Selective BCL-2 Inhibition by ABT-199 Causes On-Target Cell Death in Acute Myeloid Leukemia


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INTRODUCTION

Acute myeloid leukemia (AML) is a hematopoietic neoplasm characterized by the rapid, clonal growth of the myeloid lineage of blood cells. The disease affects approximately 14,000 adults in the United States each year and unfortunately, despite recent advances in the treatment of AML, 10,400 people die from their disease (1). Most patients with AML become resistant to chemotherapy at some point in their course and succumb to their disease. Therefore, it is necessary to prevent chemoresistance or enhance chemosensitivity in a selective fashion to lead to a higher cure rate and a lower toxic burden.

A novel strategy to treat cancer cells is to directly stimulate the mitochondrial apoptotic pathway. The mitochondrial apoptotic pathway is regulated by the B-cell leukemia/lymphoma 2 (BCL-2) family of proteins. These proteins respond to upstream activator and effector proteins to drive mitochondrial permeabilization (MOMP) and the release of cytochrome c, a hallmark of mitochondrial apoptosis (2). The family consists of proapoptotic BH3-only proteins, proapoptotic multi-domain effector proteins, and antiapoptotic proteins which together act as a rheostat to control MOMP. The BH3-only proteins are further subdivided into two groups based on function—the “activators” (BID, BIM, and PUMA) and “sensitizers” (such as BAD, BIK, HRK, and NOXA; refs. 3–5). The activators bind to and inhibit the antiapoptotics (such as BCL-2, BCL-XL, and MCL-1), and also directly interact with the effector proteins, causing the proteins to oligomerize at the mitochondria and cause MOMP (6, 7). However, the BH3-only sensitizers can only bind to the antiapoptotics, which allows for the release of the activator and effector proteins to drive MOMP (3).

Navitoclax (ABT-263), which functions as a small-molecule mimetic of the BH3 domain of the BH3-only sensitzer protein BAD, efficiently binds to BCL-2, BCL-XL, and BCL-W, releasing bound proapoptotic proteins and causing MOMP in BCL-2–dependent cancer cells (8). In early clinical trials, navitoclax showed potency in the treatment of chronic lymphocytic leukemia (CLL) and small-cell lung cancer (9, 10). However, treatment with navitoclax causes on-target, dose-limiting thrombocytopenia because platelets are dependent on the antiapoptotic protein BCL-XL for their survival (11). The dose-dependent thrombocytopenia limited the use of navitoclax in many malignancies, particularly leukemias where patients often present with preexisting thrombocytopenia. This prompted the development of ABT-199, a modified BH3-mimetic derivative of ABT-263 which maintains specificity for BCL-2, but lacks affinity for BCL-XL (12). The remodeled drug has shown cancer-killing efficacy in CLL in vivo, in Myc-driven lymphomas in mice, and in estrogen receptor–positive breast cancer while sparing platelets (12–14).

AML bulk and stem cells are dependent on BCL-2 for survival, and BCL-2 inhibition by ABT-737 (an in vitro tool compound with activity very similar to navitoclax) causes cell death in AML cells (15). Importantly, BCL-2 inhibition

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RESULTS

ABT-199 Kills AML Cell Lines Potently and Quickly In Vitro and In Vivo

As an initial test of the potential utility of ABT-199 in AML, we exposed AML cell lines to increasing concentrations of ABT-199 for 48 hours and then determined the IC_{50} values. Comparisons were made with ABT-737. As shown in Fig. 1A, the IC_{50} of ABT-199 ranged from <10 nmol/L to >1,000 nmol/L, and sensitivity to ABT-737 roughly tracked with sensitivity to ABT-199. It is notable that in sensitive AML cell lines (IC_{50} < 0.1 μmol/L), ABT-199 is more potent than ABT-737 (Supplementary Fig. S1A and S1B), probably due to ABT-199’s 5-fold higher affinity to BCL-2 protein (12). Prior experience with CLL, a disease for which excellent clinical activity of ABT-199 has been observed, has revealed that CLL cells are killed in an on-target fashion, and that the killing was evident within 4 hours (21). Therefore, we tested whether ABT-199 could rapidly induce apoptosis in a sensitive AML cell line, MOLM-13. In Fig. 1B and C, it can be seen that cell growth is inhibited and cell apoptosis is observed within just a few hours of exposure to ABT-199.

To demonstrate that the efficacy seen was consistent with tolerable in vivo delivery of ABT-199, we tested the effect of ABT-199 on an aggressive mouse xenograft model of MOLM-13. Nonobese diabetic/severe combined immunodeficient gamma (NSG) mice were injected with luciferase-labeled MOLM-13 cells and monitored by bioluminescence imaging (BLI) for tumor development. After confirmation of AML engraftment in the bone marrow (Fig. 1D, day 4), the mice were treated with ABT-199 (100 mg/kg) by daily oral gavage for 2 weeks. Serial BLI images showed that ABT-199 treatment markedly inhibited leukemia progression, which translated into prolonged overall survival when compared with vehicle-treated mice (P = 0.0004; Fig. 1E). ABT-199 treated mice also carried significantly lower leukemia burden in bone marrow, spleen, and liver as indicated by hematoxylin and eosin staining (H&E, Fig. 1F) and immunohistochemical analysis of human CD45 (Fig. 1G).

