Maturation Stage of T-cell Acute Lymphoblastic Leukemia Determines BCL-2 versus BCL-XL Dependence and Sensitivity to ABT-199

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Acute lymphoblastic leukemia (ALL) is a hematopoietic malignancy derived from immature B-lymphoid and T-lymphoid cells (T-ALL). In T-ALL, there is an early T-cell progenitor (ETP) subgroup that has a very high risk for relapse. In this study, we used mitochondrial BH3 profiling to determine antiapoptotic protein dependencies in T-ALL. We found that T-ALL cell lines and primary patient samples are dependent upon BCL-XL, except when the cancer bears an ETP phenotype, in which case it is BCL-2 dependent. These distinctions directly relate to differential sensitivity to the BH3 mimetics ABT-263 and ABT-199, both in vitro and in vivo. We thus describe for the first time a change of antiapoptotic protein dependence that is related to the differentiation stage of the leukemic clone. Our findings demonstrate that BCL-2 is a clinically relevant target for therapeutic intervention with ABT-199 in ETP-ALL.

**SIGNIFICANCE:** ETP T-ALL is a treatment-resistant subtype of T-ALL for which novel targeted therapies are urgently needed. We have discovered, through BH3 profiling, that ETP-ALL is BCL-2 dependent and is very sensitive to in vitro and in vivo treatment with ABT-199, a drug well tolerated in clinical trials. Cancer Discov; 4(9); 1074–87. © 2014 AACR.

**INTRODUCTION**

Acute lymphoblastic leukemia (ALL) is the most common malignancy in childhood. In the era of combination chemotherapy, outcomes for pediatric ALL have markedly improved, but 20% of pediatric patients die of the disease, and this number increases to 50% in adults (1). In the majority of ALL cases, the malignant clone is derived from a B-lymphoid progenitor. However, in nearly 20% of pediatric and a larger percentage of adult ALL cases, the malignant clone arises from a T cell (T-ALL). Robust markers of prognosis were lacking in T-ALL, but recent work has identified differentiation arrest at very early stages of T-cell development, known as the early T-cell progenitor (ETP) phenotype, as a predictor of poor clinical outcome (2). ETP-ALL occurs following clonal expansion of a recently emigrated thymocyte, which has the capacity for multilineage differentiation. Distinct biomarkers have been used to identify these cases, including a characteristic immunophenotype, gene expression signature, or the absence of biallelic TCRγ locus deletion (ABD), a marker of differentiation arrest before V(D)J recombination specific to this locus (2, 3). Each of these biomarkers identifies an overlapping subset of cases with differentiation arrest at very early stages of T-cell development that have very poor outcomes, despite contemporary intensified therapeutic regimens. Consequently, there is a need for more effective treatment options for patients to improve the response, along with reducing the deleterious side effects of the cytotoxic agents.

In the past decade, numerous research studies have shown that hematopoietic cancers, including B-ALL, are dependent on antiapoptotic proteins such as BCL-2 for survival (4, 5). The antiapoptotic B-cell lymphoma-2 (BCL-2) protein was originally identified at the breakpoint of t(14;18) translocation in B-cell lymphoma (6). This led to the discovery of a large family of both proapoptotic and antiapoptotic proteins that control commitment to cell death via the mitochondrial pathway of apoptosis. The family of proteins is distinguished by the presence of one or more BCL-2 homology (BH) domains that regulate interactions among the members of this protein family (7). The multidomain proapoptotic proteins BAX and BAK are essential for mitochondrial apoptosis and are directly activated by activator BH3-only proteins (BIM, BID, and potentially PUMA; refs. 8–11). Active BAX and BAK homo-oligomerize and insert in the mitochondrial membrane, causing mitochondrial outer membrane permeabilization (MOMP) and release of cytochrome c, committing the cell to programmed cell death (12). The antiapoptotic proteins, including BCL-2, MCL-1, BCL-W, BCL-XL, and BFL-1, function by binding and sequestering the prodeath proteins, including the BH3-only proteins, and active monomeric BAX or BAK to prevent MOMP and death from occurring (13, 14). A class of BH3-only proteins, called sensitizers (BAD, NOXA, BMF, BIK, PUMA, and HRK), cannot directly
activate BAX and BAK, but they can bind selectively to the antiapoptotic proteins, releasing bound prodeath proteins and thereby initiating death (8). The individual BH3-only sensitizers have binding preferences for the antiapoptotic proteins that can be used to detect antiapoptotic dependencies. For example, NOXA selectively binds to MCL-1, whereas HRK preferentially binds to BCL-XL (14–16).

