Monocytic Differentiation and AHR Signaling as Primary Nodes of BET Inhibitor Response in Acute Myeloid Leukemia

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INTRODUCTION

Acute myeloid leukemia (AML) is an aggressive hematologic malignancy, diagnosed primarily in elderly patients. Many patients cannot tolerate the intensive chemotherapy regimen (cytarabine + anthracycline), which has been a standard of care for >40 years (1). These patients rely on alternative treatment strategies, such as targeted small-molecule inhibitors. Recently, combinations of the BCL2 inhibitor venetoclax with hypomethylating agents were approved for treatment of patients unfit for chemotherapy, but not all patients respond to this therapeutic regimen (2). Most native treatment strategies, such as targeted small-molecule inhibitors, will develop resistance and relapse. Therefore, understanding molecular mechanisms driving drug resistance is critical for the development of drug combinations that yield durable remissions and extend survival.

AML is a heterogeneous cancer that is primarily driven by four classes of mutations: (i) activation of proliferative and antiapoptotic genes, (ii) block of differentiation, (iii) epigenetic regulators, and (iv) splicing machinery (3–5). As such, we have seen the implementation of many small-molecule inhibitors targeting these pathways (6).

The bromodomain and extra-terminal domain (BET) protein family consists of bromodomain containing the proteins BRD2, BRD3, BRD4, and BRDT, which interact with acetylated histone tails to facilitate many downstream functions, such as chromatin remodeling and transcriptional regulation. Epigenetic inhibitors targeting BET family proteins (BETi) have recently come to the forefront of development due to evident cytotoxicity in hematologic settings (7–11). BRD4 binds acetylated histone tail and recruits positive transcription elongation factor b (P-TEFb) to enhancer regions to mediate the phosphorylation of the C-terminal domain of RNA pol II, required for elongation of the nascent mRNA (12, 13). BRD4 also acts as a histone acetyltransferase (HAT; ref. 14), an atypical kinase (15), and interacts with splicing machinery (16). Previous RNAi studies identified BRD4 loss as a potent inhibitor of leukemic growth (17). BET proteins have also been linked to driving leukemia disease by recruiting transcription machinery to MYC and BCL2 promoters (18, 19). BETi treatment in leukemia cells has been shown to dramatically reduce transcription of these oncogenes and induce cell death (8, 18, 20). Clinically, the BETi OTX-015 achieved complete remissions in a small subset of patients who failed alternative therapies. However, many patients were unresponsive to OTX-015, prompting the investigation of potential intrinsic resistance mechanisms to BETi (21).

Several studies have reported on a wide scope of genetic resistance mechanisms to BETi involving autophagy (22), WNT signaling driven by leukemia stem cells and...
transcriptional plasticity (23, 24), and PP2A (25). Further, Bell and colleagues have shown that nongenetic resistance can arise upon BETi exposure (26). These studies prompted us to mine the Beat AML functional genomic data set and also utilize genome-wide CRISPR screens to further understand drivers of resistance to BETi in AML and identify therapeutically druggable dependencies.

RESULTS

Monocytic Markers Correlate with BETi Sensitivity in AML Patient Samples

To identify potential genetically driven BETi resistance mechanisms, we correlated ex vivo drug-sensitivity data for three BETi (JQ1, OTX-015, and CPI-6010) against recurrent genetic mutations using the Beat AML cohort, an integrative data set containing ex vivo drug-sensitivity analyses, exome sequencing, and RNA sequencing for over 500 primary AML patient specimens (3). Of the entire database, we were able to obtain BETi ex vivo data for 173 unique patient samples. Interestingly, we found no genetic mutations or cyrogeneric patterns that significantly correlated with resistance or sensitivity to any of the three BETi (Supplementary Fig. S1A–S1F). In recent work by our lab and others, similar explorations of response to venetoclax identified leukemic differentiation state as a primary determinant of sensitivity and resistance (27–30). Accordingly, because no mutations correlated with BETi response, we next asked whether BETi sensitivity and resistance correlated with expression of cell-surface markers that are known indicators of cell differentiation state. Indeed, within the Beat AML database, ex vivo responses to JQ1, OTX-015, and CPI-0610 in 173 primary human patient samples were significantly correlated with expression of monocytic markers such as CSF1R, VCAN, CD33, ITGAL, and LILRA1 (Fig. 1A and B). In addition, we found high congruence of drug-sensitivity versus surface marker expression correlations across all three BETi (Fig. 1C). Historically, AML cases have been classified based on the French–American–British (FAB) M0 to M7 classification system, where M0, M1, and M2 represent tumors comprised of minimally or undifferentiated cells, and M4 and M5 represent tumors of a myelomonocytic or monocytic cell state. As expected, we also found that CSF1R, VCAN, LILRA1, and LILRB1 were highly expressed in monocytic leukemia FAB subtypes (M4–M5) compared with undifferentiated cases (M0–M2; Fig. 1D–G). Together, these findings indicate that BETi may be more efficacious in differentiated leukemias and highlight a novel vulnerability for these leukemias.

