Preclinical Anticancer Efficacy of BET Bromodomain Inhibitors Is Determined by the Apoptotic Response

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Abstract

Small-molecule inhibitors of the bromodomain and extraterminal (BET) family of proteins are being tested in clinical trials for a variety of cancers, but patient selection strategies remain limited. This challenge is partly attributed to the heterogeneous responses elicited by BET inhibition (BETi), including cellular differentiation, senescence, and death. In this study, we performed phenotypic and gene-expression analyses of treatment-naive and engineered tolerant cell lines representing human melanoma and leukemia to elucidate the dominant features defining response to BETi. We found that de novo and acquired tolerance to BETi is driven by the robustness of the apoptotic response, and that genetic or pharmacologic manipulation of the apoptotic signaling network can modify the phenotypic response to BETi. We further reveal that the expression signatures of the apoptotic genes BCL2, BCL2L1, and BAD significantly predict response to BETi. Taken together, our findings highlight the apoptotic program as a determinant of response to BETi, and provide a molecular basis for patient stratification and combination therapy development.

Introduction

The development and progression of tumors is driven by altered transcriptional programs and underlying tumor-specific chromatin states. BET proteins are highly enriched and functionally essential at gene control elements that drive the expression of critical oncogenes such as MYC (1). As such, BET inhibition affects many pathways critical for cancer cell growth, leading to broad efficacy in pre-clinical disease models (2–5). However, at the molecular and phenotypic level, response to BETi can be quite heterogeneous, making it unclear what the critical drivers are for defining patient stratification strategies. A deeper understanding of the pathways responsible for maximal response to BETi will, therefore, refine these strategies and allow for effective patient selection.

Materials and Methods

Cell line information

Viability analysis was carried out at the MGH Center for Molecular Therapeutics (245 cell line panel), which authenticates cell lines by SNP genotyping. CA46, CHL1, CHP212, Colo783, Colo829, Daudi, H929, HCC1143, HCT116, HCT15, HL-60, HT, HT29, KS62, Kasumi-1, KOPN8, LP-1, MC116, MDAMB231, Mewo, MOLT4, MV411, Namalwa, OPM-2, Raji, Ramos, REH, RPMI8226, SKMEL28, SKNAS, SKN-DZ, STF486, SU-DHL-4, SU-DHL-5, SU-DHL-6, SU-DHL-8, SW48, SW480, SW620, THP-1, U266, WSU-DLCL2, and Z-138 were obtained from ATCC. AMO-1, DOHH2, IGR1, IGR39, JIN-3, KARPA-S22, KMS-12PE, Melho, ML2, MOLM-13, MOLP-8, MOLT16, NB4, OCI-AML2, OCI-AML3, OCI-AML5, OCI-LY-19, PL21, RC-R8, RL, Set-2, U-2932, and U2940 were obtained from DSMZ. KMM-1, KMS-26, KMS-28PE were obtained from HSRRB/忉B. All cell lines were authenticated using SNP genotyping with RNA-sequencing data (Supplementary Tables S3 and S4).

Generation of BETi-tolerant cell lines

A375 cells (ATCC) were cultured in 1 μmol/L CPI203 for approximately 90 days. NOMO-1 cells (DSMZ) were cultured with CPI203 (increasing concentrations) for approximately 9 months. Live cells were periodically enriched by centrifugation over Ficoll-Paque (GE Healthcare). Following selection, cells were maintained in 1 μmol/L CPI203. Parental and BETi-tolerant cells were authenticated using SNP genotyping with RNA-sequencing data (Supplementary Tables S3 and S4).

Analysis of gene expression in BETi-sensitive and -insensitive cell lines

Cells were treated with CPI203 (3–4 days) and viability was assessed [CellTiter Glo (Promega) or resazurin (Sigma)]. Expression values were obtained from the Cancer Cell Line Encyclopedia (6). For qRT-PCR, ΔCt values for BCL2 and BCL2L1 were obtained by subtracting Ct PPIB from Ct BCL2 or BCL2L1. ΔΔCt values were
calculated by subtracting the median $\Delta C_t$ across all cell lines (Supplementary Data) from the $C_t$ value in each cell line, and $\log_2$ fold change was calculated as $\log_2 \left( \frac{2^{\Delta C_t}}{C_0} \right)$.

**Cell cycle, viability, and apoptosis analyses**

Cells were analyzed for cell-cycle distribution and viable cell number as described previously (5).