BH3 profiling is a tool we developed to understand the interactions of the BCL-2 family of proteins (14). In the past, we have demonstrated that BH3 profiling can correctly identify BCL-2– and MCL-1–dependent cancers (17). BH3 profiling can also measure how “primed” cancer cells are for death, a property that correlates with in vivo sensitivity to chemotherapy (18, 19). The assay involves adding peptides generated from the BH3 domain of BH3-only proteins to permeabilized cells and measuring the loss of mitochondrial potential following MOMP, using the fluorescent dye JC-1 (14, 20). For example, if a cell is dependent on BCL-2 for survival, the addition of the BAD peptide would release proapoptotic proteins from BCL-2, leading to the activation of BAX/BAK and loss of mitochondrial potential. The BH3 mimetic small molecules ABT-737 and ABT-263 (navitoclax) bind to BCL-2, BCL-XL, and BCL-W in a manner analogous to the BAD BH3 domain (21–23). Navitoclax has shown promising monotherapy results in clinical trials for chronic lymphocytic leukemia (24). However, platelets are dependent upon BCL-XL for survival, and inhibition of BCL-XL by ABT-263 causes a rapid induction of apoptosis and peripheral clearance of platelets that has limited the clinical use of ABT-263 (22, 24, 25). To circumvent the thrombocytopenia, AbbVie reengineered ABT-263 to make the BCL-2–selective inhibitor ABT-199, which still has nanomolar binding affinity to BCL-2 and has been shown to spare platelets both in vitro and in vivo (26). Indeed, the BCL-2–specific BH3 mimetic has shown efficacy in CLL in vivo, along with preclinical activity in estrogen-positive breast cancer, acute myelogenous leukemia, and MYC-driven B-cell lymphomas (26–29).

Inhibition of BCL-2 (and BCL-XL/BCL-W) with ABT-737/ABT-263 is sufficient as a monotherapy to kill B-cell leukaemic cells both in vitro and in primagrafts (4, 30). Here, we used BH3 profiling of both primary samples and cell lines and measured apoptotic sensitivity to the BH3 mimetics to ABT-263 and ABT-199 to delineate antiapoptotic protein dependencies in T-ALL. We found that whether a cell was dependent primarily on BCL-2 or on BCL-XL was determined by the differentiation stage of the leukemia, with the immature ETP-ALL demonstrating selective dependence on BCL-2 and sensitivity to ABT-199. This is the first demonstration that the maturation stage of the malignancy can determine the antiapoptotic protein dependence and sensitivity to targeted therapy in a clinically relevant cancer.

RESULTS
BH3 Profiling Reveals BCL-XL Dependence in Most T-ALL Cell Lines

To evaluate BCL-2 and BCL-XL dependence in T-ALL cell lines, we performed BH3 profiling. To distinguish between BCL-2 and BCL-XL dependence, we took advantage of the different binding affinities of the BAD and HRK BH3 peptides (Fig. 1A; ref. 14). We have found that in cells that are primarily BCL-XL dependent, the BAD and HRK peptides give roughly an equal signal. However, in a BCL-2–dependent cell, the BAD peptide gives a stronger response signal than HRK, as HRK does not interact with high affinity with BCL-2. The majority of T-ALL cell lines are dependent on BCL-XL (Fig. 1B). The T-ALL cell line that seems to be the most dependent on BCL-2 is LOUCY. Here, the BAD peptide caused a more robust mitochondrial depolarization than the HRK peptide, indicating a principal dependence on BCL-2. Notably, the LOUCY cell line is distinguished by having an ETP phenotype (31), whereas the other cell lines are typical T-ALL cell lines. We then asked whether the T-ALL cell lines are equally sensitive to ABT-263 (which binds BCL-2/BCL-XL/BCL-W) and ABT-199 (which binds BCL-2). We treated the cell lines with a range of doses of ABT-199 and ABT-263 and graphed the IC50 values (Supplementary Fig. S1). T-ALL cell lines, consistent with their BCL-XL dependence observed by BH3 profiling, are killed more efficiently by ABT-263 than by the BCL-2 selective inhibitor ABT-199 (Fig. 1C and D). Notably, however, the LOUCY cell line was quite sensitive to ABT-199, consistent with its dependence on BCL-2, and with a similar observation for this cell line (32). We then analyzed the protein expression of BCL-2 and BCL-XL by Western blotting. It is notable that only for the LOUCY cell line is the signal from BCL-2 higher than that for BCL-XL, congruent with the results above (Fig. 1E and F). Overall, these results suggest that T-ALL is largely BCL-XL dependent, but that the lone T-ALL cell line with an early T-cell progenitor phenotype is more BCL-2 dependent.

