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

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1

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2

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3

Abstract:

Acute Lymphoblastic Leukemia (ALL) is a hematopoietic malignancy derived from immature B- 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 utilized mitochondrial BH3 profiling to determine anti-apoptotic 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 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 anti-apoptotic 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:

Early T-Cell precursor 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 treatment with ABT-199 both *in vitro* and *in vivo*, a drug well-tolerated in clinical trials.

4

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 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 prior to 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, which 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 anti-apoptotic proteins like BCL-2 for survival (4,5). The anti-apoptotic 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 pro- and anti-apoptotic 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

5

BCL-2 homology (BH) domains that regulate interactions among the members of this protein family (7). The multi-domain pro-apoptotic proteins BAX and BAK are essential for mitochondrial apoptosis and are directly activated by activator BH3-only proteins (BIM, BID and potentially PUMA) (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 anti-apoptotic proteins including BCL-2, MCL-1, BCL-w, BCL-XL and BFL-1 function by binding and sequestering the pro-death 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, HRK) cannot directly activate BAX and BAK but they can bind selectively to the anti-apoptotic proteins releasing bound pro-death proteins and thereby initiating death (8). The individual BH3-only sensitizers have binding preferences for the anti-apoptotic proteins that can be utilized to detect anti-apoptotic dependencies. For example, NOXA selectively binds MCL-1, while HRK preferentially binds 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 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 addition of the BAD peptide would release pro-apoptotic proteins from BCL-2 leading to 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

6

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 re-engineered 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 mylogenous 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 cancer leukemic cells both *in vitro* and in primagrafts (4,30). Here, we utilized BH3 profiling of both primary samples and cell lines and measured apoptotic sensitivity to the two BH3 mimetics ABT-263 and ABT-199 to delineate anti-apoptotic 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 anti-apoptotic 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)(14). We have found that in cells that are primarily BCL-XL dependent, the BAD and HRK peptides give roughly an equal signal.

7

However, in a BCL-2 dependent cell, the BAD peptide gives a stronger response signal than HRK, since 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 appears to be 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, LOUCY is distinguished by having an ETP phenotype (31), while the other cell lines are typical T-ALL cell lines. We then asked if 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 IC_{50} values (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, 1D). 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 blot. 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, 1F). 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 where 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

8

to the BAD peptide but more variably sensitive to the HRK peptide (Fig. 2A). Where high HRK sensitivity was observed, we imputed BCL-XL dependence; where low HRK sensitivity was observed, we imputed BCL-2 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 status was defined immunophenotypically by an absence of (<5%) CD1a- and CD8 expression along with a weak CD5a expression (<75%) and the presence (>25%) of a stem-cell or myeloid marker (CD34, CD117, CD13, CD33, CD11b, CD65 or HLA-DR) (2). When we plotted sensitivity to the BAD BH3 peptide against, sensitivity to the HRK peptide, the cases clustered according to ETP-ALL or typical T-ALL status, suggesting maturation state specific dependence on individual anti-apoptotic proteins (Fig. 2B). Upon comparison with Fig. 2A, it became clear that, consistent with our hypothesis, ETP-ALL is more BCL-2 dependent compared to typical T-ALL, which is more BCL-XL dependent. This is evident by a lower response to the HRK peptide in the ETP cases, while there is no difference in the BAD peptide response between the two groups (Fig. 2C, 2D). As a simple metric of BCL-2 dependence we utilized the BAD minus the HRK response, which is greater in the ETP samples compared to 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 negative) stage of differentiation of normal T cells while typical T-ALL resembles the more mature double positive (CD4 and CD8 positive) stage of differentiation (see Fig S2) (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

9

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, the expression declines during the differentiation until the single positive $CD4^+$ or $CD8^+$ stage where 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, 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 an analogous change in expression pattern to explain the BCL-2 dependence of ETP-ALL. We analyzed published mRNA datasets for BCL-2 family expression between ETP-ALL and typical T-ALL (38). We found that indeed, BCL-2 is also highly expressed at the mRNA level in ETP samples compared to typical T-ALL samples (Fig. 3C). Consistently, BCL-2 protein levels are increased in the ETP-ALL cell line LOUCY and in the relapsed ETP-ALL patient sample (T-ALL-x-11) whereas, the BCL-XL protein predominates in the typical human T-ALL cell lines and patient samples (T-ALL-x-4, TALL-x-9, T-ALL-x-1 and T-ALL –x-2)(Fig. 3D,E).