**Gene-expression profiling**

Total RNA was purified as described previously (5) and RNA sequencing and alignments were performed at Ocean Ridge Biosciences. RNA-seq data have been deposited at GEO as GSE69383.

**Comparison of apoptotic and cell-cycle arrest response with GI50**

Cell lines were treated with CPI203 (4 days) and viability assessed (resazurin; Sigma). The median of the values for %subG1 and %G1 increase (relative to DMSO) across all cell lines was calculated; Z-scores were defined as the number of SDs of the values in each cell line from the median.

**Lentiviral shRNA transduction**

Lentiviral shRNA constructs targeting BCL2L1 and BCL2 were obtained from Sigma and transduced according to the manufacturer’s instructions.

**BH3 profiling**

BH3 profiling was performed as described previously (7). BH3 peptides were obtained from Anaspec (BIM: cat#62439, BAD: cat. #64082) or Abgent (HRK: cat. #SP1016a).

**Statistical analysis**

GraphPad Prism was used for all statistical calculations. Unless otherwise noted, $P$ values were calculated by parametric, unpaired two-tailed $t$ tests. $P$ values are indicated as $^*$, 0.01–0.05; **, 0.001–0.01; ***, 0.0001–0.001; and ****, <0.0001. For linear regression $R^2$ is the square of the Pearson coefficient $r$, and the $P$ value indicates the probability that the slope does not differ from zero.

**Compounds**

CPI203 has been described previously (8). ABT-737 and ABT-199 were obtained from Selleck Chemicals.

Additional details can be found in Supplemental Information.

**Results and Discussion**

Alteration of the apoptotic response in models of acquired BETi tolerance

Long-term culture of A375 (melanoma) and NOMO-1 (acute myelogenous leukemia) in the BET inhibitor CPI203 generated cells that could proliferate in either CPI203 or the distinct BET inhibitor JQ1. Two BETi-tolerant A375 clones, A375-t1 and
A375-t2, revealed a modest shift in the GI50 values for BETi (Fig. 1A, left; Supplementary Fig. S1A and Supplementary Fig. S1C, left). BETi-tolerant NOMO-1-t cells proliferated at concentrations of BETi approximately 30-fold higher than their original GI50 concentrations (Fig. 1A, right; and Supplementary Fig. S1C, right) and surprisingly grew best in the presence of CPI203. Both BETi-tolerant cell lines A375-t1 and NOMO-1-t displayed a blunted apoptotic response to BETi relative to parental cells (Fig. 1B; Supplementary Fig. S1B and S1D). Notably, A375-t1 and NOMO-1-t were equally or more sensitive than parental cells to both cytarabine and doxorubicin, which suggests that they are not resistant to all apoptosis-inducing agents and points toward the use of BETi in combination with cytotoxic chemotherapies (Supplementary Fig. S1E–S1F).

Consistent with these phenotypes, through gene-expression profiling we observed increased expression of the BCL2 family member BCL2L1 (encoding BCL2L1, formerly known as BCL-xL) in both A375-t and NOMO-1-t compared with parental cells treated with CPI203 (Fig. 1C; Supplementary Fig. S2A–S2C). In BETi-treated NOMO-1-t cells, BCL2 was also upregulated compared with BETi-treated parental cells (Fig. 1C; Supplementary Fig. S2C). MCL1 and BCL2A1 were reduced in both tolerant A375 and NOMO-1 cells, highlighting the selective alteration of specific BCL2 family members. In contrast with recent work pointing to the Wnt pathway as a determinant of response to BETi (9, 10), we did not observe increased expression of Wnt target genes in either A375 or NOMO-1 BETi resistance models.

BCL2L1 and BCL2 are critical mediators of BETi sensitivity and resistance
Given the expression changes noted above, we focused on the functional roles of BCL2L1 in A375-t1 and NOMO-1-t cells, and BCL2 in NOMO-1-t cells given that BCL2 was expressed at very low levels in A375-t1 (Fig. 1C; Supplementary Fig. S2B). Knockdown of BCL2L1 in A375-t1 cells, and of BCL2 or BCL2L1 in NOMO-1-t cells, led to dramatically reduced proliferation and significantly increased apoptosis in

