L.D.B. and P.Y. performed the microarray experiments; M.R. helped with FACS analysis; B.P. analysed results and the heat-maps; W.D.F. and E.F.L. did the Biacore measurements; K.J.C., C.J.V., and S.C. generated the vavP-Mcl-1 and vavP-Bcl-2/Bax- or Bak-deficient mice; C.H.W., D.J.A., and M.J.C.L. designed and performed the protein redistribution essays; A.W.R. gave useful advice; and D.M., S.L.K., D.C.S.H., and P.B. designed the project and wrote the paper.

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