Relative Mitochondrial Priming of Myeloblasts and Normal HSCs Determines Chemotherapeutic Success in AML

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http://dx.doi.org/10.1016/j.cell.2012.08.038

SUMMARY

Despite decades of successful use of cytotoxic chemotherapy in acute myelogenous leukemia (AML), the biological basis for its differential success among individuals and for the existence of a therapeutic index has remained obscure. Rather than taking a genetic approach favored by many, we took a functional approach to ask how differential mitochondrial readiness for apoptosis (“priming”) might explain individual variation in clinical behavior. We found that mitochondrial priming measured by BH3 profiling was a determinant of initial response to induction chemotherapy, relapse after remission, and requirement for allogeneic bone marrow transplantation. Differential priming between malignant myeloblasts and normal hematopoietic stem cells supports a mitochondrial basis to the therapeutic index for chemotherapy. BH3 profiling identified BCL-2 inhibition as a targeted strategy likely to have a useful therapeutic index. BH3 profiling refines predictive information provided by conventional biomarkers currently in use and thus may itself have utility as a clinical predictive biomarker.

INTRODUCTION

Though the majority of current cancer research has focused on novel targeted therapies, empirically derived conventional chemotherapy, largely targeting DNA and microtubules, has cured millions of cancer patients over the last 5 decades. A better understanding of why these therapies work can help us more wisely utilize them presently and better exploit targeted therapies in the future. Acute myeloid leukemia (AML) is a malignancy primarily of adults in which a malignant myeloid clone in the bone marrow is arrested in development and proliferates abnormally. A highly successful empirically derived treatment scheme combining cytarabine with an anthracycline has yielded a 70% remission rate, greater overall survival, and even cures for what is otherwise a fatal disease (Fernandez et al., 2009). The only curative option for patients who are resistant to or relapse after this induction regimen is allogeneic bone marrow or stem cell transplantation (Allo-SCT), which consists of an intensive preparatory chemotherapeutic regimen followed by introduction of donor hematopoietic stem cells (HSCs) (Schlenk et al., 2008). The success of the allogeneic approach is thought to depend on an immunologic graft-versus-leukemia effect rather than direct chemotherapeutic cytotoxicity for success.

The risk of induction-related death increases with age, yet alternatives to high-dose chemotherapy have modest efficacy (Appelbaum et al., 2006; Sekeres and Stone, 2002). Patients at high risk of relapse after induction of a complete remission are typically referred for allogeneic transplantation because chemotherapy alone is usually insufficient to ensure a durable remission in those cases (Schlenk et al., 2008). However, due to treatment-related mortality and graft-versus-host disease, allogeneic transplantation bears considerable risks and should be used only for patients who are at high risk of relapse with standard chemotherapy. Thus, predicting how well a patient will respond to chemotherapy and the risk of relapse is essential in deciding the best treatment course for each individual patient. Currently, prognostic factors based on cytogenetic abnormalities and gene mutations govern the use of allogeneic transplantation (Döhner et al., 2010). Current strategies in AML treatment are based on meticulous clinical observations rather than on a biological understanding of differential response to standard chemotherapeutic regimens. We propose here that the basis of differential response and clinical outcome after chemotherapy in AML lies in the intrinsic mitochondrial priming of the AML cells.

Mitochondrial priming is controlled by the BCL-2 family of proteins (Brunelle and Letai, 2009; Brunelle et al., 2009; Cerro et al., 2006; Deng et al., 2007; Letai, 2008; Ni Chonghaille et al., 2011; Ryan et al., 2010). This family consists of proapoptotic and antiapoptotic members. If proapoptotic members overwhelm the antiapoptotic members, the threshold of death is crossed and the cell dies. The BCL-2 family consists of four groups of proteins containing at least one of four homology
domains called the BH domains (BH1–BH4) (Brunelle and Letai, 2009; Danial and Korsmeyer, 2004). The first group consists of proapoptotic multidomain “effector” members Bax and Bak. Once activated, these proteins homo-oligomerize to induce mitochondrial outer membrane permeabilization (MOMP) (Wei et al., 2000; Wei et al., 2001), which results in the release of cytochrome c (and other proapoptotic factors) from the mitochondria and loss of mitochondrial transmembrane potential (Kluck et al., 1997). In the cytosol, cytochrome c cooperates in the formation of a multienzyme apoptotic complex that initiates a cascade of proteolysis executed by caspases (Zou et al., 1999). Bim and Bid (and perhaps PUMA) proteins contain the BH3 domain (“BH3 only”) and are proapoptotic “activators” of Bax and Bak (Gavathiotis et al., 2008; Wei et al., 2000). Antiapoptotic members like BCL-2, BCL-XL, BCL-w, BFL-1, and MCL-1 contain multiple BH domains and can inhibit by sequestration both the multidomain effectors and BH3-only activator proteins (Certo et al., 2006; Cheng et al., 2001; Willis et al., 2005). The last class consists of BH3-only proteins (Puma, Bmf, Bad, Noxa, and Hrk) referred to as “sensitizers” because they lack the ability to directly activate Bax/Bak (Certo et al., 2006; Letai et al., 2002). However, they sensitize cells to death by antagonizing antiapoptotic members. Sensitizer proteins have unique binding specificity to the antiapoptotic proteins and thus can only inhibit certain antiapoptotic members (Certo et al., 2006; Chen et al., 2005; Kuwana et al., 2005; Opferman et al., 2003). Cellular stress caused by chemotherapeutic agents induces the relative increase of BH3-only proteins. In cells highly primed for death, this overwhelms antiapoptotic members and results in cell death. In less primed cells, antiapoptotic reserves prevent death.