ABT-199 Sensitivity Correlates with BCL-2 Protein Level

Next, we tested whether there were correlates of cell line sensitivity to ABT-199 that supported an on-target action of killing via competition for the BH3 binding site of BCL-2. Relative levels of several BCL-2 family proteins were measured by Western blot analysis and densitometry (Fig. 2A). Spearman analysis was performed to evaluate the correlation between IC_{50} values and protein expression. Levels of BCL-2 correlated with sensitivity to ABT-199, whereas levels of BCL-XL inversely correlated with ABT-199 sensitivity (Fig. 2B). Levels of MCL-1 demonstrated a trend to anticorrelation with sensitivity to ABT-199, but the trend was not statistically significant (Fig. 2B). These observations supported the on-target effects of ABT-199.

The OCI-AML3 cell line was relatively insensitive to ABT-199 and ABT-737 (Fig. 1A). A quantitative immunoblot showed that OCI-AML3 cells had high expression of BCL-2 and MCL-1 and a relatively low level of BCL-XL (Fig. 2A). If ABT-199 is a BH3 mimetic specific for BCL-2, then MCL-1 knockdown should significantly sensitize OCI-AML3 cells to this compound. To test this hypothesis, MCL-1 protein level was reduced by 85% in OCI-AML3 cells by lentiviral transduction with a previously validated MCL-1–specific shRNA, without affecting BCL-2 protein expression (Fig. 2C). Indeed, MCL-1 knockdown greatly increased sensitivity to ABT-199 (Fig. 2D) as well as to ABT-737 (Supplemental Fig. S2).

HL-60 cells with high levels of BCL-2 protein and relatively low BCL-XL and MCL-1 expression are very sensitive to ABT-199 (Fig. 1A). BCL-XL overexpression conferred resistance to ABT-199 in HL-60 cells, whereas BCL-2 overexpression made HL-60 cells moderately resistant to ABT-199 (Fig. 2E and F). All these results are consistent with a killing mechanism operating via selective targeting of BCL-2 in AML cells.

ABT-199–Resistant MOLM-13 Cells Express Lower BCL-2 Levels Compared with the Parental Cells

Although ABT-199 effectively induced apoptosis in MOLM-13 cells, a fraction of MOLM-13 cells remained alive after 24 hours of treatment with 50 nmol/L ABT-199 (Fig. 1C). To determine whether surviving cells represent a subclone with low target (BCL-2) expression levels, we selected ABT-199–resistant MOLM-13 cells by culturing parental cells in medium containing 50 nmol/L ABT-199. Intracellular flow cytometry was performed to measure cellular BCL-2 protein in the parental and ABT-199–resistant MOLM-13 cells. Although no distinct subpopulations were observed, the BCL-2–associated median fluorescence intensity (MFI) of the parental MOLM-13 cells distributed between...
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**Figure 1.** Selective inhibition of BCL-2 by ABT-199 kills AML cell lines quickly and effectively. A, AML cell lines were treated with ABT-199 or ABT-737 for 48 hours. Calcusyn software was used to calculate the IC₅₀ values based on the number of viable cells (i.e., Annexin V⁻ /PI⁻) determined by fluorescence-activated cell sorting (FACS) analysis. B, MOLM-13 AML cells were treated with indicated concentrations of ABT-199. Apoptosis induction was determined by Annexin V/PI flow cytometry. C, viable (i.e., Annexin V⁻ /PI⁻) cell counts were quantified by FACS analysis using CountBright counting beads. D, serial bioluminescence images of mice bearing MOLM-13 tumors treated with the vehicle or ABT-199 (treatment started on day 4, administered by oral gavage at dose of 100 mg/kg). E, Kaplan–Meier survival curves for mice treated as described in D (n = 7/arm). Statistical significance was calculated using Log rank (Mantel-Cox) test (P < 0.0004). F, H&E staining of histologic sections of liver, spleen, and bone marrow 15 days after leukemia cell injection. Age- and sex-matched mice without tumor were used as controls. Representative MOLM-13 cells are indicated by arrows. Representative engraftment areas are circled in green. All pictures were taken under the same magnification; scale bar, 50 μm. G, immunohistochemical staining of histologic sections of liver, spleen, and bone marrow with human CD45 (hCD45) antibody 15 days after leukemia cell injection. Scale bar, 50 μm.

approximately 1 and 20 (Supplementary Fig. S3A), indicating a heterogeneity of BCL-2 expression in the parental cells. In sum, resistant cells expressed lower levels of BCL-2 protein compared with the parental cells (resistant cells, MFI = 3.48; parental cells, MFI = 5.51; P < 0.0001, Supplementary Fig. S3B).