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

10

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). Similar to the COG samples analyzed, we found that the DFCI samples were sensitive to BAD peptide but that there was greater variability in the response to the HRK peptide (Fig. S4A). We categorized the BAD and the 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, 4B, 4C and Fig. S4B). To test our hypothesis that ETP-ALL is BCL2 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) (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, S5). When the response to ABT-263 and ABT-199 is categorized based on maturation stage, it is evident that ETP-ALL is BCL-2 dependent and sensitive to ABT-199 (Fig. 4E, 4F). As an index of selective BCL-2 dependence, we employed 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 from an ETP-ALL and typical T-ALL patients. Patient-

derived xenografts (PDX) 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 distinct BCL2 and BCL-XL dependence correlates to maturation stage (Fig. 5A-D). To examine the clinical efficacy of ABT-199 or 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 two 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 two weeks (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 (Fig. S6E-H). The reasons for this effect are unclear as both compounds eliminated leukemic cells in the ETP-ALL PDX model (Fig. S6A-D). The *in vivo* data support our *in vitro* studies, which revealed the ETP-ALL subtype to be BCL-2 dependent while the typical T-ALL sample exhibits BCL-XL dependence (Fig. 5B, 5F).

Discussion

In the last decade it has come to light that lymphoid malignancies utilize the anti-apoptotic 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 anti-apoptotic protein and inhibition of the function of the protein alone is

12

sufficient to kill the cells (41). Consequently, the anti-apoptotic proteins have become attractive targets for therapeutic intervention (21). Anti-apoptotic proteins are required for normal maturation of the hematopoietic compartment, and dependence of normal blood cells on anti-apoptotic proteins can vary with maturation state (20,42,43). Therefore, we decided to thoroughly investigate the anti-apoptotic 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 (44,45).

Nonetheless, there was some heterogeneity in T-ALL, with a subset of cases demonstrating dependence on BCL-2 instead of BCL-XL. Here we have made the clinically relevant observation that the BCL-2 dependent samples are nearly all cases of ETP-ALL, a poor-risk subset of pediatric T-ALL. While 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 patient-derived xenograft 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. 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 where

13

thrombocytopenia is a common co-morbidity. 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 suggests a therapeutic strategy for these treatment resistant patients. While tumor lysis syndrome was observed in early clinical testing of ABT-199 in chronic lymphocytic leukemia (CLL), and clinical trials were halted, trials have been re-started for over a year and continue in acute myelogenous leukemia, CLL, and non-Hodgkins lymphoma.

We report for the first time that the maturation state of a malignancy can determine the specific anti-apoptotic 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 (34,46). 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) (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 versus typical T-ALL cases (Fig. 3)(38). Previous, hierarchical analysis of T-ALL primary samples by gene expression revealed clustering of samples by transcription factors, the LYL^{+} cluster which lack the expression of CD4^{-}/CD8^{-} (DN immature phenotype) had high BCL-2 expression compared to the other clusters that were positive for both CD4^{+} and CD8^{+} (double-positive stage) (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 (47). We hypothesize that transformation of an early T-cell progenitor retains its BCL2 dependence, whereas the CD4^{+} and CD8^{+} positive 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

14

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 (31,38,48-50). In this respect it is notable that acute myeloid leukemia is BCL-2 dependent and sensitive to pharmacological 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 that 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 that of the stem or progenitor cell. Extending our observations here suggest 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 most approaches to deriving predictive biomarkers rely on static measurements of genetics, even whole genomes or whole transcriptomes. Our data suggests that focused phenotypic observations following targeted perturbations of cancer cells may be a very useful alternative in the pursuit of precision medicine.