We define priming functionally as the magnitude of response of mitochondria to proapoptotic peptides derived from the BH3 domains of BH3-only proteins (Deng et al., 2007; Ni Chonghaille et al., 2011; Ryan et al., 2010). In practice, we measure this as the release of cytochrome c or the loss of mitochondrial transmembrane potential caused by standardized doses of BH3 peptides in an assay we call BH3 profiling. The greater the loss of mitochondrial transmembrane potential caused by the BH3-only peptides, the more cells are primed for death. Loss of potential caused by BH3 peptides like BIM that promiscuously inhibits all the antiapoptotic members provides a measure of overall priming (Ni Chonghaille et al., 2011). Sensitizer BH3 peptides like BAD, NOXA, and HRK inhibit only specific antiapoptotic members and thus provide a measurement of dependence on the antiapoptotic proteins they inhibit (Brunelle et al., 2009; Certo et al., 2006). For instance, mitochondrial response to the NOXA BH3 peptide is an indication of dependence on MCL-1, whereas mitochondrial response to BAD BH3 peptide is an indication of dependence on BCL-2, BCL-w, or BCL-XL.

Conventional chemotherapeutic agents generally kill via the mitochondrial apoptotic pathway. We have previously found that increased priming is the basis for differential clinical response in several cancers, including multiple myeloma, acute lymphocytic leukemia, ovarian cancer, and AML (Ni Chonghaille et al., 2011). In the case of AML, we found that in a preliminary series of 15 patients, achieving remission was related to the degree of mitochondrial priming. Here, we expand upon those results to explain a wide range of clinical phenomena associated with AML that have previously lacked elucidation of a biological mechanism. We find that differential priming determines not only the initial response rate but also risk of relapse. Using AML cell lines, we demonstrate that increasing mitochondrial priming enhances chemosensitivity. We determine that the source of a therapeutic index in AML depends upon AML cells being more primed than normal hematopoietic stem cells (HSC). We find that low priming identifies a subset of patients with a very high risk of relapse for whom allogeneic stem cell transplantation is likely required for cure. Finally, we use BH3 profiling to identify dependence on BCL-2 present even in chemorefractory myeloblasts but not in normal HSC, suggesting another potential avenue to rescue low-primed AML cases. Our results demonstrate that the functional information about mitochondrial apoptotic priming provided by BH3 profiling not only is useful in defining biological mechanisms in AML but also can be potentially exploited as a clinical predictive biomarker in AML.

RESULTS

Mitochondrial Priming Is a Major Determinant of Topoisomerase II Inhibitor Efficacy

We first asked whether there was a relationship between pretreatment mitochondrial priming and cellular response to chemotherapeutic drugs used clinically in AML. To answer this question, we BH3 profiled AML cell lines to determine their priming (Figure 1A) and treated each line with the drugs. We noted that one cell line, KG-1, had potent drug efflux activity that was not inhibited by verapamil, so we excluded it from further analysis (Figure S1A available online). We denote priming as the percentage of mitochondrial charge loss caused by the BIM BH3 peptide. We found that 0.3 μM of BIM BH3 peptide gave the appropriate dynamic range to compare the priming of the various AML lines (Figure 1A). Mitochondrial priming best correlated with cellular response to the drugs etoposide, daunorubicin, and mitoxantrone (Figures 1B–1H and S1D–S1F). It is notable that these three drugs are all topoisomerase II inhibitors, suggesting that reliance on mitochondrial priming for killing is a property of this class of drugs (Nitiss, 2009). In contrast, araC and clofarabine are nucleoside analogs, and azacitidine and decitabine are hypomethylating agents, suggesting that killing of AML cells by these classes of agents may be less reliant on mitochondrial priming. Notably, proliferation rate, often cited as a key determinant of chemotherapeutic response, did not correlate with chemosensitivity (Figures S1G–S1M). We tested whether expression of individual BCL-2 family proteins correlated with chemosensitivity. Of the seven tested, correlation with BAX levels achieved statistical significance, though not if a Bonferroni correction for multiple hypothesis testing were applied (Figures S1H–S1N). Note that the correlation of BAX with etoposide response is not as good as it is for BH3 profiling. Finally, there is not a good correlation between BAX expression and BH3 profiling (Figure S1O), suggesting that variability in BAX expression alone cannot explain differences in priming. Overall, these results suggest that monogenic predictors provide less predictive information than the functional approach of BH3 profiling.
Perturbation of Mitochondria Alters Chemosensitivity of AML Cells