Genome-Wide CRISPR Screen Identifies Monocytic Differentiation Regulators of BETi Resistance

To further study mechanisms of resistance to BETi in AML, we performed a genome-wide CRISPR resistance screen in OCI-AML2 cells under selection of the BETi CPI-0610. Distribution of single-guide RNAs (sgRNA) in deep-sequestered libraries from drug-treated cells was compared with DMSO-treated controls using edgeR (31). To prioritize hits, we used a tiering structure that we previously developed for CRISPR screens (32). This scheme organizes top candidates into three tiers based on evidence (determined by the number of sgRNA guide hits per gene), concordance (indicated by the agreement across the set of guides for a given gene), and discovery (based on expanding effect size threshold). Using this prioritization scheme and focusing on tiered genes with a mid-log fold change > 1.5, CPI-0610 (Fig. 2A) selected cells showed enrichment for guides targeting hematopoietic transcription factors (TF; e.g., FOSL2, JUNB; Fig. 2B) and aryl-hydrocarbon signaling (e.g., AHR, ARNT; Fig. 2C) compared with DMSO. We ran a parallel screen using overlay of a related BETi, JQ1, instead of CPI-0610. Although the results of this screen did not yield hits that were as statistically significant as the CPI-0610 screen with no genes assigned to a tier, we focused attention to genes with a mid-log fold change > 2. Under this threshold, we also identified resistance-enriched guides that targeted genes in the same category of TFs known to regulate myelopoiesis (SPL1, FOS, CREB1; Supplementary Fig. S2A and S2B). To prioritize hits for follow-up analysis, we assessed enrichment of hits from the two screens in a pathway context. We took the combined list of gene hits from both screens and seeded a STRING (Search Tool for Retrieval of Interacting Genes/Proteins; ref. 33) network to investigate associations between this union of candidate genes from both screens (Fig. 2D; Supplementary Fig. S2C). In addition, we took these same hits and analyzed them via Gene Ontology Cellular Component Ontology and found high concordance between genes from both screens under shared ontological components (Supplementary Fig. S2D). Using both methods, a core set of gene hits—SPL1, FOS, JUNB, AHR, and ARNT—were mapped in close proximity across multiple pathway annotations, and these genes were chosen for downstream validation.