Typical T-ALL Is Dependent on BCL-XL, whereas ETP-ALL Is Dependent on BCL-2

Our results from the T-ALL cell lines provoked the hypothesis that primary patient-derived T-ALL samples are usually dependent on BCL-XL, except for samples in which the clone harbors an ETP phenotype, in which case they are dependent on BCL-2. To test this hypothesis, we obtained 26 primary pediatric T-ALL clinical specimens collected at the time of diagnosis from patients enrolled on a Clinical Oncology Group (COG) clinical trial. Initially blinded to maturation state, we BH3 profiled the samples and found that the samples were consistently very sensitive to the HRK peptide but more variably sensitive to the BAD peptide (Fig. 2A). Where high HRK sensitivity was observed, we imputed BCL-2 dependence; where low HRK sensitivity was observed, we imputed BCL-XL dependence. Following completion of the profiling of the 26 primary samples, it was revealed to us that 10 of the samples were ETP-ALL. ETP-ALL demonstrated selective dependence on BCL-2 and sensitivity to ABT-199. This is the first demonstration that the maturation stage of the malignancy can determine the antiapoptotic protein dependence and sensitivity to targeted therapy in a clinically relevant cancer.
ETP-ALL Is BCL-2 Dependent and Sensitive to ABT-199

**Figure 1.** BH3 profiling and in vitro testing of ABT-263 and ABT-199 reveals BCL-XL dependencies in T-ALL. **A,** the binding affinities of BH3 peptides BAD and HRK for the antiapoptotic BCL-2 family. Red, high-affinity binding; green, nondetectable binding measured by a fluorescence polarization assay (14). **B,** the BAD and HRK responses from the BH3 profiles of T-ALL cell lines are plotted. The means ± SDs of three independent experiments are graphed. **C,** the cell lines were treated with a six-point dose range from 1 nmol/L to 10 µmol/L of ABT-263 and ABT-199 for 48 hours, and apoptosis was measured by Annexin V and propidium iodide (PI) staining. The average of three independent experiments was used to generate dose–response curves in GraphPad Prism. The IC_{50} in µmol/L is graphed for each cell line. **D,** there is a statistical difference between the IC_{50} for ABT-263 and ABT-199 in the T-ALL lines. The ETP cell line LOUCY is shown in red. **E,** Western blot analysis shows expression of BCL-2 and BCL-XL in the T-ALL cell lines. **F,** the mean ratio ± SEM of BCL-2 expression divided by BCL-XL expression measured by densitometry of three independent plots is graphed.
is more BCL-2 dependent compared with typical T-ALL, which is more BCL-XL dependent. This is evident by a lower response to the HRK peptide in the ETP cases, whereas there is no difference in the BAD peptide response between the two groups (Fig. 2C and D). As a simple metric of BCL-2 dependence, we used the BAD minus the HRK response, which is greater in the ETP samples than in the typical T-ALL samples (Fig. 2E). Overall, this suggests that BH3 profiling can distinguish typical T-ALL samples that are BCL-XL dependent from the more immature ETP-ALL samples that are BCL-2 dependent.