Figure 2.
Increased expression of BCL2 family members mediates BETi tolerance. A and B, shRNA-transduced cells were treated with 1 μmol/L CPI203 (A, A375-t1 for 11 days; B, NOMO-1-t for 4 days) before assessing viability and the percentage of sub-G1. Values represent the mean and SEM [A, n = 2; B, n = 2 (shLuc and shBCL2) or n = 5 (shBCL2L1)]. P values are relative to shLuc. *, P < 0.05; **, P < 0.01; ***, P < 0.001 by parametric, unpaired two-tailed t tests. C, cells were cotreated with ABT-737 and 1 μmol/L CPI203 or DMSO (11 days). The percentage of growth in CPI203 is indicated relative to DMSO. Values represent the mean and SEM (n = 3, P values are relative to 0 μmol/L ABT-737); ***, P < 0.01, ****, P < 0.001 by parametric, unpaired two-tailed t tests. D, cells were treated with 0.25 μmol/L CPI203 and the indicated concentration of ABT-737 (4 days). The percentage of growth in ABT-737 is relative to CPI203-treated cells. Values represent the mean and SEM (n = 6, P values are relative to 0 μmol/L ABT-737). ****, P < 0.0001 by parametric, unpaired two-tailed t tests.
the presence of CPI203 (Fig. 2A and B and Supplementary Fig. S3B–S3C). In addition, overexpression of BCL2L1 in parental A375 cells resulted in a blunted apoptotic response to BETi (Supplementary Fig. S3A).

Treatment of A375-t1 and NOMO-1-t with ABT-737, an inhibitor of BCL2, BCL2L1, and BCL2L2 (11), concurrently with CPI203 led to growth suppression and induction of apoptosis, while affecting parental cells to a lesser extent (Fig. 2C and D; Supplementary Fig. S3D–S3E). Consistent with their sustained expression of BCL2, NOMO-1-t cells also showed growth inhibition with the BCL2-specific inhibitor, ABT-199 (Supplementary Fig. S3F; ref. 12). In both models, the degree of apoptosis induction in tolerant cells cotreated with CPI203 and ABT-737 was comparable with parental cells treated with BETi alone, suggesting that pharmacologic inhibition of BCL2 family members can abrogate tolerance to BETi. Notably, combination of the BET inhibitor JQ1 with ABT-199 showed synergistic effects on growth inhibition (13), highlighting the potential of combining BETi with BH3 peptidomimetics.

Engagement of the apoptotic program is associated with robust phenotypic response to BET inhibition

Next, we tested whether the apoptotic program correlates with de novo sensitivity to BETi inhibitors by profiling 51 hematologic cell lines that we termed “sensitive” or “insensitive” based on a GI50 cutoff value of 0.25 μmol/L CPI203. As shown in Fig. 3A, the magnitude of apoptosis, but not the cytostatic response, is highly correlated with sensitivity to BET inhibition. This is consistent with previous work showing that the induction of apoptosis is often associated with preclinical efficacy (2, 3, 5, 14–16).

Given that BETi regulates the expression of antiapoptotic factors like BCL2 (2), we tested whether apoptosis is associated with a change in the expression of key anti- and proapoptotic factors after CPI203 treatment. A clear trend toward downregulation of antiapoptotic factors and upregulation of proapoptotic factors was observed in cell lines that undergo apoptosis (Fig. 3B, top), and a more robust transcriptional response is significantly correlated with the magnitude of the apoptotic response to CPI203 (Fig. 3B, bottom). To functionally test whether these
changes in gene expression were associated with increased apoptotic signaling, we used BH3 profiling with the BIM BH3 peptide to assess mitochondrial “priming” (7, 17). In two cell lines that show a robust apoptotic response to BET inhibition (MV411 and Kasumi-1), preincubation with CPI203 induced measurable increases in mitochondrial priming, which was not observed in cell lines with a minimal apoptotic response (Daudi and SUDHL5; Fig. 3C; Supplementary Fig. S4A).