Experimental procedures

Cell lines and treatments

All of the media were supplemented with 10% heat inactivated fetal bovine serum (Gibco, Carlsbad, CA, USA) 10 mM L-Glutamine and 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 IMDM media (Gibco) and supplemented with 100 ng/mL stem cell factor, 20 ng/mL IL-3 for 6hrs (all supplements obtained from Peprotech, New Jersey, USA). The cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2. The cell lines (PF382, MOLT4, LOUCY,

15

P12ITCHIWAKI, CEM-CCRF, JURKAT) were a kind gift from Dr. Alejandro Gutierrez and were authenticated in August 2012 by short tandem repeat (STR) DNA fingerprinting using 9 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 x 10^4 in 24 wells plates and after 24hrs cells were treated with ABT-199 and ABT-263 (Abbott) for 48 hrs.

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 a 70 μM are plated in triplicate on a black 384 well plate. Cells are gently permeabilized with 0.005% digitonin and loaded with the fluorescent mitochondrial dye JC-1. The cells are plated on top of the peptides at 2.5 *10^4 cells per well. The loss of mitochondrial potential is measured on the Tecan Saffire^2 at an excitation of 545nM and an emission of 590nM over 3 hours. Percentage mitochondrial depolarization, for the peptides is calculated by normalization to the solvent only control DMSO (0%) and the positive control 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 Children's Oncology Group or Dana-Farber Cancer Institute Acute Lymphoblastic Leukemia Consortium clinical trials, Ficoll-purified and viably frozen. All samples were collected with informed consent and institutional review board 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 which measures the absence of CD1a, CD8, 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. 1.8 x 10^6

16

viable tumor cells were immediately used for BH3 profiling. The mitochondrial depolarization was calculated at 60 min. At the same time 3×10^6 cells were used to seed 2×10^5 per well into a 24 well plate in IMDM media supplemented with stem cell factor (SCF) and IL-3 (PeproTech) for treatment with ABT-199 and ABT-263 for 6hrs. When maturation state was known, patients were grouped based on whether the ETP-ALL or typical T-ALL. Investigators performing BH3 profiling assay on ALL specimens were blinded to maturation stage at the time 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 staining. Briefly, cells were washed in PBS and resuspended in Annexin Binding Buffer (10mm HEPES Ph7.4, 140 mM Nacl, and 2.5 mM CaCl). Cells were then stained with 0.5mg/ml Annexin V FITC and 0.5mg/ml propidium iodide for 15 minutes prior to analyzing on the Fortessa (BD Pharmingen). Results are 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 versus Typical T-ALL (38) was also assessed and represented as a heat map using gene pattern.

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-CD19, anti-CD44 and anti-CD25 at 1:100 dilution for 30 min on ice. Cells were pelleted and washed in 1% BSA-PBS before fixation in 2% formaldehyde for 15 min. Cells were resuspended in staining buffer (1% BSA, 2% FBS, 0.1% Saponin and 1.5mM NaN3 in PBS) and appropriate Hamster or Rabbit IgG for blocking for 5 min. The cells were then stained with BCl-2 PE or BCL-XL 488 and along with IgG controls

17

overnight. Following washing the cells were resuspended in PBS and analyzed on the Fortessa for BCL-2 and BCL-XL expression during T-cell differentiation.

Western blotting

Cells were lysed as previously described. 30 μg of protein 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-Xl (Cell Signaling); BCL-2 6C8 (Trevigen) MCL-1 (Santa Cruz). Secondary antibodies used were horseradish peroxidase conjugates of either anti-mouse, anti-hamster or anti-rabbit.