Expression of BCL-2 and BCL-XL during Maturation of T Cells

By gene expression and immunophenotype, ETP-ALL most closely resembles the early thymic double-negative (CD4– and CD8–) stage of differentiation of normal T cells, whereas typical T-ALL resembles the more mature double-positive (CD4+ and CD8+) stage of differentiation (see Supplementary Fig. S2; refs. 2, 33). Notably, it has been shown that there is a reciprocal pattern of BCL-2 and BCL-XL expression in T-cell development in which early double-negative T cells express abundant BCL-2 and little BCL-XL, whereas more mature double-positive cells express little BCL-2 and abundant BCL-XL (13, 34–36). To investigate BCL-2 and BCL-XL expression in T-cell populations with slightly greater refinement, we measured BCL-2 and BCL-XL protein levels by flow cytometry (Fig. 3A). Consistent with prior results, BCL-2 is highly expressed at the DN1 early progenitor stage of T-cell differentiation (CD44+ /CD25−/CD4−/CD8−) that corresponds to ETP in human T cells, and the expression declines during differentiation until the single-positive CD4+ or CD8+ stage, in which BCL-2 is again highly expressed. In contrast, BCL-XL is expressed at the highest levels at the double-positive CD4+ and CD8+ stage of differentiation (Fig. 3A and Supplementary Fig. S3). To validate these results in human thymocytes, we exploited an existing mRNA dataset for the human thymus (37). Similarly, BCL-2 mRNA is highly expressed at the early T-cell progenitor stage in human thymocytes, declining at the double-positive CD4+ and CD8+ stage of differentiation (Fig. 3B). Thus, this phenomenon of switching between BCL-2 and BCL-XL dependence occurs during both mouse and human thymocyte development. Our hypothesis was that a comparison of immature ETP with more mature typical T-ALL would reveal
Figure 3. BCL-2 and BCL-XL expression alters with maturation stage of the T cell. BCL-2 and BCL-XL protein expression was measured by FACS analysis, and the gating strategy for distinguishing the different stages of T-cell differentiation is shown in Supplementary Fig. S3. **A**, the expression of BCL-2 and BCL-XL is normalized to the double-positive stage and expressed in mean fluorescent units (MFU); the experiment was repeated three times and the mean ± SD is graphed. **B**, the mRNA expression of BCL-2 is shown for primary human cells at the listed stages of differentiation. These data are modified from the online database (37). **C**, the mRNA expression of BCL-2 and BCL-XL in both ETP-ALL and typical T-ALL is graphically depicted in a heat map, with red indicating high expression and blue indicating low expression. The data are modified from the published online database (38). **D**, the BCL-2/BCL-XL and MCL-1 protein levels are shown for the typical human T-ALL cell lines and samples (TALL-x-4, -9, -1, and -2) as well as the ETP-ALL cell line LOUCY and the relapsed ETP-ALL sample (TALL-x-11). **E**, ratio of BCL-2:BCL-XL protein is shown for the cell lines and primary samples examined in D.
BH3 Profiling Reveals a Dependence on BCL-2 in ETP-ALL and Sensitivity to ABT-199

The BH3 profiling data of the primary pediatric T-ALL samples provided by COG suggested that selective BCL-2 dependence could be distinguished from BCL-XL dependence in primary T-ALL cells based on maturation state. To validate this result, we analyzed a separate cohort of pediatric and adult primary T-ALL patient samples collected at the time of diagnosis from patients enrolled on clinical trials at the Dana-Farber Cancer Institute (DFCI, Boston, MA). Similar to the COG samples analyzed, we found that the DFCI samples were sensitive to the BAD peptide, but that there was greater variability in the response to the HRK peptide (Supplementary Fig. S4A). We categorized the BAD and HRK peptide responses based on ETP-ALL versus typical T-ALL, as assessed by immunophenotypic analysis (Fig. 4A). Similar to the COG samples, ETP-ALL appears to be BCL-2 dependent by BH3 profiling (Fig. 4A–C and Supplementary Fig. S4B).

To test our hypothesis that ETP-ALL is BCL-2 dependent, we treated the DFCI T-ALL primary samples in a short-term culture with the BH3 mimetics ABT-263 (inhibits BCL-2/BCL-XL/BCL-W) and ABT-199 (BCL-2–specific inhibitor; Supplementary Fig. S4). Consistent with the human T-ALL cell lines examined (Fig. 1B–D) and the BCL-XL dependence observed by BH3 profiling, the primary T-ALL samples were more sensitive to ABT-263 treatment than treatment with the BCL-2-selective BH3 mimetic ABT-199 (Fig. 4D and Supplementary Fig. S5). When the response to ABT-263 and ABT-199 is categorized on the basis of maturation stage, it is evident that ETP-ALL is BCL-2 dependent and sensitive to ABT-199 (Fig. 4E and F). As an index of selective BCL-2 dependence, we used BAD minus HRK peptide response. Mitochondrial BCL-2 dependence negatively correlated with sensitivity to ABT-199 treatment with a Spearman \( r \) of −0.67 and a \( P \) value of 0.0037 (Fig. 4G). These findings suggest that selective BCL-2 dependence can be distinguished from BCL-XL dependence in primary T-ALL cells by BH3 profiling, and that this distinction has important consequences for the choice of BH3 mimetic treatment.

BH3 Profiling Predicts In Vivo Response of Patient-Derived T-ALL Primagrafts to ABT-199