**ABT-199 Operates Selectively on BCL-2-Dependent Mitochondria**

If ABT-199 is killing cancer cells via displacement of pro-apoptotic proteins from BCL-2, it should be operating on mitochondria. As one would expect if this were the case, we
observed a correlation between direct mitochondrial toxicity and cellular toxicity for ABT-199 and ABT-737 in the 12 cell lines studied in Fig. 1A (Fig. 3A and B). We also tested whether detection of BCL-2 dependence using mitochondrial exposure to the BAD BH3 peptide correlated with cellular sensitivity to these agents. We found that although there was a good correlation between mitochondrial sensitivity to the BAD peptide and cellular drug sensitivity for the most-sensitive cell lines, there was a group of relatively drug-resistant cell lines that still demonstrated mitochondrial sensitivity to the BAD BH3 peptide (Fig. 3C and D). A clue to the reason for this observation was revealed by the tendency of these cell lines to have mitochondria that were also quite sensitive to the HRK peptide, an indicator of BCL-XL dependence. To ensure that we were studying BCL-2 dependence specifically, especially in these less drug-sensitive cells, we made a correction, by subtracting the HRK signal from the BAD signal. In Fig. 3E and F, we used this modified metric to observe a good correlation between mitochondrial BCL-2 dependence and cellular sensitivity.

**ABT-199 Efficiently Kills Primary AML Myeloblasts**

We observed that ABT-199 selectively kills BCL-2 dependent cell lines; therefore, we next wanted to test the sensitivity of primary patient AML samples to treatment with ABT-199. AML myeloblasts from patient bone marrow or peripheral blood were exposed to ABT-199 for 48 hours in minimum essential medium alpha supplemented with cytokines. It is notable that the primary cells were quite sensitive, with median IC_{50} values less than 10 nmol/L (Fig. 4A). Note that
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Figure 3. ABT-199 functions selectively on BCL-2–dependent mitochondria in AML cell lines. A, the IC_{50} values of AML cell lines treated with ABT-737 from Fig. 1A were correlated with the mitochondrial response of ABT-737 (1 μmol/L). Mitochondrial response was measured by JC1-based BH3 profiling. B, IC_{50} values of cell lines treated with ABT-199 from Fig. 1A were correlated with the mitochondrial response of mitochondrial ABT-199 (0.1 μmol/L). C, IC_{50} values of AML cells treated with ABT-737 were correlated with the response to the BAD BH3 (80 μmol/L). The mitochondrial responses to the BAD BH3 peptide were measured by JC1-based BH3 profiling. D, IC_{50} values of AML cells treated with ABT-199 from Fig. 1A were correlated with the mitochondrial response of the BAD BH3 (80 μmol/L) – HRK BH3 (80 μmol/L) peptide. Statistical correlation was performed using a one-tailed Spearman ρ using GraphPad Prism 6.
Figure 4. ABT-199 efficiently kills primary AML myeloblasts as a single agent. A, IC₅₀ determination for ABT-199 and ABT-737 treatment of primary AML samples. Fresh mononuclear cells from patients with AML were isolated from bone marrow or peripheral blood and treated with ABT-199 and ABT-737 for 48 hours. The IC₅₀ values were calculated on the basis of viable (i.e., Annexin V⁻/PI⁻) cell numbers determined by fluorescence-activated cell sorting (FACS) analysis. Samples with ABT-199 IC₅₀ < 0.1 μmol/L were defined as “sensitive,” whereas those with ABT-199 IC₅₀ > 1 μmol/L were defined as “resistant.” B, frozen primary AML myeloblasts were thawed and treated with ABT-199 and ABT-263 for 8 hours in the absence of FBS. Viability was assessed by Annexin V⁻/PI⁻ via FACS analysis, and IC₅₀ values were calculated using GraphPad Prism software. C, thawed primary AML samples were treated for 2 hours with 1 to 1000 nmol/L of ABT-199 and viability was assessed by Annexin V⁻/PI⁻ by FACS analysis. D, nonparametric Spearman correlation analysis shows a significant (P = 0.017) negative correlation between ABT-199 IC₅₀ values and BCL-2 protein levels. E, a nonsignificant (P = 0.069) positive correlation was observed between ABT-199 IC₅₀ values and BCL-XL protein levels. F, boxplots represent the quartiles and range of log₂ values of mRNA expression for BCL-2 genes in different subgroups of AML and normal bone marrows. The median is indicated by the black line in each box. Numbers on top indicate number of patients in each specified subgroup. Differences in gene expression with P values ≤ 0.005 were considered statistically significant, as denoted by * . G, AML patient samples treated with 100 nmol/L ABT-199 for 24 hours were subjected to FACS analysis of specific apoptosis based on Annexin V staining in bulk AML myeloblasts and CD34⁺/CD38⁻/CD123⁺ leukemia stem/progenitor cells (LSPC). Symbols represent individual patient samples. P value was determined via paired t test.
this is significantly lower than the IC$_{50}$ observed in the AML cell lines (Fig. 1A). Note also that this is comparable to the sensitivity observed for ex vivo exposure of CLL cells, a disease in which ABT-199 has shown clinical activity in most patients treated (21).