Patient-derived xenograft studies

Primary human T-ALL cells were obtained from children with T-ALL enrolled on clinical trials of the Dana-Farber Cancer Institute. 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 stored in liquid nitrogen. NSG mice were purchased from Jackson Laboratory or were a generous gift from Leonard D. Shultz, Michael A. Brehm and Dale L. Greiner. NSG mice were maintained on a regimen of acidified antibiotic water. Fresh or frozen leukemic blasts were expanded in NSG mice by transplanting $0.5–5 \times 10^6$ cells via intravenous injection. Human engraftment in the spleen and bone marrow was greater than 80% by staining for human CD45 (BD Bioscience). For *in vivo* xenograft studies, NSG mice were injected with 1×10^6 leukemic blasts via intravenous injection and bled weekly to determine the percentage of circulating human CD45-positive cells in the peripheral blood. Once the leukemic burden reached 65% in the periphery, mice were randomized to receive vehicle 10% ethanol, 30% PEG, 60% Phosal-50 (a gift from American Lecithin Company), ABT-199 or ABT-263 (100mg/kg daily by oral gavage). Mice received two weeks of treatment and

18

were sacrificed to assess disease burden. All mouse procedures used in this study were approved by the University of Massachusetts Medical School Institutional Animal Care and Use Committee.

Flow Cytometry

Flow cytometric analysis was performed on a BD LSRII, using FITC and 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 was analyzed using FlowJo software (Tree Star).

Data analysis and Statistics

Graphpad prism 6.0 software (GraphPad Software, San Diego, CA) was used for all statistical analysis. The IC_{50} for the dose response curves following ABT-263 and ABT-199 treatment was calculated using linear regression curve fit (Log inhibitor v's normalized response). Comparison of the IC_{50} response of ABT-263 versus ABT-199 for the primary patient derived samples was calculated using Mann Whitney non-parametric test. Correlation of the ABT-199 response to the BAD minus HRK peptide response was performed using the non-parametric Spearman r correlation test with a two-sided T-test for significance. The flow cytometry data was analyzed using FACS DIVA version 6.1.1 (BD Pharmingen). Student *t* tests were performed on *in vivo* results with Welch's correction using GraphPad Prism software (version 5.0)

Acknowledgements

19

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22

Figure Legends

Figure 1: BH3 profiling and *in vitro* testing of ABT-263 and ABT-199 reveals BCL-XL dependencies in T-ALL.

The binding affinities of BH3 peptides BAD and HRK for the anti-apoptotic BCL-2 family. Red indicates high affinity binding, green is non-detectable binding measured by fluorescence polarization Assay (14) (A). The BAD and HRK responses from the BH3 profiles of T-ALL cell lines are plotted. The mean +/- standard deviations of three independent experiments are graphed (B). The cell lines were treated with a six-point dose range from 1nM to 10μM of ABT-263 and ABT-199 for 48 hrs and apoptosis was measured by Annexin V and Propidium Iodide staining. The average of three independent experiments was used to generate dose response curves in graphpad prism. The IC_{50} in μM is graphed for each cell line (C). There is a statistical difference between the IC_{50} for ABT-263 versus ABT-199 in the T-ALL lines. The ETP cell line LOUCY is shown in red (D). Western blot analysis shows expression of BCL-2 and BCL-XL in the T-ALL cell lines (E). The mean ratio +/- SEM of BCL-2 expression divided by BCL-XL expression measured by densitometry of three independent plots is graphed (F).

Figure 2: BH3 profiling reveals BCL-2 dependencies in primary ETP COG T-ALL samples.

BH3 profiling of pediatric COG T-ALL primary samples prior to initiating treatment. The mean of the BAD and HRK responses are graphed +/- standard deviation from three replicate wells (A). A dot plot of the BAD peptide response versus the HRK peptide response is graphed. The ETP cases as defined by immunophenotypic analysis are marked in red (B). The yellow color indicates probable BCL-2 dependence while the blue color indicates probable BCL-XL dependence. The BAD peptide response (C), the HRK peptide response (D), and BAD minus

23

HRK peptide response was graphed for ETP versus typical T-ALL samples (E). Statistical significance was calculated using the non-parametric Mann-Whitney test.