If priming truly causes as well as correlates with chemosensitivity, then increasing mitochondrial priming should increase chemosensitivity. To test this, we lowered antiapoptotic BCL-2 expression in the MOLM13 AML line by using shRNA knockdown (Figure 2A). The MOLM13 BCL-2 partial knockdown produced increased priming as measured by BH3 profiling (Figure 2B). The increase in priming was associated with increased killing by etoposide, daunorubicin, and mitoxantrone in proportion to the quality of the knockdown (Figures 2C–2E).

In an alternative approach to directly altering mitochondrial priming, we used the small molecule BCL-2 antagonist ABT-737 (Oltersdorf et al., 2005). Only the two lines that are least sensitive to the BAD BH3 peptide, OCI-AML2 and THP-1, could be primed by ABT-737 without being killed by the inhibitor. As expected, treatment of OCI-AML2 and THP-1 cell lines with ABT-737 caused an increase in priming (Figures 2F and 2H), and this increase in priming resulted in a concomitant increase in chemosensitivity (Figures 2G and 2I). Thus, specific perturbation of mitochondrial priming by two distinct methods resulted in a change in chemosensitivity. These results support the concept

Figure 1. Mitochondrial Priming Predicts Response to Topoisomerase II Inhibitors

(A–H) BH3 profiling response of each AML cell line to the BH3 peptides. Priming is measured by using 0.3 μM Bim response, the percent mitochondrial depolarization induced by the Bim BH3 peptide. Priming is compared with killing by nucleoside analogs (A) araC and (B) clofarabine, topoisomerase II inhibitors (D) daunorubicin, (E) etoposide, and (F) mitoxantrone, and DNA demethylating agents (G) azacytidine and (H) decitabine. All are one-tailed Spearman correlations. Treatment with topoisomerase II inhibitors were done in the presence of 20 μM verapamil to exclude the effects of drug pumps. See also Figure S1 and Table S1.
that mitochondrial priming is not merely correlative but is actually causative of chemosensitivity.

BH3 Profiling of Primary Blood and Bone Marrow Samples

We next wanted to test whether priming was similarly deterministic in the clinical setting. We first needed to develop a reliable method of BH3 profiling normal and malignant cells in heterogeneous patient blood and bone marrow samples. Often, only a minority of nucleated cells in the bone marrow are AML myeloblasts. For this reason we adapted our BH3 profiling method to identify cells of interest by FACS analysis (Lacombe et al., 1997; Ni Chonghaile et al., 2011; Ryan et al., 2010) (Figure 3A). We combined a patient AML sample with normal leukocytes to establish the AML blast gate by using CD45 staining and side scatter (Figure S2A). We validated that AML myeloblasts are CD45<sup>lo</sup> and SSC<sup>lo/mid</sup> by FACS sort followed by histological verification (Figure 3B). The digitonin used to permit peptide access to mitochondria did not affect the gating of our AML blasts (Figure S2B). JC-1 dye was used to detect mitochondrial charge loss caused by BH3 peptides upon initiation of MOMP on the PE channel (Cossarizza et al., 1993) (Figure S2C). Most patient bone marrow and blood samples were available only as viably frozen cells in 10% DMSO. We found that the freezing process in 10% DMSO does not change the priming of the cells as long as cell viability is high after thawing (Figure S2D). Moreover, the same-patient sample frozen in two different media also showed comparable priming readouts (Figure S2E).
Higher Pretreatment Mitochondrial Priming of Patient AML Correlates with Clinical Induction Success

We used two different sources of clinically annotated AML samples: Dana-Farber Cancer Institute and Memorial Sloan Kettering. All samples were frozen prior to BH3 profiling. Investigators performing BH3 profiling were blinded to clinical response. In the primary test of our hypothesis that mitochondrial priming determines response to chemotherapy in AML, the myeloblasts of patients who subsequently attained a complete remission (CR) in response to induction therapy were more primed than patients who did not achieve a CR (Figure 4A and S3A). We considered induction therapies to be those that included a topoisomerase II inhibitor like daunorubicin, idarubicin, mitoxantrone, or etoposide, essential components of most standard AML induction regimens.

In those patients where sufficient follow up was available, we examined long-term duration of response, defined as 5 year disease-free survival after completion of induction therapy (Figure 4B). Because we wanted to evaluate outcome after chemotherapy alone, we excluded patients who received an allogeneic bone marrow or stem cell transplant after induction therapy. We found that those who obtained a CR and were cured were more primed than those that underwent a CR and then relapsed. The latter were in turn more primed than those who did not achieve CR (Figure S3C). This suggests that differences in proliferation rate in vivo did not drive the differences in response.