ETP-ALL is a treatment-resistant subtype of T-ALL for which novel targeted therapies are urgently needed. To determine whether our BH3 profiling results have in vivo relevance, we established patient-derived xenografts (PDX) from ETP-ALL and typical T-ALL patients. PDXs are state-of-the-art preclinical models that recapitulate the original tumor heterogeneity and have been shown to accurately reflect patient responses to chemotherapy (39). BH3 profiling and compound sensitivity tests on these patient samples confirmed our prior findings, showing that distinct BCL-2 and BCL-XL dependence correlates with maturation stage (Fig. 5A–D). To examine the clinical efficacy of ABT-199 or ABT-263 treatment in vivo, mice with high leukemia burdens (65% CD45+ blasts) were randomized to one of three groups; vehicle, ABT-199, or ABT-263 compounds were administered daily for 2 weeks. After the treatment period, mice were then sacrificed, and leukemic burden in mouse blood, spleen, and bone marrow was determined (Fig. 5E). Consistent with our BH3 profiling, the ETP-ALL PDXs were sensitive to ABT-199 or ABT-263 treatment, whereas the typical T-ALL PDX was distinctly more sensitive to ABT-263 than to the BCL-2-selective compound ABT-199 (Fig. 5F–I). The exquisite sensitivity of the primary ETP PDX to the clinically well-tolerated ABT-199 is noteworthy, with leukemia essentially undetectable in blood, bone marrow, and spleen following ABT-199 monotherapy for 2 weeks (Supplementary Fig. S6A–D). Although treatment with ABT-263 or ABT-199 significantly reduced the number of human leukemic cells at all sites examined, leukemic burden in the spleen of the T-ALL PDX was least affected (Supplementary Fig. S6E–H).

DISCUSSION

In the past decade, it has come to light that lymphoid malignancies use the antiapoptotic BCL-2 family of proteins to maintain viability in the face of stress signals generated during oncogenesis (4, 6, 40). In effect, in many cases, the leukemia cells become dependent on the expression of the antiapoptotic protein, and inhibition of the function of the protein alone is sufficient to kill the cells (5). Consequently, the antiapoptotic proteins have become attractive targets for therapeutic intervention (21). Antiapoptotic proteins are required for normal maturation of the hematopoietic compartment, and dependence of normal blood cells on antiapoptotic proteins can vary with maturation stage (20, 41, 42). Therefore, we decided to thoroughly investigate the antiapoptotic protein dependencies of T-ALL in both pediatric and adult patient–derived samples.

We found that human T-ALL cell lines and two distinct cohorts of primary patient samples are largely dependent on BCL-XL. This was evident from the robust response observed upon treatment with the BAD peptide, which binds to BCL-2/BCL-XL/BCL-W, or the HRK peptide, which binds to BCL-XL. To our knowledge, this is the first demonstration of a consistent primary dependence on BCL-XL for survival in a hematologic malignancy. Previously, BCL-XL dependence has been generally considered to be a property of solid tumors, not hematologic tumors (43, 44).

Nonetheless, there was some heterogeneity in T-ALL, with a subset of cases demonstrating dependence on BCL-2
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instead of BCL-XL. Here we have made the clinically relevant observation that BCL-2 dependence is present in nearly all cases of ETP-ALL, a poor-risk subset of pediatric T-ALL. Although this distinction was first made via BH3 profiling, important confirmatory evidence was provided by the selective sensitivity of ETP-ALL to ABT-199, a BCL-2-selective small-molecule BH3 mimetic. Preclinical testing in PDX models of ETP-ALL showed that ABT-199 treatment dramatically reduced the disease burden in the bone marrow, spleen, and the peripheral blood, consistent with its BCL-2 dependence (Fig. 5). The distinction between sensitivity to ABT-199 and ABT-263 has clinical significance.

Figure 4. BH3 profiling reveals BCL-2 dependence in ETP-ALL in a separate cohort of DFCI patient samples. A, BH3 profiling of pediatric and adult DFCI T-ALL primary samples before initiating treatment. A dot plot of BAD peptide versus HRK peptide is graphed; ETP-ALL are marked in red, and green indicates that ETP status was not determined. Yellow indicates putative BCL-2 dependence, whereas blue indicates putative BCL-XL dependence. The BAD peptide response (B) and the HRK peptide response (C) are plotted for ETP-ALL versus typical T-ALL samples. Statistical significance was calculated using the nonparametric Mann–Whitney test. The primary DFCI T-ALL samples were treated with a six-point dose range from 1 nmol/L to 10 μmol/L of ABT-263 and ABT-199 for 6 hours, and apoptosis was measured by Annexin V and PI staining. The IC50 of ABT-263 and ABT-199 are graphed (D). The ABT-263 (E) and ABT-199 (F) response was compared between the typical T-ALL and ETP-ALL samples. The IC50 for ABT-199 is correlated with the BAD minus the HRK peptide percentage mitochondrial depolarization (G). Correlation was calculated using the nonparametric Spearman r test. Red marks the ETP-ALL cases, green marks cases with undetermined ETP status, and black marks typical T-ALL.
Therapy with ABT-263, a BH3 mimetic that inhibits BCL-XL as well as BCL-2, has been limited in the clinic due to the on-target thrombocytopenia that resulted from the BCL-XL dependence of platelets (22, 25). This toxicity is particularly important in cases of acute leukemia, in which thrombocytopenia is a common comorbidity. In contrast, clinical trials of ABT-199 have thus far demonstrated little or no thrombocytopenia in patients with hematologic malignancies (26). Thus, our data demonstrating that ABT-199 treatment eliminates human ETP-ALL cells in vivo is clinically relevant and suggest a therapeutic strategy for these treatment-resistant patients. Although tumor lysis syndrome was observed in the early clinical testing of ABT-199 in chronic lymphocytic leukemia (CLL), and clinical trials were halted, trials have been restarted for more than a year and continue in acute myelogenous leukemia, CLL, and non-Hodgkin lymphoma.