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, the gating strategy for distinguishing the different stages of T-cell differentiation is shown in (Fig. S3).The expression of BCL-2 and BCL-XL is normalized to the double positive stage, the experiment was repeated three times and mean +/- SD is graphed (A). The mRNA expression of BCL-2 is shown for primary human cells at the listed stages of differentiation. This data is modified from the online database (37) (B). The mRNA expression of BCL-2 and BCL-XL in both ETP and typical T-ALL is graphically depicted in a heat map with red indicating high expression and blue indicating low expression. The data is modified from the published online database (C) (38). (D) The BCL-2/BCL-XL and MCL-1 protein levels are shown for the typical human T-ALL cell lines and samples (T-ALL-x-4,-9,-1,-2) as well as the ETP-ALL cell line LOUCY and the relapsed ETP-ALL sample (T-ALL-x-11). (E) Ratio of BCL-2/BCL-XL protein is shown for the cell lines and primary samples examined in D.

Figure 4: BH3 profiling reveals BCL-2 dependence in ETP-ALL in a separate cohort of DFCI patient samples

BH3 profiling of pediatric and adult DFCI T-ALL primary samples prior to initiating treatment. A dot plot of BAD peptide versus HRK peptide is graphed, ETP are marked in red and green indicates ETP status was not determined (A). The yellow color indicates putative BCL-2 dependence while the blue color indicates putative BCL-XL dependence. The BAD peptide response (B) and the HRK peptide response (C) are plotted for ETP versus typical T-ALL samples. Statistical significance was calculated using the non-parametric Mann-Whitney test. The primary DFCI T-ALL samples were treated with a six-point dose range from 1nM to 10μM of

24

ABT-263 and ABT-199 for 6 hrs and apoptosis was measured by Annexin V and Propidium Iodide staining. The IC_{50} 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 IC_{50} for ABT-199 is correlated to the BAD minus the HRK peptide % mitochondrial depolarization (G). Correlation was calculated using the non-parametric Spearman r test. Red marks the ETP cases, green marks cases with undetermined ETP status and black marks typical T-ALL.

Figure 5: Patient-derived xenograft of ETP-ALL is very sensitive to *in vivo* treatment with ABT-199 while typical T-ALL is relatively resistant.

Primagrafts were generated from two primary T-ALL samples one sample was identified as ETP by immunophenotypic analysis (TALL-x-11) while 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 Propidium iodide following 6 hrs of treatment for the ETP-ALL sample. The % 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, the BAD and HRK response from the BH3 profile are graphed (C) and % survival following ABT-199 and ABT-263 treatment (D). 1 X 10^6 cells of either the ETP-ALL sample or the typical T-ALL samples were injected in the tail vein of NSG mice until an engraftment of 65% human CD45^{+} cells (E). The animals were then randomized into vehicle, ABT-199 or ABT-263 treatment (100mg/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 (F) and in the bone marrow (G) at the end of the two-weeks of treatment in the ETP-ALL sample. Similar *in vivo* experiments were carried out with the typical T-ALL sample with total human CD45^{+} in the blood (H) and in the bone marrow (I) measurements shown following two-weeks of treatment.