If priming is a determinant of response, we would expect to see selection for decreased priming of AML cells after relapse. In our cohort, we identified cases for which there was a CR followed by a relapse. Of these, six cases were identified for which paired samples were available, one obtained before treatment and one after relapse (Figure 4C). We indeed observed in vivo selection for decreased mitochondrial priming in cases of relapsed AML where the relapsed samples tended to be less primed than the initial samples (p = 0.031). As a control for time or chemotherapy causing these changes in the absence of selection, we examined myeloblast samples before and after chemotherapy in patients who did not enter a complete remission, and found no evidence of decreased priming. This suggests that in the relapsed samples we are observing true selection rather than a paradoxical direct chemotherapy effect because response is required to observe the selection for decreased priming.

BH3 Profiling Further Refines Prognostic Information from Cytogenetics and Genetics

The most widely used prognostic system currently in use for AML is that of the European LeukemiaNet (Döhner et al., 2010). This classification system is based on classical cytogenetic results as well as possession of a nucleophosmin (NPM1) or FLT-3 internal tandem duplication (ITD) mutation to classify patients in one of four prognostic groups: favorable, intermediate I, intermediate II, and poor. Although of demonstrated prognostic
utility, the ELN criteria leave considerable dispersion in outcome in each of the groups. Restricting our analysis to those patients from 4A for whom we had cytogenetic data, we segregated patients as favorable, intermediate (combining intermediate I and II), or poor risk according to this system. As expected, clinical outcome was best in the favorable group, worse in the intermediate, and worst in the poor risk group. However, as expected, even within each of these groups, there was a mixture of the clinical responses: CR (cured), CR (relapse), and no CR. We asked whether BH3 profiling could better refine the prognostic information provided by the ELN criteria, even within these genetically defined groups. In each case it could. In the favorable group, BH3 profiling could distinguish between CR (cured) and CR (relapse) (Figure 4D). In the intermediate group, BH3 profiling could distinguish between those destined for cure, relapse, or no CR (Figure 4E). Finally, BH3 profiling could distinguish between those destined to relapse and those who would be refractory to initial therapy in the poor category (Figure 4F).

Cytogenetic abnormalities have long been demonstrated to provide prognostic information about both short- and long-term clinical outcomes in AML (Mrózek et al., 2004). However, the important physiological effects of major chromosomal alterations that confer altered prognosis and chemotherapy response are obscure. We hypothesize that poor-risk cytogenetic abnormalities are related to low mitochondrial priming, resulting in relative chemoresistance. Monosomy 7 is the one cytogenetic abnormality present in sufficient abundance in our sample for us to test this hypothesis. We therefore examined the mitochondrial priming of myeloblasts containing monosomy 7 and found that they were significantly less primed than responsive AML samples lacking monosomy 7 (Figure S3B). Low mitochondrial priming may provide for the first time a physiological mechanism to connect this poor-risk cytogenetic finding to poor chemotherapy response in AML.

**Priming of Myeloblast Relative to Hematopoietic Stem Cell Determines Therapeutic Index**

If poorly primed AML is less sensitive to chemotherapy, what is limiting oncologists from just giving higher doses of chemotherapy to the poorly primed population? In the clinic, drug

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**Figure 4. Relative Priming of AML Determines Clinical Outcome**

(A) Preinduction priming as measured by Bim 0.1 μM depolarization of patient AML samples compared with clinical response to induction therapy specified as CR or no CR.

(B) Patients who achieve CR are separated into cured (no relapse and no transplantation) or relapse (relapse after initial CR). Highly primed patients having better clinical outcome.

(C) Sequential samples were obtained from the same patients at two different dates. “Sensitive” patients initially obtained a CR and then relapsed. “Refractory” patients never achieved a CR and had a second biopsy taken after induction. Decrease in priming after relapse is statistically significant based on a one-tailed Wilcoxon matched-pair signed rank test with a p value of 0.03. No such significant trend was observed for the refractory sequential samples.

(D–F) Patients were grouped based on their ELN relapse risk factors, and clinical outcome was compared with priming of primary AML samples. Patients with normal cytogenetics lacking NPM1 and FLT3 information were classified as intermediate risk. Significant differences were calculated by using a nominal one-tailed Mann-Whitney test. *p < 0.05, **p < 0.005, ***p < 0.0005. All data represent mean ± SD. See also Figure S3 and Table S2.
dosing is limited by the tolerance of critical normal cells. Induction chemotherapy regimens for AML have evolved over the past few decades based mainly on empiric observations of what drugs were effective and what doses were tolerable. By far, the most common dose limiting toxicity is bone marrow toxicity. Because HSCs are responsible for regrowth of the ablated bone marrow, we wondered whether the therapeutic index of induction chemotherapy was dependent on the difference in chemosensitivity between normal HSCs and AML myeloblasts.