Screen Validation

Because both the JQ1 and CPI screens pointed to the same pathways but yielded less agreement at the level of individual genes, we wanted to determine whether these specific gene knockout events were truly causing differential resistance to the two drugs or whether targeting of these genes yields more of a pan-BETi resistance phenotype. Accordingly, we designed two sgRNAs per gene to target SPL1, FOS, or JUNB or nontargeting. Knockouts were validated by Tracking of Indels by DEcomposition (TIDE) analysis or Western blot (Supplementary Tables S1 and S2; Supplementary Fig. S3A). Sensitivity to BETi (JQ1 and CPI-0610) was determined by MTS assay after selection ofOCI-AML2 cells with control or gene-targeting sgRNAs. With this approach, we found that loss of SPL1, FOS, and JUNB resulted in pan-BETi resistance (Fig. 3A–C). Small-molecule AHR inhibitors and activators are readily available and, thus, to investigate altered AHR signaling in mediating BETi resistance, we tested the combination of CPI-0610 with either an AHR inhibitor (AHRi), CH-223191, or an AHR agonist, FICZ. As expected, the AHR antagonized CPI-0610 cytotoxicity in OCI-AML2s (Fig. 3D), with a zero interaction potency (ZIP) synergy score of −3.493, whereas FICZ enhanced these effects, with a ZIP synergy score of 4.425 (Fig. 3E). We validated these findings further by knocking out AHR and ARNT with single sgRNAs and observed multi-BETi resistance (Supplementary Fig. S3A and S3B; Supplementary Table S3). Convergent lines of evidence suggest that hematopoietic TFs (34, 35) and AHR (36, 37) recruit histone-modifying machinery to active transcription sites and interact with the P-TEFb complex (38, 39). The subsequent hyperacetylation at these regions increases
dependency on BRD4 and induces monocytic differentiation. Therefore, we hypothesized that monocytic leukemias, which characteristically have high expression of these genes, may exhibit greater sensitivity to BETi, and that undifferentiated blasts will be intrinsically resistant to BETi due to decreased expression of these genes.

**BETi-Resistant Cells Exhibit Decreased Markers of Leukemic Differentiation, and Forced Myeloid Differentiation Increases Sensitivity to BETi**

Given our proposed mechanism, which posited that BETi sensitivity is driven by monocytic differentiation, we asked whether acquired BETi-resistant (BETi-R) cells had reduced marks of myeloid differentiation. We generated JQ1- and CPI-0610–resistant (JQ1-R and CPI-0610-R) OCI-AML2 cell models by serially passaging them under increasing selective pressure of BETi and reached IC50 values 5-fold higher than parental OCI-AML2 cells (JQ1: 52 nmol/L parental, 1.8 μmol/L resistant; CPI-0610: 170 nmol/L parental, 1.1 μmol/L resistant; Fig. 4A). Immunophenotyping of OCI-AML2 parental and BETi-R cells revealed a significant reduction in myeloid differentiation marker CD33 expression in BETi-R cells at baseline (Fig. 4B). To test whether BETi treatment selects for less differentiated clones, we treated both BETi-naive and BETi-R OCI-AML2 and observed decreased CD33 expression in both BETi-naive and BETi-R cells in a...
Whole-genome CRISPR screen identifies hematopoietic TFs and AHR signaling as drivers of BETi resistance. Cas9-expressing OCI-AML2 were lentivirally transduced with a genome-wide CRISPR library containing an average of five guides per gene, collectively targeting 18,010 human genes (32, 77). Cells were treated with 500 nmol/L CPI-0610 or vehicle (DMSO) for 21 days, followed by DNA harvest and PCR amplification of sgRNA guide sequences. A, Volcano plot comparing enrichment of sgRNAs relative to DMSO control in CPI-0610-treated OCI-AML2 cells versus \(-\log_{10}\) transformed median P value after 21 days, with corresponding significance tiers. Each dot represents a combined sgRNA knockout (KO) enrichment score (combining approximately five to six unique sgRNAs targeting the same gene) relative to control versus corresponding P value. Significance of each sgRNA was determined via edgeR (RRID: SCR_012802) after trimmed mean of M values (TMM; ref. 78) normalization. Briefly, considering only significant sgRNAs (FDR \(\leq 0.05\)) genes were classified into five ordered groups. Tier 1 genes had more than one significant sgRNA, a minimum \(\log_{2}\) fold change \(\geq 2\) and 75% of sgRNAs per gene present, and concordance among sgRNAs per gene \(\geq 75\%\); tier 2 hits had \(\log_{2}\) fold change \(\geq 2\) but only a single significant sgRNA. Enriched hits not satisfying these criteria were classified into the unassigned group. The x-axis corresponds to median \(\log_{2}\) fold change (CPI treated/control treated) sgRNA counts. The y-axis corresponds to \(-\log_{10}\) median P value. FC, fold change. B, Enrichment of cells containing the relative sgRNA KO relative to DMSO control (roughly five to six unique sgRNAs per gene KO for Yusa library) targeting hematopoietic TFs JUNB (left) and FOSL2 (right) in CPI-0610–treated OCI-AML2. Each line represents the guide counts of the relevant unique sgRNA KOs in control or CPI-0610–treated cells. C, Enrichment of cells containing the relative sgRNA KO targeting aryl-hydrocarbon receptor signaling components AHR (left) and ARNT (right) in CPI-0610–treated OCI-AML2. Each line represents the guide counts of the relevant sgRNA KOs in control or CPI-0610–treated cells. D, A STRING (33) network, which is used to model known or predicted protein–protein interactions from custom gene lists, was seeded with significant hits from both the CPI-0610 and JQ1 CRISPR screens, and identifies a concordant network of enriched hits of interest across both JQ1 and CPI-0610 CRISPR screens centered on hematopoietic TFs, AHR, and histone-modifying machinery. Size of node denotes tier (largest = tier 1, smallest = unassigned); color denotes gene screen of origin.