Because prolonged ex vivo culture by itself can promote sensitivity to apoptosis of AML myeloblasts, we wanted to test whether we would see sensitivity at shorter time points as well. Another potential confounding element is that the AML class of compounds tends to be highly bound by serum proteins (22). We found that culturing AML cell lines or primary cells in the absence of serum for 8 hours did not alter the mitochondrial priming or BCL-2 dependence, compared with culture in the presence of serum (Supplementary Fig. S4). Therefore, we tested the sensitivity of AML myeloblasts to ABT-199 at 8 hours in the absence of serum (Fig. 4B). Clinical and genetic data for both sets of AML samples is available in Supplementary Table S1. Again, AML myeloblasts proved to be sensitive to ABT-199 with a median IC$_{50}$ of 20 nmol/L. Indeed, when we reduced exposure time further, to 2 hours, we could still see induction of apoptosis by ABT-199 in AML myeloblasts (Fig. 4C). Similarly rapid induction of cell death has been observed for the clinically sensitive CLL, consistent with a direct action of ABT-199 on AML myeloblast mitochondria, promoting apoptosis in the absence of a requirement for additional cell signaling extrinsic to the mitochondria.

Upon testing of additional cryopreserved AML patient samples, including AML cells with diploid cytogenetics and mutations in FLT3, NRAS, and NPM1 genes, 20 of 25 (80%) were sensitive to ABT-199 (100 nmol/L), and 5 samples were resistant to both ABT-199 and ABT-737 (Supplementary Table S2). However, samples from patients with complex cytogenetics and JAK2 mutation ($n = 9$) were largely insensitive to ABT-199 (1/9, or 11.1% response rate; $P = 0.0005$ by two-tailed Fisher exact test). Furthermore, we found no correlation between ABT-199 sensitivity and FAB classification (Supplementary Fig. S5A) or NPM1 (Supplementary Fig. S5B) or FLT3 mutational status (Supplementary Fig. S5C). There was no difference in ABT-199 sensitivity between samples sensitive or resistant to conventional induction chemotherapy (Supplementary Fig. S5D), consistent with prior findings with ABT-737 (16).

We next tested whether sensitivity to ABT-199 correlated with protein expression for primary AML myeloblasts. As we found with AML cell lines, sensitivity to ABT-199 correlated directly with BCL-2 expression and inversely with BCL-XL expression as measured by quantitative Western blot analysis (Fig. 4D and E). No significant relationship was observed between MCL-1 level and sensitivity to ABT-199 in primary AML myeloblasts (Supplementary Fig. S6). AML myeloblasts also demonstrate BCL-2 mRNA expression than normal bone marrow (Fig. 4F). Higher expression of mRNA for MCL-1, BCL-XL, and BIM in AML myeloblasts was not observed (Supplementary Fig. S7).

**ABT-199 Induces Apoptosis in AML Stem/Progenitor Cells**

We next tested whether ABT-199 is capable of inducing cell death not only in AML blasts, but also in the phenotypically defined AML stem/progenitor cells characterized by the CD34$^+$CD38$^+$CD123$^+$ immunophenotype (23). Samples from 6 ABT-199–sensitive patients with AML with high blast counts were incubated with ABT-199 or ABT-737 for 24 hours, and apoptosis induction was determined by Annexin V flow cytometry in electronically gated AML blasts (CD45$^{dim}$SSC$^{low}$) and AML stem/progenitor cells (CD45$^{dim}$SSC$^{low}$ CD34$^+$CD38$^+$CD123$^+$). ABT-199 (Fig. 4G) and ABT-737 (Supplementary Fig. S8) induced apoptotic cell death in both bulk AML blasts and AML stem/progenitor cells.

**BH3 Profiling Predicts AML Myeloblast Killing by ABT-199**

We next tested whether ABT-199 killed primary AML myeloblasts in an on-target, BCL-2–dependent fashion. If this was the case, we would expect that mitochondria sensitive to the BAD BH3 peptide should also be directly sensitive to ABT-199. Indeed, we found an extremely tight correlation between mitochondrial sensitivity to BAD BH3 and ABT-199 across 30 independent patient samples (Fig. 5A). Supporting the on-target effect of this class of drugs, a similar correlation was found for ABT-263 (Supplementary Fig. S9). No such correlation was observed for the comparison of the BCL-XL–selective peptide HRK BH3 and the IC$_{50}$ of ABT-199, supporting BCL-2–selective action of ABT-199 (Fig. 5B). We observed a weak anticorrelation between cellular sensitivity to ABT-199 and sensitivity to the MCL-1–selective peptide NOXA BH3 (Fig. 5C). This suggests that there is a minor tendency for MCL-1–dependent mitochondria to be less sensitive to ABT-199.

In other diseases, BH3 profiling has proven a useful tool for predicting the cytotoxic effect of BH3-mimetic small molecules (24, 25). Here we tested whether BH3 profiling using the BAD BH3 peptide predicted cytotoxicity from ABT-199 and found that the correlation was very good (Fig. 5D). In addition, the mitochondrial effect of ABT-199 correlated well with the cytotoxic effect (Fig. 5E), again supporting a direct mitochondrial effect of ABT-199, consistent with a mechanism of action of direct competition for the BH3 binding site of BCL-2 on mitochondria.