We proceeded to test the hypothesis that the therapeutic index of conventional induction chemotherapy in AML is based on the relative mitochondrial priming of normal HSCs and AML myeloblasts. That is, AML patients whose myeloblasts are less primed than normal HSCs would have a lower rate of clinical success, whereas those whose AML are more primed than normal HSCs would have a higher rate of clinical success. To test this, we measured the priming of normal human HSCs with the method used for our FACS BH3 profile of patients who did not achieve a CR. The priming of HSCs represents the index of conventional induction chemotherapy in AML. The biological significance of this finding is that it suggests that apoptosis is irrelevant to the mechanism of graft-versus-leukemia killing of myeloblasts in vivo in humans. Furthermore, it suggests that given the otherwise poor prognosis of AML, there are subpopulations that are of dominant importance in determining clinical outcome.

**Poorly Primed AML Requires Allogeneic Transplantation for Long-Term Survival**

When we examined the patients who attained long-term survival after induction therapy without discriminating based on postinduction therapy, it became clear that some patients were cured despite presenting with very poorly primed myeloblasts. We noted that some AML cases that failed to achieve CR were relatively highly primed. We therefore used the priming of HSCs as an index to categorize AML cells into highly primed (more primed than HSCs) or low primed (less primed than HSCs). In patients for whom we have at least 3 years clinical follow up, we compared overall survival based on whether the preinduction myeloblasts were more or less primed than the mean of normal HSCs. Patients with highly primed AML achieve a much greater overall survival than patients with low-primed AML. This finding supports the concept that the mitochondrial priming of HSC may be important in establishing the maximum tolerated doses of induction therapy and hence the therapeutic index of induction therapy in AML.

Myeloblasts can comprise a heterogeneous population as demonstrated by differential expression of cell-surface antigens CD34 and CD38 (Lapidot et al., 1994). We noted that some AML patients that were cured underwent allogeneic transplantation. The biological significance of this finding is that it suggests that apoptosis is irrelevant to the mechanism of graft-versus-leukemia killing of myeloblasts in vivo in humans. Furthermore, it suggests that given the otherwise poor prognosis of AML, there are subpopulations that are of dominant importance in determining clinical outcome.

**Figure 5. Priming of AML Relative to HSC Priming Determines Clinical Outcome**

(A) The priming of normal HSCs is compared with priming of cured, relapse, and no CR patients by using a one-tailed Mann-Whitney test. Data represent mean ± SD. (B) Kaplan-Meier survival curves based on pretreatment priming. Using the priming of normal HSCs as a cut-off, patients were categorized as high primed or low primed. Patients with AML that are high primed have significantly better overall survival than low-primed patients.

"p < 0.005, ***p < 0.0005. See also Figure S4."
allogeneic transplant is not a guarantee of long-term survival in patients presenting with poorly primed AML, it seems to be a requirement thereof.

**Chemosensitive and Chemoresistant Myeloblasts Are More Sensitive To BCL-2 Antagonism Than Normal HSCs**

Although allogeneic bone marrow stem cell transplantation apparently is an attractive option for low-primed chemoresistant AML patients, its application is often limited by the age and comorbidities of the patient, as well as the availability of a suitable allograft. We therefore asked whether BH3 profiling could detect other differences between AML myeloblasts and HSCs that would suggest alternative, less toxic therapeutic strategies that could be exploited. Although we have so far focused on priming measures based on the promiscuously interactive BIM BH3 peptide, other BH3 peptides used in the BH3 profile inhibit only specific antiapoptotic BCL-2 family proteins. Mitochondrial dysfunction induced by such peptides is therefore a measure of specific dependence on the antiapoptotic protein with which they inhibit (Certo et al., 2006). For instance, we have previously shown that MOMP induced by the BAD BH3 peptide indicates dependence on BCL-2, BCL-XL, or BCL-w.

When we examined BH3 profiling results for our AML cell lines, we found that all AML cell line mitochondria were more sensitive to the BAD BH3 peptide than the NOXA BH3 (which antagonizes MCL-1) or HRK BH3 (which antagonizes BCL-XL) peptides (Figure 7A). This suggests a specific dependence on BCL-2 or BCL-w for these cells’ survival. Prior work has indicated that BCL-2 is the key protein (Konopleva et al., 2006). To directly test whether this observed mitochondrial dependence corresponded to a cellular dependence, we exposed the cell lines to ABT-737, which antagonizes function of BCL-2, BCL-w, and BCL-XL (Oltersdorff et al., 2005). We found that the mitochondrial response to the BAD BH3 peptide correlated well with cellular killing by ABT-737 (Figures 7B and S5A). This result demonstrates that BH3 profiling can detect antiapoptotic dependencies that we can exploit pharmacologically to predictably kill AML cells.