We report for the first time that the maturation state of a malignancy can determine the specific antiapoptotic protein on which it depends for survival. Previous reports in the literature have shown that as T cells mature in the thymus, they differentially depend on either BCL-2 or BCL-XL (34, 45). At an immature stage (DN1 in mice), or ETP in humans, the cells are dependent on BCL-2; this switches to BCL-XL dependency during the double-positive stage, and then following both positive and negative thymic selection, the dependency switches back to BCL-2 at the mature single-positive stage (CD4/CD8; refs. 13, 20, 34–36). Indeed, the observation of increased BCL-2 expression in ETP-ALL is validated by analysis of published gene expression profiling of ETP-ALL versus typical T-ALL cases (Fig. 3; ref. 38). Previous hierarchical analysis of T-ALL primary samples by gene expression revealed clustering of samples by transcription factors; the LYL1 cluster, which lacks the expression of CD4+/CD8+ (DN immature phenotype), had high BCL-2 expression compared with the other clusters that were positive for both CD4 and CD8 (double-positive stage; ref. 33). It was recently shown that normal B and T cells undergoing differentiation in the mouse display differential degrees of sensitivity to ABT-199, depending on the stage of maturation (46). We hypothesize

Figure 5. PDX of ETP-ALL is very sensitive to in vivo treatment with ABT-199, whereas typical T-ALL is relatively resistant. Primagrafts were generated from two primary T-ALL samples; one sample was identified as ETP-ALL by immunophenotypic analysis (TALL-x-11), whereas the other was identified as typical T-ALL (TALL-x-2). The mean BAD and HRK peptide response of triplicate wells are plotted ± SD for the ETP-ALL sample (A). The in vitro response to ABT-199 and ABT-263 was measured using Annexin V and PI following 6 hours of treatment for the ETP-ALL sample. The percentage of survival is graphed on the dose–response curve, and the subsequent IC50 values are listed (B). Similar results are shown for the typical T-ALL sample, and the BAD and HRK response from the BH3 profile are graphed (C) and percentage survival following ABT-199 and ABT-263 treatment (D). Cells (1 × 10⁹) of either the ETP-ALL sample or the typical T-ALL sample were injected in the tail vein of NOD scid gamma (NSG) mice until an engraftment of 65% leukemic blasts in the periphery. The animals were then randomized into vehicle, ABT-199, or ABT-263 treatment (100 mg/kg by oral gavage daily). The in vivo response to ABT-199 and ABT-263 was measured by counting the total human CD45+ leukocytes in the blood (continued on following page)
that transformation of an early T-cell progenitor retains its BCL-2 dependence, whereas the CD4+ and CD8+ leukemic blasts are more dependent on BCL-XL (Fig. 6). Genetic analysis of ETP-ALL shows a broad diversity in the underlying genetic alterations; however, certain genetic alterations have been shown to be similar to myeloid malignancies (e.g., FLT3, JAK3, IDH1/2, and PRC2 complex mutations), and gene expression profiling suggests that ETP-ALL may be a stem cell leukemia (31, 38, 47–49). In this respect, it is notable that acute myeloid leukemia is BCL-2 dependent and sensitive to pharmacologic inhibition by ABT-199 (19, 28).

Differences in differentiation state can be present not only between different patients’ tumors but also within a single tumor. This is perhaps most relevant to the presence of less-differentiated cancer stem cells in some tumors. In such malignancies, which may include ALL, it is possible that the bulk of the tumor may have a dependence on an anti-apoptotic protein that is different from the dependence of the stem or progenitor cell. Extending our observations here suggests that this difference may have important therapeutic implications in many other cancers, particularly those composed of cells of heterogeneous differentiation state.

In this age of cancer genomics, it is not surprising that most approaches to deriving predictive biomarkers rely on static measurements of genetics, even whole genomes, or whole transcriptomes. Our data suggest that focused phenotypic observations following targeted perturbations of cancer cells may be a very useful alternative in the pursuit of precision medicine.