The results reported above suggest that in highly sensitive cells, BETi treatment disproportionately modulates apoptotic signaling by altering the expression of anti- and proapoptotic genes. Whether these changes in gene expression are the direct result of release

Figure 4.
Basal expression of apoptotic factors predicts BETi response. A, expression (RMA) is shown relative to BETi sensitivity (GI50 < 0.25 μmol/L CPI203). B, mitochondrial depolarization (JC-1 fluorescence) from the indicated cell lines treated with a BAD BH3 peptide (50 μmol/L). Values are the mean of n = 12 (n = 8 for SUDHL4); error is shown in Supplementary Fig. S4B. C, high or low was defined as the expression in the top or bottom third of all cell lines. Dashed line, the overall response rate (28%). Values represent the mean and SEM (P values were determined by two-tailed Fisher exact t test). ***, P < 0.0001 by parametric, unpaired two-tailed t test. D, expression of BCL2 and BCL2L1 (qRT-PCR) are predictive of phenotypic response to BETi. High or low expression was defined as above or below the median (P = 0.002 by two-tailed Fisher exact t test). The overall response rate is indicated in the graph by a dashed line (66%). **, P < 0.01 by parametric, unpaired two-tailed t tests.
of BET family proteins from chromatin is not clear. Recent work has reported that highly BRD4-occupied gene control elements define loci that are highly BET dependent, which includes BCL2 family genes (1). However, we have found that high BRD4 occupancy and release upon BETi treatment are not universal predictors of downregulation of any given gene (data not shown). It is likely that identification of predictive features of BET transcriptional regulation will facilitate our ability to forecast a robust apoptotic response to BET inhibition.

**Basal expression levels of BCL2, BCL2L1, and BAD serve as predictive biomarkers for BETi treatment**

Given our functional data, we tested whether the baseline mRNA expression level of apoptotic factors (Supplementary Table S1) could predict phenotypic response to BETi in a panel of 245 cell lines (Supplementary Table S2; Supplementary Information). We found that five genes, BCL2, BCL2L2, BCL2L1, BAD, and BCLAF1, were differentially expressed with high significance, with BCL2 being the most differentially expressed gene (Supplementary Table S1, Fig. 4A). We reasoned that BETi may selectively target cells that are dependent on BCL2, and tested for BCL2 dependence in a subset of cell lines using mitochondrial exposure to the BAD BH3 peptide (18). We observed that mitochondrial depolarization in response to BAD peptide is correlated with the expression of BCL2 in a set of 6 cell lines (Fig. 4B, top), consistent with published data (18), and that the degree of mitochondrial depolarization was inversely correlated with the GI50 of BETi (Fig. 4B, bottom). There was no significant mitochondrial depolarization in response to the HRK BH3 peptide, arguing against a contribution of BCL2L1 to the BAD depolarization response (Supplementary Fig. S4B). As both inhibitors seem to preferentially target cell lines that are dependent on BCL2 for survival, we noted that the GI50 values of CPI203 and ABT-199 are significantly correlated (Supplementary Fig. S4C, ref. 12).

We next tested whether the expression levels of these apoptotic mediators could predict a robust response to BET inhibition. Selection based on the expression of a one or two genes significantly increased the response rate, with selection of cells with high BCL2 expression and low expression of either BCL2L1 or BAD improving the response rate to 65% or 75%, respectively (Fig. 4C and Supplementary Fig. S5A). Importantly, selection based on these factors was not merely enriching for cell lines of hematologic origin, as these selection criteria increased the response rate of isolated hematologic or solid tumor cell lines, and of acute lymphoblastic leukemia cell lines (Supplementary Fig. S5B–S5D). We also noted that selection based on these biomarkers selected for the most sensitive tumor subtypes, which may point to indications beyond hematologic malignancies that will benefit from BETi (Supplementary Fig. S4D). Finally, the enrichment of phenotypically sensitive cell lines was observed when gene expression was measured by qRT-PCR or Western blotting (Fig. 4D; Supplementary Fig. S6), indicating that these criteria may be used with clinically relevant methods.

Although they have tremendous promise, BET inhibitors also bring new challenges for selecting patient populations and for determining optimal drug combinations. This work identifies the apoptotic signaling network and the expression of factors such as BCL2, BCL2L1, and BAD as patient selection biomarkers that will help BET inhibitors to reach their full therapeutic potential.

**Disclosure of Potential Conflicts of Interest**

N.E. Follmer is a senior scientist in Merck & Co., Inc. B.M. Bryant has ownership interest (including patents) in Constellation Pharmaceuticals. P. Greninger is a head of research and has ownership interest (including patents) in Constellation Pharmaceuticals. J.A. Mertz has ownership interest (including patents) in Constellation Pharmaceuticals. R.J. Sims III is a vice president of research, reports receiving other commercial research support, and has ownership interest (including patents) in Constellation Pharmaceuticals. No potential conflicts of interest were disclosed by other authors.

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