Next, we compared the response of mitochondria of primary AML cells from both sensitive and refractory AML patients with the response of HSCs to BAD, NOXA, and HRK BH3 peptides. Most AML mitochondria from both refractory and sensitive patients responded significantly more to the BAD BH3 peptide than did that of the HSCs (Figure 7C). It is noteworthy that increased mitochondrial sensitivity to BAD BH3 was also found in the CD34+ CD38− subpopulation of myeloblasts, the subpopulation most commonly thought to harbor AML stem cells (Figure 7D). Taken in conjunction with an overall weak response to the HRK BH3 peptide (Figure 7E), these results suggest that AML cells, both bulk and CD34+CD38−, regardless of their clinical response to standard induction chemotherapy, are usually more dependent on BCL-2 than are normal HSCs.

In contrast, mitochondria from HSCs were significantly more sensitive to the NOXA BH3 peptide than were the majority of the AML cells (Figure 7F). This suggests that normal human HSCs are selectively more dependent on MCL-1 for survival than are most AML cells. These results are congruent with the demonstration of MCL-1 dependence of murine HSCs obtained from mouse genetic models (Opferman et al., 2005). However, this represents the first demonstration of MCL-1 dependence in human HSCs. Note that BH3 profiling also identified a subset of AML cases that were MCL-1 dependent, which could likely benefit from MCL-1 directed therapy should such a drug become available. This illustrates a strength of the BH3 profiling tool, that it can provide important information about genetic dependencies and the apoptotic pathway without requiring the sort of genetic manipulation used in mice that is impossible in primary human tissues.

Recall that all of the refractory AML patient cells were poorly primed. Because most of these refractory cells are sensitive to the BAD BH3 peptide, this suggests that BCL-2 inhibition might benefit even refractory low-primed AML, offering a potential non-transplant alternative to these poor prognosis patients. These results prompted us to compare sensitivity of AML and HSCs to treatment with ABT-737 (Figures 7G and S5B). As predicted by the BH3 profiling results, primary AML cells, whether sensitive or refractory to induction, were more sensitive to ABT-737 than were normal HSCs. Two of the AML samples we tested were poorly primed, chemorefractory AML cells (blue dots), both of which were highly sensitive to ABT-737 killing. We also found that cellular response to ABT-737 correlated with mitochondrial sensitivity to the BAD BH3 peptide (Figure 7H). Interestingly, cellular sensitivity to ABT-737 correlated inversely to mitochondrial dependency on MCL-1 as measured by the response to our Noxa peptide (Figure 7I). Various groups have observed
MCL-1 can promote resistance to ABT-737 (Konopleva et al., 2006; van Delft et al., 2006). Thus, BH3 profiling can detect resistance as well as sensitivity to ABT-737. In summary, these results suggest that there is a useful therapeutic index for BCL-2 inhibition between malignant myeloblasts and normal HSCs that can be exploited. Significantly, BCL-2 inhibition offers a therapeutic index even in those patients who are poorly primed and respond poorly to conventional chemotherapy. These results suggest a promising therapeutic intervention for AML patients with poor conventional options, and furthermore, BH3 profiling could provide a predictive biomarker of potential utility in guiding BCL-2 directed therapy.

Figure 7. BCL-2 Independence of HSCs Provides a Therapeutic Window for ABT-737
(A) BH3 profiling responses to BH3 peptides show BCL-2 dependency in all AML lines.
(B) Comparison of IC_{50} killing by ABT-737 with BAD peptide response for each line after 24 hr of treatment. Correlation determined by a one-tailed Spearman correlation.
(C) Most AML cells from both sensitive and refractory patients are responsive to the BAD peptide, whereas HSCs are not.
(D) BAD peptide response of primary CD34^+CD38^- AML population is more pronounced than HSC response.
(E) HSCs and most primary AML are not responsive to the HRK peptide.
(F) HSCs are responsive to the NOXA peptide but most primary AML are not.
(G) Primary AML cells are significantly more sensitive to 1 μM ABT-737 than HSCs after 9 hr of treatment. Blue dots represent two low-primed AML refractory to standard induction.
(H and I) Greater ABT-737 sensitivity correlates with greater BAD BH3 peptide sensitivity (H) and also correlates with less NOXA BH3 peptide sensitivity (I).
*p < 0.05, **p < 0.005, ***p < 0.0005; viability = annexin V-/PI- population. Data represent mean ± SD. See also Figure S5.
DISCUSSION

Over the past 4 decades, since effective chemotherapy regimens for AML were introduced, several common observations have dominated thinking about AML therapy. These observations include: (1) some cases have excellent responses to chemotherapy, whereas others do not; (2) patients who relapse after initial response are unlikely to be cured by chemotherapy alone; (3) there is an unexplained therapeutic index for induction chemotherapy; and (4) allogeneic transplant is required to rescue relapsed and poor risk patients. Our results suggest that differences in pretreatment mitochondrial priming, as measured by BH3 profiling, provide a biological explanation for these heretofore unexplained clinical observations.