To determine acquired vulnerabilities in BETi-R cells, we performed a genome-wide CRISPR dropout screen on JQ1-R OCI-AML2 cells to generate knockout events that resensitize JQ1-R cells to BETi. Hits were identified by comparing depleted sgRNAs from JQ1-treated cells to DMSO. Using STRING analyses, we identified a subnetwork consisting of cell-cycle genes (CDK2 and CDK6) and antiapoptotic genes (BCL2, ROCK1, and BIRC2; Fig. 5A and B). Independently derived sgRNA guides targeting BCL2 resensitized both JQ1-R and CPI-0610-R OCI-AML2 cells to BETi (Fig. 5C). Data collected from the Beat AML biorepository, bloodspot.eu, and data deposited by Pei and colleagues (29) show that SPI1, FOS, FOSL2, JUNB, and AHR are highly expressed in monocytic leukemias, whereas BCL2 and CDK2/4/6 are enriched in undifferentiated blasts (Supplementary Fig. S4A-S4F). Collectively, these data further support our hypothesis that forced differentiation can enhance sensitivity to BETi.

Genome-Wide CRISPR Screening in BETi-R Cells Identifies BCL2 and CDK2/6 as Resensitizing to BETi

BETi-R cells exhibited decreased levels of JUNB, MYC, CDKN1A, CDK6, and CDK2, and preserved transcripts of CDKN1B, ROCK1, and BIRC2, consistent with forced differentiation (Fig. 5D and E). In addition, doxycycline-inducible PU.1 overexpression in HL-60s sensitized to multiple BETi (Fig. 4F). Lastly, to answer whether BETi directly target differentiation programs and, thus, support the notion that BETi may modulate the process of differentiation, we added BETi concomitantly with ATRA in HL-60s and found that differentiation was significantly attenuated, as seen by a significant reduction in CD38 expression and morphologic changes consistent with myeloid differentiation after 72 hours (Fig. 4G). Taken together, these findings validate that BETi treatment selects for less differentiated clones and that forced differentiation can enhance sensitivity to BETi.
that BETi sensitivity and resistance are tethered to differentiation state and identify potentially targetable vulnerabilities BCL2 and CDK2/6.

**BETi-R AML Cells Are Sensitive to BCL2 Inhibitors and the Combination of BETi + BCL2 Inhibitor Specifically Rescues Intrinsically BCL2 Inhibitor–Resistant Monocytic AMLs**