**METHODS**

**Cell Lines and Treatments**

All of the media were supplemented with 10% heat-inactivated FBS (Gibco), 10 mmol/L l-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. RPMI-1640 was used for the culture of all the cell lines. The primary ALL samples were grown in Iscove’s modified Dulbecco’s medium (IMDM) media (Gibco) and supplemented with 100 ng/mL stem cell factor and 20 ng/mL IL3 for 6 hours (all supplements obtained from PeproTech). The cells were cultured at 37°C in a humidified atmosphere of 5% CO2. The cell lines (PF382, MOLT4, LOUCY, P12-ICHIKAWA, CEM-CCRF, and JURKAT) were authenticated in August 2012 by short tandem repeat (STR) DNA fingerprinting using nine STR Loci and Amelogenin for gender identification (including D21S11, THO1, TPOX, VWA, Amelogenin, CSF1PO, D16S539, D7S820, D13S317, and D5S818). Cell lines were seeded at 5 × 10^4 in 24-well plates, and after 24 hours, cells were treated with ABT-199 and ABT-263 (Abbott) for 48 hours.
BH3 Profiling

The sequence of the BH3-only peptides used and method of synthesis are as previously described (20). BH3 profiling was performed using plate-based fluorimetry. Briefly, BH3 peptides at 70 μmol/L were plated in triplicate on a black 384-well plate. Cells were gently permeabilized with 0.005% digitonin and loaded with the fluorescent mitochondrial dye JC-1. The cells were plated on top of the peptides at 2.5 × 10^4 cells per well. The loss of mitochondrial potential was measured on the Tecan Saffire2 at an excitation of 545 nm and an emission of 590 nm over 3 hours. The percentage of mitochondrial depolarization for the peptides was calculated by normalization to the solvent-only control DMSO (0%) and the positive control carbonyl cyanide-4-(trifluoromethoxy)phenyl hydrazone (FCCP; 100%) at 60 minutes.

Primary Adult and Pediatric ALL Patient BH3 Profiles and Treatments

Viably frozen pediatric ALL samples were obtained at the time of diagnosis from patients enrolled on the COG or DFCI Acute Lymphoblastic Leukemia Consortium clinical trials, Ficoll purified, and viably frozen. All samples were collected with informed consent and Institutional Review Board (IRB) approval of the respective institutions. Immunophenotyping of T-ALL samples for ETP status was performed in the laboratory of Brent Wood at the University of Washington, using a carefully validated assay that measures the absence of CD1a, CD8, and weak CD5 and the presence of a stem or myeloid marker (CD34, CD117, CD13, CD33, CD11b, CD65, or HLA-DR). Both the adult and pediatric samples were treated similarly. After thawing, cell viability was assessed by Trypan blue exclusion. Viable tumor cells (1.8 × 10^6) were immediately used for BH3 profiling. Mitochondrial depolarization was calculated at 60 minutes. At the same time, 3 × 10^6 cells were used to seed 2 × 10^5 per well into a 24-well plate in IMDM supplemented with stem cell factor and IL3 (PeproTech) for treatment with ABT-199 and ABT-263 for 6 hours. When maturation state was known, patients were grouped based on whether they were ETP-ALL or typical T-ALL. Investigators performing BH3 profiling assay on ALL specimens were blinded to maturation stage when the assay was performed.

Annexin V/Propidium Iodide Staining

Following treatment with ABT-263 or ABT-199, apoptosis was assessed by Annexin V/propidium iodide (PI) staining. Briefly, cells were washed in PBS and resuspended in Annexin Binding Buffer (10 mmol/L HEPES pH 7.4, 140 mmol/L NaCl, and 2.5 mmol/L CaCl). Cells were then stained with 0.5 mg/mL Annexin V-FITC and 0.5 mg/mL PI for 15 minutes before analyzing on the Fortessa (BD Pharminogen). Results were normalized to the DMSO-only control and dose–response curves were graphed using GraphPad Prism.

mRNA Expression

Published mRNA data on BCL-2 expression in human thymocytes (37) and of ETP-ALL versus typical T-ALL (38) were also assessed and represented as a heat map using GenePattern.
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**Intracellular Protein Staining Analyzed by FACS**

Mouse thymocytes were counted and suspended in 1% BSA/PBS at 10^7 cells/mL and stained with anti-CD19, anti-CD8, anti-CD4, anti-CD12, anti-CD44, and anti-CD25 at a 1:100 dilution for 30 minutes on ice. Cells were pelleted and washed in 1% BSA/PBS before fixation in 2% formaldehyde for 15 minutes. Cells were resuspended in staining buffer (1% BSA, 2% FBS, 0.1% Saponin, and 1.5 mM/L NaN₃ in PBS) and appropriate Hamster or Rabbit IgG for blocking for 5 minutes. The cells were then stained with BCL-2 phcoerythrin or BCL-XXL 488 and along with IgG controls overnight. Following washing, the cells were resuspended in PBS and analyzed on the Fortessa for BCL-2 and BCL-XXL expression during T-cell differentiation.