**BH3 Profiling Predicts Response to ABT-199 in an AML Xenograft Model**

Tumor xenograft models established by inoculation of cancer cell lines into immunodeficient mice have been used widely for testing novel therapies. However, cultured tumor cells can undergo changes in their gene expression patterns after prolonged passage in in vitro culture. Therefore, the preclinical results obtained from patient-tumor–derived xenograft (PDX) models may offer superior modeling of the human disease, especially for testing target-oriented therapies. We have shown that ABT-199 was very effective in a murine AML cell line xenograft model (Fig. 1E). As a more clinically relevant test of ABT-199’s antileukemic efficacy in vivo, NSG mice were injected with primary AML cells from two different patients (R and S) and monitored for leukemia engraftment by measurements of human CD45$^+$ cells in peripheral blood. After confirmation of AML engraftment, the mice were randomly divided into vehicle and treatment groups. Treated mice received ABT-199 for 2 weeks, after which all the mice
Figure 5. BH3 profiling predicts AML myeloblast killing by ABT-199. A, intracellular BH3 (iBH3) profiling was performed on thawed primary AML cells using the BAD BH3 (80 μmol/L) and ABT-199 (1 μmol/L). The mitochondrial sensitivity to BAD BH3 and ABT-199 were positively correlated. B, there is no correlation between the IC₅₀ of primary AML samples from Fig. 4B with the BCL-XL–specific BH3 peptide HRK (80 μmol/L). C, the IC₅₀ of primary AML samples from Fig. 4B were correlated with the NOXA (80 μmol/L), an MCL-1–specific NOXA BH3 peptide. D, the ABT-199 IC₅₀ of primary AML samples from Fig. 4B were correlated with the BAD BH3 peptide (80 μmol/L). E, the ABT-199 IC₅₀ from Fig. 4B was correlated with the ABT-199 mitochondrial response (1 μmol/L). All correlations were tested using a one-tailed Spearman r correlation using GraphPad Prism software.
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were sacrificed, and bone marrows were examined for AML tumor burden by human CD45 flow cytometry. Fluorescence-activated cell sorting (FACS) analysis showed that 2 weeks of ABT-199 treatment significantly reduced leukemia burden in murine bone marrow in mice injected with cells from patient S [mean, 70 ± 16% human CD45+ cells in the bone marrow of control mice (n = 9) and 32.7 ± 12% in ABT-199–treated mice (n = 11, P = 0.0004; Fig. 6A)]. We did not observe a decrease in tumor burden in mice injected with cells from patient R [mean 70.3 ± 8.1% human CD45+ cells in the bone marrow of control mice (n = 8) and 74.3 ± 6.4% in ABT-199–treated mice (n = 8, P = 0.1930; Fig. 6B)].

Because we observed a difference in response in the xenograft model following ABT-199 treatment, we asked whether the response to ABT-199 could be predicted by BH3 profiling. In blinded fashion, pretreatment AML myeloblasts from each model were subjected to BH3 profiling in which mitochondria were exposed to the BAD BH3 peptide as well as ABT-199 itself. We found that mitochondria from AML myeloblasts from patient S released more cytochrome c compared with patient R (Fig. 6C). These results provide evidence that ABT-199 kills AML myeloblasts by the expected mechanism of inhibition of mitochondrial BCL-2. Furthermore, these results suggest that BH3 profiling might predict the response of AML primary cells to ABT-199 in vivo.

**DISCUSSION**

Relapsed AML is a difficult cancer to treat effectively; therefore, there is need for improved treatment options for refractory AML. Here, we show that selective inhibition of BCL-2 by ABT-199 kills AML cell lines and primary patient cells both ex vivo and in vivo mouse xenografts as a single agent in the low nanomolar range. The concentrations used in our studies here are in the 0.001–1 μM range, a range readily achievable in clinical trials where serum concentrations of 3–4 μg/mL have been observed (21). Moreover, the drug acts very quickly in vitro, killing cells within 2 hours of drug exposure. We also show that, as in CLL, ABT-199 functions on-target at the mitochondria. This is consistent with the observation that AML myeloblasts from chemorefractory patients showed no difference in their BCL-2 dependence, as measured by BH3 profiling, or sensitivity to ABT-199 compared with chemosensitive cells (16).

Our in vitro results suggest there will be heterogeneity in clinical response (IC50 values ranged from 0.43 to >1,000 nmol/L), so that a predictive biomarker would be of great utility. Here we present four methods that may be predictive of clinical response to ABT-199. The first method is cytogenetics. The cellular death response to ABT-199 appears to be largely independent of cytogenetic and genetic mutation status, except perhaps for complex karyotype and JAK2 mutation, suggesting that treatment with ABT-199 could be useful for patients who have poor prognostic factors. The utility of cytogenetics as a more general predictive biomarker for response to ABT-199 needs to be examined across many more samples.

A second method is ex vivo short-term culture of the primary patient samples with ABT-199. The disadvantage of this method is that it is difficult to reliably culture primary AML cells for the requisite time frame to observe cell death. We observed that even after a short 8-hour culture, there could be upwards of 60% spontaneous apoptotic death in the control untreated primary AML cells. Therefore, it would not be ideal to rely on an in vitro cell death assay where many samples could be potentially lost due to spontaneous cell death during culture.

The third predictive biomarker method is to measure BCL-2 levels by Western blot analysis. We show that increased expression of BCL-2 is associated with increased sensitivity to ABT-199. However, given the complex interactions of the BCL-2 family members, individual measurements of the various antiapoptotics alone may not provide accurate data on the in vivo biology of the antiapoptotic dependencies in AML. Many of the BCL-2 family members are regulated by post-translational modifications and interactions with other proteins. These types of interactions are difficult to capture in static Western blot measurements.