The observation that some patients achieve a complete remission and maintain it after chemotherapy alone, whereas others do not achieve remission or relapse, has lacked a biological explanation. Although cytogenetics have proven valuable as prognostic indicators of this behavior, it should be noted that cytogenetics have behaved mainly as empirically derived markers, with little power to explain the biological mechanism underlying differential killing of myeloblasts between patients. In this study, we tested the hypothesis that the differential mitochondrial tendency to apoptosis, or priming, measured by BH3 profiling explains the differential cellular and clinical response to cytotoxic chemotherapy. Our results support this hypothesis, as reflected in both initial response (Figure 4A) and longer term freedom from relapse (Figure 4B). This lends additional support to the concept that mitochondrial priming is an important determinant of clinical chemosensitivity that we supported in prior work in other cancers, including multiple myeloma, acute lymphoblastic leukemia, and ovarian cancer (Ni Chonghaile et al., 2011).

Another important observation is that patients who relapse after an initial complete remission due to induction chemotherapy are unlikely to achieve long-term remissions from subsequent chemotherapy, no matter what combination of agents is used. It is known that relapsed AML tends to be broadly more chemoresistant than that of the initial presentation, but the mechanism underlying this chemoresistance has been unclear. Our results suggest that selection for reduced mitochondrial priming in relapsed AML may well be an important determinant of this chemoresistant phenotype (Figure 4C).

Success of induction chemotherapy depends on a therapeutic index. That is, there must be a feature of myeloblasts that renders them selectively more chemosensitive than normal tissues. Perhaps surprisingly, given its centrality to the treatment of AML, the biologic basis of this feature remains poorly understood. There is no obvious AML-specific target exploited by standard induction chemotherapy, as it acts primarily to damage DNA, a target present in normal as well as malignant cells. We found that mitochondrial priming was a key determinant of the therapeutic index between myeloblasts and normal cells (Figure 5) and therefore one answer to the question, “why does chemotherapy work”? We could not identify a BCL-2 family protein whose level replicated the performance of BH3 profiling, supporting the concept that priming is likely the result of the simultaneous contribution of many proteins, perhaps even including some outside the BCL-2 family.

Two important points go beyond the elucidation of biological mechanisms of clinical behavior of AML and into potential clinical application. The first is the identification of a therapeutic index and a potential predictive biomarker for BCL-2 inhibition. We have made the observation not only of myeloblast sensitivity but also relative HSC insensitivity to BCL-2 inhibition. Others have previously made a similar observation (Konopleva et al., 2006). However, we demonstrated, based on our mitochondrial BH3 profiling studies, that this is an on-target effect, based in the mitochondrion, and further, that BH3 profiling is a potential predictive biomarker. Significantly, we found that BCL-2 dependence is observed even in cases where there was a poor response to conventional induction chemotherapy, indicating a potential strategy to rescue this difficult to treat population. Notably, identifying this therapeutic index and validating a predictive biomarker is of more than purely scientific interest because clinical BCL-2 inhibition is now a practicable clinical approach. Currently, Abbott Laboratories has two drugs in clinical trials that directly target BCL-2, ABT-263, and ABT-199 (Roberts et al., 2012; Tse et al., 2008; Wilson et al., 2010). Both are orally available counterparts of ABT-737. ABT-263 causes thrombocytopenia as an off-target toxicity due to its high-affinity binding of BCL-XL, a protein required for platelet survival. This toxicity may limit testing of ABT-263 in AML because patients very commonly present with existing thrombocytopenia. However, ABT-199 has greater selectivity for BCL-2 and lower affinity for BCL-XL, so the chances are better for achieving in vivo BCL-2 antagonism without exacerbating thrombocytopenia.

The second important advance is our identification of BH3 profiling as a potential predictive biomarker in AML, not only for BCL-2 inhibition but also for conventional chemotherapy. The understandable enthusiasm for BCL-2-targeted therapy notwithstanding, we expect that conventional chemotherapy and allogeneic bone marrow and stem cell transplantation, with their demonstrated curative potential, will remain a mainstay of AML therapy for many years to come. Therefore, it is worth considering how our findings could be used to better direct use of these modalities. There are two important dilemmas often encountered in the treatment of AML patients. In those under 60, what is the best postremission strategy? In other words, who should receive an Allo-SCT in first complete remission? Allo-SCT has the potential to cure patients who are at high risk of relapse, but it bears a higher treatment-related mortality and can be accompanied by years of chronic graft-versus-host disease. Therefore, the optimal strategy is to identify those that are most likely to relapse after complete remission, and selectively direct them to Allo-SCT. Currently, predictive tools such as those used by the ELN employ a combination of genetic and cytogenetic markers to perform such prediction. Although useful, these still appear to be imprecise tools, and we have found that BH3 profiling can actually improve the prognostic capabilities of the conventional prognostic approach (Figures 4D–4F) and by itself identify a subpopulation that apparently requires ALLO-SCT for cure (Figure 6).