**Western Blotting**

Cells were lysed as previously described. Protein (30 µg) was loaded on NuPAGE 10% Bis-Tris polyacrylamide gels (Invitrogen). The following antibodies were used to detect proteins on the membrane: Actin (Chemicon; MAB1501), BCL-XXL (Cell Signaling Technology), BCL-XXL 6C8 (Trevigen), and MCL-1 (Santa Cruz Biotechnology). Secondary antibodies used were horseradish peroxidase conjugates of either anti-mouse, anti-hamster, or anti-rabbit.

**PDX Studies**

Primary human T-ALL cells were obtained from children with T-ALL enrolled on clinical trials at DFCI. Samples were collected with informed consent and with approval of the IRB. Patient consent forms were required from all patients or their legal guardians (if minors) for all samples collected for the study. Leukemic blasts were isolated from peripheral blood or bone marrow samples by Ficoll-Hypaque centrifugation and were cryopreserved in FBS containing 10% DMSO and in liquid nitrogen. NOD scid gamma (NSG) mice were purchased from The Jackson Laboratory or were a generous gift from Leonard D. Shultz (Jackson Laboratory), Michael A. Brehm (University of Massachusetts Medical School, Worcester, MA), and Dale L. Greiner (University of Massachusetts Medical School, Worcester, MA). NSG mice were maintained on a regimen of acidified antibiotic water. Fresh or frozen leukemic blasts were expanded in NSG mice by transplanting 0.5 to 5 x10^6 cells via intravenous injection. Human engraftment in the spleen and bone marrow was greater than 80% by staining for human CD45 (BD Biosciences). For flow cytometry analysis of blood, spleens, and bone marrow was greater than 80% by staining for human CD45 (BD Biosciences). For **Flow Cytometry**

Flow cytometric analysis was performed on a BD LSRII, using FITC- and allophycocyanin (APC)- conjugated antibodies against human CD45 and mouse CD45 (from BD Pharmingen). For flow cytometric analysis of blood, spleens, and bone marrow, single-cell solutions were generated, and red blood cell lysis was performed before staining. Data were analyzed using the FlowJo software (TreeStar).

**Data and Statistical Analyses**

The GraphPad Prism 6.0 software (GraphPad Software) was used for all statistical analysis. The IC₅₀ for the dose–response curves following ABT-263 and ABT-199 treatment was calculated using linear regression curve fit (Log inhibitor vs. normalized response). Comparison of the IC₅₀ response of ABT-263 versus ABT-199 for the primary patient-derived samples was carried out using the Mann–Whitney nonparametric test. Correlation of the ABT-199 response to the BAD minus HRK peptide response was performed using the nonparametric Spearman r correlation test with a two-sided t test for significance. The flow cytometry data were analyzed using FACSDiva version 6.1.1 (BD Pharmingen). Student t tests were performed on in vivo results with Welch correction using the GraphPad Prism software (version 5.0).

**Disclosure of Potential Conflicts of Interest**

B. Wood reports receiving a commercial research grant from Becton-Dickinson. R. Stone is a consultant/advisory board member for AbbVie. A. Letai reports receiving a commercial research grant from and is a consultant/advisory board member for AbbVie. No potential conflicts of interest were disclosed by the other authors.

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Conception and design: T. Ni Chonghaile, A. Gutierrez, A. Letai Development of methodology: T. Ni Chonghaile, J. Ryan, M.A. Kelliher, A. Letai Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Ni Chonghaile, J.E. Roderick, C. Glenfield, S.E. Sallan, L.B. Silverman, M.L. Loh, S.P. Hunger, B. Wood, D.J. DeAngelo, R. Stone, M. Harris, A. Gutierrez, M.A. Kelliher Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T. Ni Chonghaile, J.E. Roderick, C. Glenfield, A. Gutierrez, M.A. Kelliher, A. Letai Writing, review, and/or revision of the manuscript: T. Ni Chonghaile, J. Ryan, S.E. Sallan, L.B. Silverman, M.L. Loh, S.P. Hunger, D.J. DeAngelo, R. Stone, M. Harris, A. Gutierrez, M.A. Kelliher, A. Letai Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Ryan Study supervision: M.A. Kelliher, A. Letai

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**REFERENCES**


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