The fourth method, BH3 profiling, may prove useful as a predictive biomarker. BH3 profiling is a functional assay that...
accounts for the relative amounts and interactions of all of the BCL-2 family members. We show here that the mitochondrial response to the BAD peptide as well as mitochondrial ABT-199 correlates with the \textit{ex vivo} drug treatment. Most significantly, BH3 profiling could discriminate \textit{ex vivo} sensitivity of human AML cells to ABT-199 (Fig. 6). Thus, we may be able to use BH3 profiling of pretreatment AML samples to direct ABT-199 treatment to AML cases that are most BCL-2 dependent. Although the assay is less familiar to many, it is a straightforward protocol using reagents and equipment available in most clinical and research laboratories. Moreover, results are available the same day the sample is acquired. As for the other putative biomarkers, empiric testing in the clinical setting is the only way to truly validate BH3 profiling as a useful predictive biomarker.

Although we focused largely on the expression of BCL-2 and BCL-XL in AML, we also detected the expression of MCL-1. It has recently been reported that the antiapoptotic MCL-1 is necessary for the development and sustained growth of AML (26). Because ABT-199 does not inhibit MCL-1, increased expression of MCL-1 could be a potential source of upfront resistance to BCL-2 inhibition by ABT-199. However, we show that the majority of AML patient samples tested did not show MCL-1 dependence (as indicated by the NOXA response). This suggests that although MCL-1 may be necessary for the development of AML, most cases of AML may not depend on MCL-1 for survival as much as on BCL-2. Indeed, in a pertinent direct comparison, we have found that most AML myeloblasts are more BCL-2 dependent and less MCL-1 dependent than HSCs, though there are about 20% exceptions (16). It may well be that dependence on individual antiapoptotic proteins varies with myeloid differentiation stage.

BCL-2 was discovered in lymphoid cancer cells, and much of the research on this protein has been conducted in lymphoid cells, where it is highly expressed (27). It is therefore understandable that clinical testing of ABT-199 has so far focused on lymphomas and CLL. Here we demonstrate that selective, on-target BCL-2 inhibition using a clinically active drug is a promising avenue for clinical investigation in the myeloid malignancy AML. It is particularly important to recognize that even AML myeloblasts that are resistant to conventional therapies appear to be quite sensitive to BCL-2 inhibition. Thus, BCL-2 inhibition by ABT-199 offers hope to those AML cases that most need novel therapeutic intervention. Our results strongly support the testing of ABT-199 for treatment of patients with AML, as the majority of patient samples were sensitive to the drug in \textit{ex vivo} culture. Furthermore, our results support the testing of BH3 profiling as a predictive biomarker for ABT-199 response in the clinic.

**METHODS**

**Cell Lines**

The AML cell lines were purchased from the American Type Culture Collection (ATCC) or Deutsche Sammlung von Mikroorganismen und Zellkulturen or were kindly provided by Dr. James Griffin (Dana-Farber Cancer Institute, Boston, MA). HL-60, MOLM-13, OCI-AML2, OCI-AML3, KG-1, U-937, and Kasumi-1 were validated in September 2010 by short tandem repeat DNA fingerprinting using the AmpFlSTR Identifier kit according to the manufacturer’s instructions (Applied Biosystems). HL-60 cell lines with stable overexpression of BCL-2 or BCL-XL and the control cell line with empty vector were kindly provided by Dr. Kapil N. Bhalla (The Methodist Hospital Research Institute, Houston, TX). AML cell lines were cultured in RPMI-1640 medium supplemented with 10% or 20% FBS, 10 mmol/L L-glutamine, 100 U/mL penicillin, and 10 mg/mL streptomycin. Cells were kept at 37°C in a humidified atmosphere of 5% CO₂.

**Treatment of AML Cell Lines with ABT-737 and ABT-199**

AML cells were incubated for appropriate times in RPMI-1640 medium supplemented with 10% FBS and titrated concentrations of ABT-199 or ABT-737. Viable AML cells were enumerated by flow cytometry using counting beads with concurrent Annexin-V and propidium iodide (PI) staining. IC₅₀ values were calculated using CalcuSyn software (Biosoft) based on the number of live cells (Annexin-V/PI⁻). For specific details, see Supplementary Methods.

**Quantitative Western Blot Analysis**

Cell lysates were prepared and immunoblotted as previously described (28). Antibodies used for quantitative Western blot analysis were: BCL-2 (#M0887, Dako), BCL-XL (#2764, Cell Signaling Technology), MCL-1 (#559027, BD Biosciences), α-Tubulin, and β-Actin (loading controls). Blots were scanned with Odyssey Infrared Imaging System (LI-COR Biosciences). The band intensity was quantified using Odyssey software v2.0. The ratio of band intensity of BCL-2 proteins relative to that of loading control was normalized to the ratio in untreated OCI-AML3 cells.