25

Figure 6: Schematic of T-cell differentiation and BCL-2/BCL-XL dependence in ETP-ALL and typical T-ALL

During T-cell maturation the most immature T-cells express CD34 but do not express CD4 or CD8 hence they are referred to as double negative (DN). As the cells mature they express both CD4 and CD8 to become double-positive thymocytes. Thymocytes that survive both positive and negative selection become mature CD4 or CD8 single positive T-cells. There is a reciprocal dependence on BCL-2 during the immature double negative stage, which changes to BCL-XL dependence during the double-positive (CD4+ and CD8+) stage of differentiation. Malignancy arising in an immature T-cell ETP-ALL is dependent on BCL-2 and sensitive to both the BCL-2 selective BH3 mimetic ABT-199 and ABT-263 (binds BCL-2, BCL-XL and BCL-w). In contrast malignancy arising from the more mature double-positive T-cells (typical T-ALL) is dependent on BCL-XL and selectively sensitive to the ABT-263 BH3 mimetic.

Ni Chonghaile et. al.

Figure 1

A

	BAD	HRK
BCL-2	🟥	🟩
BCL-XL	🟥	🟥
BCL-W	🟥	🟩
MCL-1	🟩	🟩
BFL-1	🟩	🟩

B

C

D

E

F

Figure 2
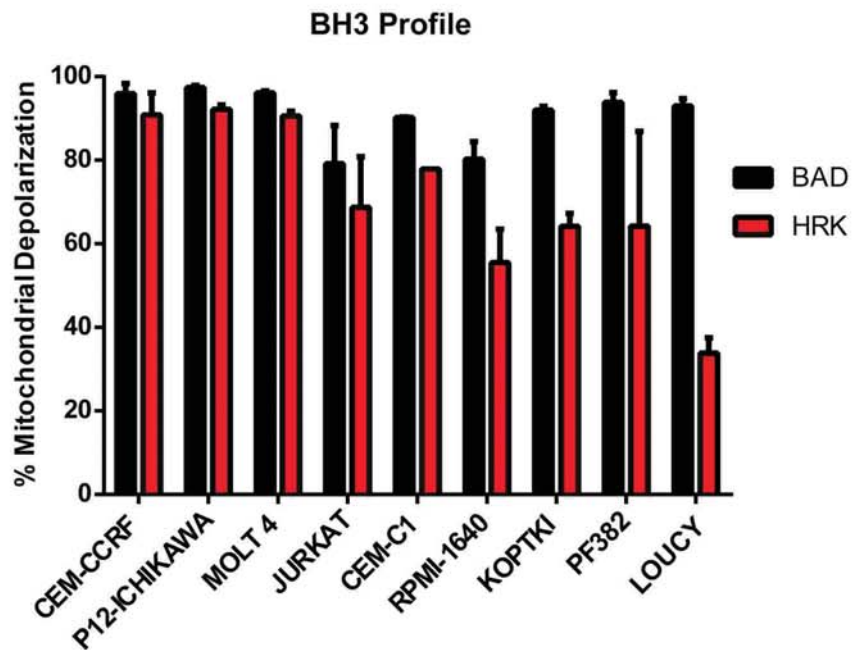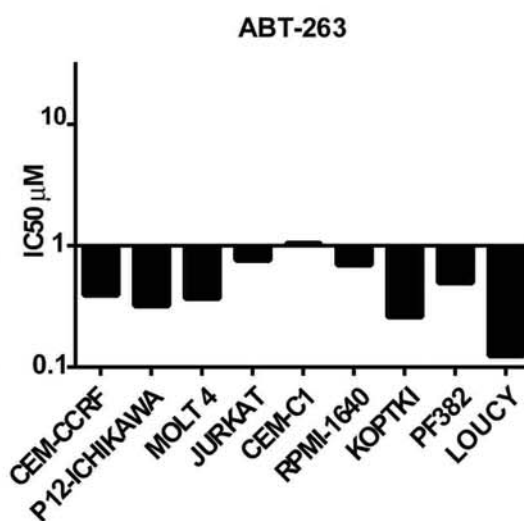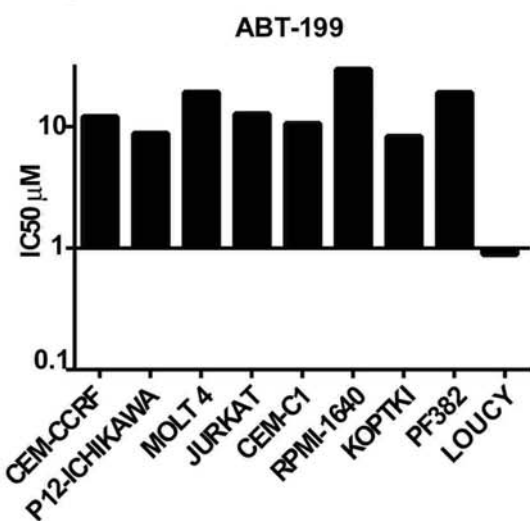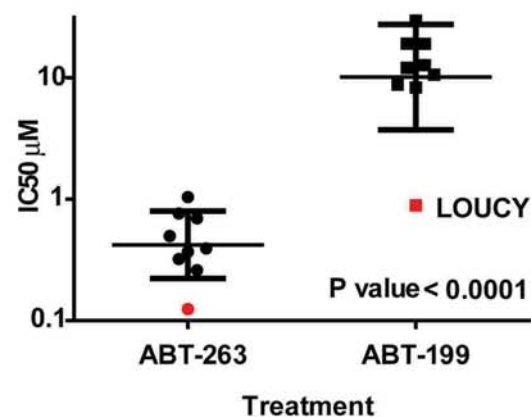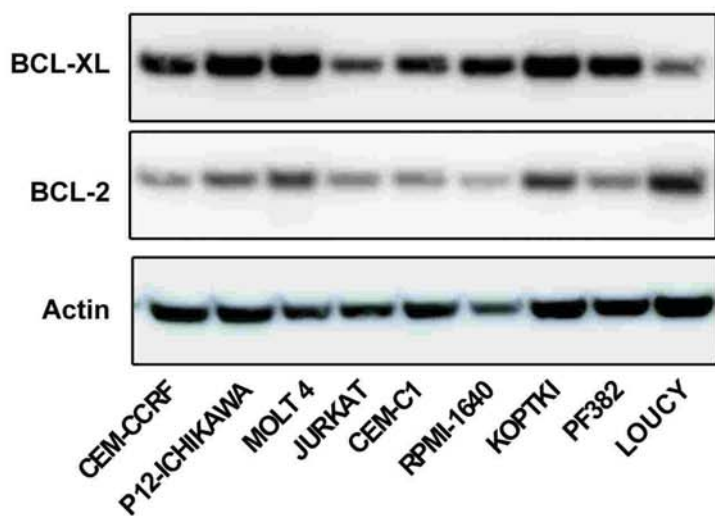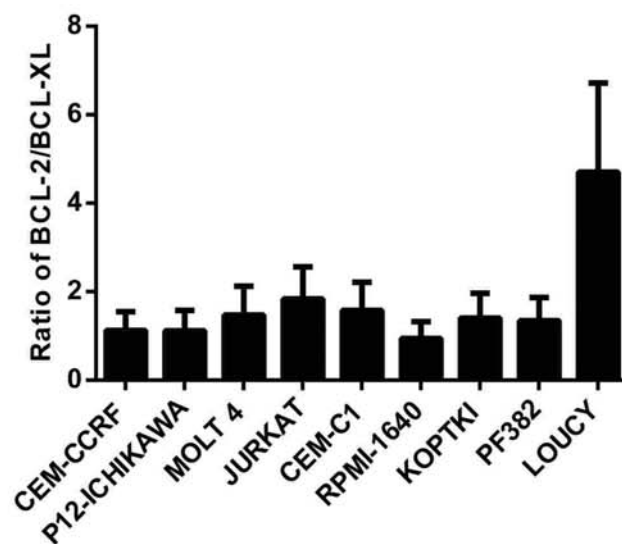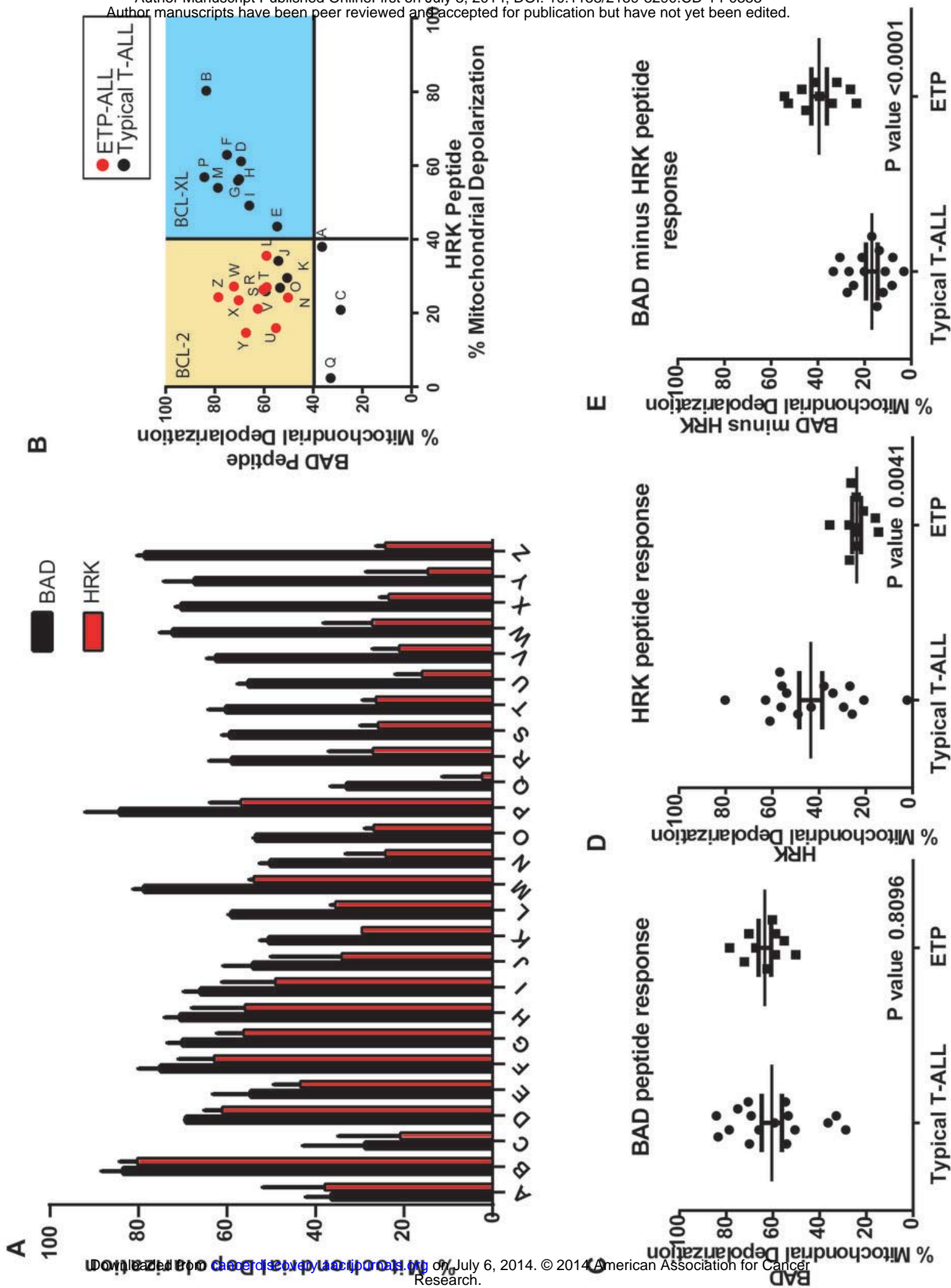
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Figure 3   Ni Chonghaile et. al.

Figure 4

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Figure 6