We and others have shown that BCL2 inhibitors (BCL2i) are more effective in undifferentiated blasts, and monocytic differentiation is a driver of BCL2i resistance in AML (27–30)—the inverse correlation that we have seen here where BETi are more effective on AML cells of monocytic differentiation state and an undifferentiated state promotes BETi resistance. Thus, we tested the efficacy of the BCL2i venetoclax in BETi-naïve and BETi-R cells and found increased sensitivity in BETi-R cells (Fig. 6A). This correlated with increased expression of BCL2, MCL1, and BCL2L1 (BCL-XL) in the BETi-R cells (Fig. 6B). In addition, the combination of venetoclax and BETi significantly increased cytotoxicity in undifferentiated c-kit+ cells in BETi-naïve and more so in BETi-R cells (Fig. 6C). Finally, AML patient samples treated ex vivo with JQ1 showed enhanced sensitivity in M4/M5 FABs but were intrinsically resistant to venetoclax, as previously published (28–30). However, the combination of JQ1 + venetoclax showed significantly enhanced sensitivity compared with venetoclax alone, specifically in matched monocytic leukemias, rescuing intrinsic BCL2i resistance due to monocytic differentiation (Fig. 6D and E).
As discussed previously, our data indicate that BETI selects for less differentiated clones, which correlates with increased CDK2/6 expression, and was sensitized by sgRNAs targeting these genes. Thus, we tested the combination of the CDK4/6 inhibitors (CDK4/6i) palbociclib or abemaciclib with JQ1, OTX-015, or CPI-203 in BETI-naive OCI-AML2 and OCI-AML3 cells and observed strong synergy (Supplementary Fig. SSA). In concordance with our proposed mechanism, we found that palbociclib resistance correlates with increased monocytic markers, inverse of BETI (Supplementary Fig. SSB), and that forced myeloid differentiation of HL-60s drove strong resistance to palbociclib (59.45 nmol/L IC_{50} undifferentiated, 3766 nmol/L IC_{50} differentiated; Supplementary Fig. SSC). In addition, AML patient samples treated with palbociclib or JQ1 + palbociclib recapitulated our findings with venetoclax, with M4/M5 FAB patient samples exhibiting resistance to single-agent palbociclib treatment but enhanced sensitivity with BETI combination treatment (Supplementary Fig. SSD). Collectively, these data suggest that the synergy shown here and by others previously between BCL2i or CDK4/6i and BETI is driven by differentiation state, and that myeloid differentiation can drive resistance to CDK4/6i and BCL2i.

**H3K27Ac Chromatin Immunoprecipitation Sequencing Stratified by FAB Subtype Reveals Enrichment of AHR Signaling and Hematopoietic TFs in Monocytic Leukemias**

Finally, to determine whether undifferentiated blasts (FAB subtype: M0–M2) have differentially acetylated histone residue profiles in comparison with monocytic leukemias (FAB subtype: M4–M5), as a consequence of differential expression