**Gene Knockdown by shRNA**

MCL-1 was knocked down by lentiviral transduction using a MCL-1–specific shRNA transfer vector targeting residues 2421–2440 on RefSeq NM_021960.4. Lentivirus was prepared by cotransfection of HEK293T cells (ATCC) with an equal molar mix of transfer vector and packaging plasmid (psPAX2 and pMD2.G, Addgene) using JetPrime transfection reagent as directed by the manufacturer (Polyplus). Fresh lentiviral supernatants were passed through 0.45-μm surfactant-free cellulose acetate membranes; polybrene was added to 8 μg/mL, and the virus stock was used at once to spinoculate OCI-AML3 cells as described before (29). Infected cells were selected with 0.5 μg/mL puromycin. In parallel, control cells were transduced using lentivirus delivering a hairpin targeting GFP in plKO.1 (Addgene). Knockdown was verified by Western blot analysis.

**Selection of Resistant MOLM-13 Cells and Measurements of BCL-2 Protein by Intracellular Flow Cytometry**

Resistant MOLM-13 cells were selected in RPMI-1640 medium supplemented with 10% FBS and 50 mmol/L of ABT-199. Every 2 days, the cells were pelleted by centrifugation and resuspended in fresh medium with 50 mmol/L ABT-199. Cell viability was monitored by Vi-CELL viability analyzer (Beckman Coulter) until MOLM-13 cells reached a viability higher than 90%. The cellular content of BCL-2 protein was measured in both parental and resistant MOLM-13 cells by flow cytometry. Briefly, 1 million highly viable cells were washed twice with PBS and fixed in 1 mL of 4% paraformaldehyde for 15 minutes on ice, followed by washing with PBS and permeabilization with 1 mL of 0.1% Triton X-100 in PBS-buffered bovine serum albumin (BSA, 1% w/v). After incubation on ice for 10 minutes, the cells were then washed with washing buffer (1% BSA in 1% PBS), resuspended in 90 μL of washing buffer, and stained with 10 μL of FITC-conjugated BCL-2 antibody or Fluorescein isothiocyanate (FITC)-conjugated IgG1 isotype control (#47053 and #X0097, DAKO). After incubation in the dark at room temperature for 30 minutes, the cells were washed again with washing buffer and analyzed by flow cytometry using Gallios Flow Cytometer (Beckman Coulter). The intensity of BCL-2–associated fluorescence was measured...
on a logarithmic scale. For each sample, 20,000 cells were analyzed for MFI using Kaluza flow analysis software (Beckman Coulter).

BH3 Profiling of Cell Lines
AML cell lines were seeded at a density of 4 × 10^5 cells/mL in 10% FBS RPMI media supplemented with 10 mmol/L L-glutamine, 100 U/mL penicillin, and 10 mg/mL streptomycin 24 hours before BH3 profiling. Two million cells of each cell line were pelleted at 400 × g for 5 minutes at room temperature and resuspended in 2 mL DTEB (135 mmol/L Trehalose, 10 mmol/L HEPES-KOH, 0.1% w/v BSA, 20 μmol/L EDTA, 20 μmol/L EGTA, 50 mmol/L KCl, 5 mmol/L succinate, final pH 7.4). Cell lines were profiled using the plate-based JC-1 BH3 profiling assay previously described (16). Cells were permeabilized with digitonin, exposed to BH3 peptides, and mitochondrial transmembrane potential loss was monitored using the ratiometric dye JC-1.

Isolation and Treatment of Primary AML Cells with ABT-263, ABT-737, and ABT-199
Primary AML cell lines were obtained by informed consent from the Dana-Farber Cancer Institute, Leukemia Group, the Pasqurrello Tissue Bank at the Dana-Farber Cancer Institute, The University of Texas MD Anderson Cancer Center, Leukemia Tissue Bank Shared Resource from the Ohio State University Comprehensive Cancer Center, and the German-Austrian Study Group according to protocols approved by the Institute’s Institutional Review Board. Samples were Ficoll purified, used immediately, or frozen in 90% FBS/10% dimethyl sulfoxide (DMSO).

Fresh (Fig. 4A, D, and E) or thawed (Figs. 4B and 5) mononuclear cells were resuspended in culture medium supplemented with cytokines. Cells were treated with ABT-199, ABT-263, or ABT-737 for the appropriate time. Cells were then washed with PBS and resuspended in Annexin binding buffer. Cell viability was assessed by FACS analysis following concurrent Annexin V and PI (or 7-AAD) staining. For more detailed methods, see Supplementary Methods.

Apoptosis of AML Stem/Progenitor Cells
AML mononuclear cells were isolated by Ficoll density centrifugation and cultured with 100 mmol/L ABT-199 or ABT-737 as described above. After 24 hours, AML cells were washed twice in Annexin binding buffer (ABB) and resuspended in 100 μL ABB containing 1:100 dilution of Annexin-V-APC (#550475), 1:50 dilution of CD45-APC-Cy7 (#557833), CD34-FTTC (#555821), CD38-PE-Cy7 (#335790), and CD123-PercP-Cy5.5 (#58714; all from BD Biosciences) for 20 minutes at room temperature in the dark. After staining, the cells were washed with ABB and resuspended in 95μL ABB containing 5μL 4′,6-diamidino-2-phenylindole (DAPI). Cells were analyzed by Gallios Flow Cytometer (Beckman Coulter). Results were expressed as percentage of specific apoptosis calculated by the formula: 100 × (% apoptosis of treated cells - % apoptosis of control cells)/(100 - % apoptosis of control cells).