Another decision-making dilemma faced by clinicians is whether to administer high-dose induction chemotherapy to newly diagnosed patients over 60 years of age. In older patients, treatment-related mortality is higher and complete remission...
rates are lower. Clinical benefit appears to be restricted to those who attain complete remission. Therefore, it would be useful to be able to predict which patients are most likely to achieve a complete remission and direct them to standard induction chemotherapy, sparing those patients unlikely to achieve remission the significant side effects. BH3 profiling apparently can identify those patients most likely to achieve a complete remission after induction chemotherapy (Figure 4). We will be testing the predictive utility of BH3 profiling in these two clinical settings in follow-up prospective clinical trials.

Our studies here do not directly demonstrate what upstream factors determine the relative priming of different cells. It is likely that activation of different oncogenes contributes because this by itself can affect sensitivity to apoptosis. It is also likely that differential activation of any of a number of tyrosine kinase-driven pathways could affect priming because it is clear that killing via inhibition of these pathways proceeds by perturbation of BCL-2 family proteins and utilization of the mitochondrial pathway of apoptosis. One strategy for improving response to chemotherapy in poorly primed AML might be to selectively increase priming in AML cells, perhaps with an agent that is highly selective but less potent than conventional chemotherapy. Once the AML is primed into a range consistent with good clinical response, then chemotherapy might be added. We tested such a strategy in vitro and showed it to work by using BCL-2 inhibition to prime AML cells and render them more sensitive to chemotherapy (Figures 2E–2H). We propose that such an approach merits testing in clinical trials and that guidance by BH3 profiling might assist with identifying useful priming agents.

There is considerable and appropriate interest in better personalization of therapy in cancer patients, including in those with AML. The vast majority of these personalization strategies are based on genetics and are directed toward targeted therapies. Even in these therapies, the gulf between genotype and phenotype can be difficult to bridge. Here, we demonstrate that BH3 profiling, an assay of mitochondrial apoptotic function, can provide information that can potentially be exploited for personalization of AML therapy in the application of BCL-2 antagonists, allogeneic bone marrow transplant, and conventional chemotherapy.

EXPERIMENTAL PROCEDURES

Cell Lines and Drug Treatments
AML cell lines were grown in 20% fetal bovine serum (FBS) in standard RPMI media for BH3 profiling and AML drug treatments. For details see Extended Experimental Procedures.

BH3 Profiling of Cell Lines
Cell lines were profiled by using the plate-based JC-1 BH3 profiling assay previously described (Ryan et al., 2010). Cells were permeabilized with digitonin, exposed to BH3 peptides, and mitochondrial transmembrane potential loss was monitored by using the ratiometric dye JC-1. For details see Extended Experimental Procedures.

JC-1 FACS-Based BH3 Profiling of AML Primary Samples
Primary AML cells were obtained from the Leukemia Group and Pasquarrello Tissue Bank at the Dana-Farber Cancer Institute under institutional review board (IRB)-approved protocol 01-206. Samples were also obtained from through Dr. Mark Frattini at Memorial Sloan-Kettering Cancer Center under IRB-approved protocols 95-091, 06-107, and 09-141. BH3 profiling was performed as previously described (Ni Chonghaile et al., 2011). For details see Extended Experimental Procedures.

Human HSC FACS
Normal human bone marrow was obtained from discarded deidentified bone marrow filters used at the Dana-Farber Cancer Institute during bone marrow isolation from healthy donors. Mononuclear cells were obtained by Ficoll. Lineage depletion was done with Miltenyi’s Human Lineage Depletion Kit to enrich for progenitor cells. The cells were further enriched with Miltenyi’s CD34 Enrichment Kit. Cells were then stained with biotin-lineage cocktail (Miltenyi), CD34-PECy7 (clone 8G12), CD38-V450 (clone HB7), CD90-APC (clone 5E10), and CD45RA-biotin (clone HH100). Biotinylated cells were detected by using streptavidin-APC-AlexaFluor750 (Invitrogen). The depletion, enrichment, and antibody staining were all done in FACS buffer on ice. Stained cells were BH3 profiled as described for primary AML. Human HSCs were identified as cells in the Lin-CD34+/CD38-CD90+/CD45RA- subpopulation (Majeti et al., 2007).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, five figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2012.08.038.

ACKNOWLEDGMENTS

The authors gratefully acknowledge support from the following sources: NIH grants F31 CA150562, P01 CA139980, and R01CA129974, Gabrielle’s Angel Foundation for Cancer Research. A.L. is a Leukemia and Lymphoma Society Scholar. We thank Abbott Laboratories for providing ABT-737. We thank Martha Wadleigh, MD, and Ilene Galinsky, RN, and the Pasquarrello Tissue Bank for help with clinical data and samples. A.L. was a cofounder and formerly served on the scientific advisory board of Eutropics Pharmaceuticals, which has a license for BH3 profiling.

Received: March 26, 2012
Revised: June 22, 2012
Accepted: August 3, 2012
Published: October 11, 2012

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