In Vivo Study of ABT-199 Efficacy in AML Mouse Models
All animal studies were conducted in accordance with the guidelines approved by the Institutional Animal Care and Use Committee at The University of Texas MD Anderson Cancer Center. Twenty female NSG mice (6-week-old, Jackson Laboratory) were intravenously injected with luciferase-labeled MOLM-13 cells (0.7 × 10^5 cells/100 μL) and randomly divided into two groups. Four days postinjection, the mice were treated with vehicle or ABT-199 (100 mg/kg body weight) daily by oral gavage for 2 weeks. For oral dosing, ABT-199 (10 mg/mL) was formulated in 60% phosal 50 propylene glycol, 30% polyethylene glycol 400, and 10% ethanol. BLI was used to monitor tumor burden on different time points. Briefly, mice were anesthetized and injected intraperitoneally with firefly luciferase substrate 1,2-luciferin and then imaged noninvasively using the IVIS-200 in vivo imaging system (PerkinElmer). Three mice from each group were sacrificed by CO2 asphyxiation after 15 days. Bone marrow, spleen, and liver were collected for H&E and immunohistochemical staining. The remaining 7 mice in each group were followed for survival.

For primary AML-derived xenograft models, NSG mice were sublethally irradiated (250 cGy) the day before intravenous injection of 10^6 PDX21 patient-derived AML cells. Three weeks following injection and after confirmation of AML engraftment, the mice were randomly divided into two groups and treated with 100 mg/kg ABT-199 or vehicle via gavage daily for 2 weeks. All mice were then sacrificed and femur bone marrow was analyzed for leukemia burden by CD45 flow cytometry (using anti-human CD45-PE antibody #555483, BD Biosciences).

Immunohistochemistry Analysis
Immunohistochemistry was performed as described previously (30). Briefly, the tissue was formalin-fixed, paraffin-embedded, sectioned into 5-μm thickness and mounted onto microscope slides. Tissue sections were then deparaffinized and rehydrated using xylene and ethanol in decreasing concentration. Samples were stained with H&E for histopathologic evaluation. For immunohistochemical staining, the tissue sections were incubated with primary antibody against human CD45 (#555480, BD Biosciences), followed by sequential incubation with biotinylated secondary antibody, peroxidase-labeled streptavidin, and 3,3′-diaminobenzidine tetrahydrochloride/H2O2 (Dako), which resulted in a brown precipitate at the antigen site. Images were taken using an optical microscope under the same magnification.

Microarray-Based Gene Expression Profiling in AML
The expression of BCL-2 family genes was determined using oligonucleotide microarrays (HG-U133 Plus 2.0, Affymetrix) in 288 AML samples comprising all cytogenetic groups, and in 103 normal samples (healthy bone marrow and non-leukemia conditions) as described in Haferlach and colleagues (31). All samples in this study were obtained from untreated patients at the time of diagnosis. Cells used for microarray analysis were collected from the purified fraction of mononuclear cells after Ficoll density centrifugation. The study design adhered to the tenets of the Declaration of Helsinki and was approved by the ethics committees of the participating institutions before its initiation. The analysis is conducted at logarithm-2 transformed gene expression intensities. Correlation analysis based on Pearson correlation coefficient and Spearman’s rank correlation coefficient was performed to identify probe sets that have consistent expression pattern corresponding to a common gene. Two-sample t test was performed for each two-group comparison, and the P value threshold of 0.005 was used to moderately correct for multiple testing.

iBH3 of Primary AML Patient Cells
Thawed cells were washed 1× with PBS and stained with 1:100 Invitrogen Live/Dead – near IR stain (#10119, Life Technologies) in FACs buffer (2% FBS, PBS) for 20 minutes on ice, washed with FACs buffer, and subsequently stained with 1:100 CD45-APC-V450 (#642275; BD Biosciences) in FACs buffer on ice for 20 minutes. Cells were pelleted at 400 × g for 5 minutes at room temperature and resuspended in DTEB. One hundred microliters of cells in DTEB were added to each tube containing twice the final concentration of each peptide in DTEB. One hundred microliters of cells in DTEB were added to each tube containing twice the final concentration of each peptide in DTEB.
LSR Fortessa flow cytometer (BD Bioscience) to quantify cytochrome c loss calculated from the MFI of Cytochrome c–Alexa488 as % Cyto c loss = 100 × (1 – ([MFI<sub>DMSO</sub> – MFI<sub>Isotype</sub>] + [MFI<sub>DMSO</sub> – MFI<sub>Isotype</sub>]))

AML blasts were identified by low-mid CD45/low SSC-A.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism software v6.0 (GraphPad). Unless otherwise indicated, the results are expressed as the mean ± SEM from at least three independent experiments. Differences with P values <0.05 were considered statistically significant.

Disclosure of Potential Conflicts of Interest

J.D. Levenson has ownership interest (including patents) in AbbVie, Inc. M. Andreeff has commercial research support from Daichi-Sankyo, has received honoraria from the speakers’ bureau of Teterologics, and is a consultant/advocacy advisory board member of Amgen and Eutropics. M. Konopleva has received a commercial research grant from AbbVie and is a consultant/advocacy advisory board member of the same. A. Letai is a consultant/advisory board member of AbbVie Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

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