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**Figure 4.** BETI-R OCI-AML2 exhibit hallmarks of decreased differentiation, and forced myeloid differentiation of HL-60s increases sensitivity to BETI. A–C, BETI-R OCI-AML2 cells were generated via incubation with serially increasing concentration of JQ1 or CPI-0610 over a several month period. A, MTS viability assays were used to assess BETI dose responses in JQ1-R (left) and CPI-R (right) OCI-AML2 cells. Error bars represent the SE margin between the six replicates. P values were calculated using a two-stage linear step-up procedure of Benjamini–Krieger–Yekutieli comparing parental to BETI-R OCI-AML2 cells. The x-axis represents BETI concentration; the y-axis represents OCI-AML2 percentage viability. B, Median fluorescence intensity (MFI) of myeloid differentiation marker CD33, as determined by flow cytometry, on untreated BETI-naive OCI-AML2 cells and BETI-R OCI-AML2 cells withdrawn from drug for 1 week. Significance determined by ordinary one-way ANOVA with multiple comparison corrections from three replicates. C, CD33 MFI, as determined by flow cytometry, of BETI-naive or BETI-R OCI-AML2 cells treated with vehicle, 300 nmol/L JQ1, or 600 nmol/L JQ1 for 72 hours. Significance determined by two-way ANOVA with multiple comparison corrections from three replicates. D, HL-60s were differentiated with 1 µmol/L ATRA for 72 hours or left undifferentiated in vehicle for 72 hours. The cells were then washed in PBS, and viability was assessed (+/−5%5) by Guava easyCyte. Drug dose–response curves, as determined by MTS viability assay, were then assessed for JQ1 (top) and CPI-0610 (bottom) comparing undifferentiated (vehicle) and ATRA-differentiated HL-60s. Error bars represent the standard error margin between the six replicates. P values were calculated using a two-stage linear step-up procedure of Benjamini–Krieger–Yekutieli comparing (vehicle-treated) cells to differentiated (ATRA-treated) cells. The x-axis represents BETI concentration; the y-axis represents HL-60 viability. E, Fold change IC_{50} values for JQ1 and CPI-0610 in undifferentiated HL-60s versus ATRA-differentiated HL-60s from D. F, Top, flow cytometry measurement of CD38 MFI, which marks HL-60 differentiation state, in naïve HL-60s treated for 72 hours with vehicle (undifferentiated), BETI, ATRA (differentiated), or simultaneously added BETI and ATRA. Significance determined by one-way ANOVA from three replicates. Bottom, Giemsa stain of HL-60 cells that were previously subjected to vehicle, BETI, ATRA, or BETI + ATRA for 72 hours. G, HEK 293T cells were used to generate inducible PU.1 virus (pINDUCER21-SPI1) with packaging vectors psPAX2 and VSVG. HL-60 cells were then lentivirally infected with the inducible virus or empty vector and GFP sorted. HL-60 cells were then induced with doxycycline (dox) for 5 days, and JQ1 and CPI-0610 IC_{50} values were determined by the MTS assay. Corresponding Western blot validating PU.1 overexpression in HL-60 cells at day 5 (bottom). The x-axis denotes control versus pINDUCER21-SPI1 cells; the y-axis denotes IC_{50} for JQ1 or CPI-0610.
of the monocytic differentiation regulators (e.g., SPI1, FOS, AHR), which recruit histone-modifying machinery, we analyzed H3K27Ac chromatin immunoprecipitation sequencing (ChIP-seq) data deposited by McKeown and colleagues (41) and called differentially acetylated regions by FAB subtypes. The undifferentiated M0 to M2 FAB subtype samples separated distinctly by principal component analysis (PCA) from the differentiated M4 and M5 subtypes (Fig. 7A). A total of 6,076 differential affinity peaks were identified between M0 to M2 and M4 and M5 samples. As expected, we observed increased acetylation at monocytic surface markers such as VCAN/LILRs and most significantly increased acetylation at the canonical AHR transcriptional target CYP1B1 (42, 43) and its repressor AHRR, which is induced during constitutive AHR signaling (refs. 44, 45; Fig. 7B). This suggests increased AHR signaling in monocytic AMLs. To further validate that enhanced AHR signaling is found in monocytic leukemias, we asked whether M4 and M5 patient samples had increased expression of canonical AHR-regulated genes CYP1B1 and CDKN1A. Indeed, both were found to be significantly increased in M4 and M5 AML (Fig. 7C). In addition, we evaluated genes coexpressed with CYP1B1 and AHR within AML patient samples in the Beat AML database and found significant positive correlations with monocytic surface markers, hematopoietic TFs previously identified within the Beat AML database, and BETi-naive whole-genome CRISPR screens as well as negative correlations with CDKs and BCL2 (Supplementary Fig. 6A and 6B). In conclusion, these data show that monocytic AML patient samples have increased binding at BRD4 targets as well as AHR. Further, we show that AHR signaling is enhanced in monocytic leukemias, validating our findings in the initial whole-genome CRISPR screens and subsequent validation with AHR agonists and antagonists.

**DISCUSSION**

BETi have shown clinical promise in a small subset of patients, but most are intrinsically resistant to BET inhibition as a monotherapy (46), which is consistent with Beat AML biorepository data, with 48 of 287 (17%) patients exhibiting *ex vivo* sensitivity (<100 nmol/L IC50, average 886 nmol/L) to the BETi JQ1 (3). Although we found no association of BETi sensitivity with known mutations, our data correlate dependency of intrinsic resistance to BETi on leukemic differentiation state. Our study indicates that primary patient samples with higher expression of genes associated with a more differentiated, monocytic phenotype exhibit enhanced sensitivity to BETi, and reveals a potential therapeutic strategy for monocytic leukemias. Consistent with the observed patient sample *ex vivo* BETi responses correlating with monocytic surface markers, our CRISPR BETi-resistance screen identified SPI1, FOS, JUNB, and AHR—genes with high expression in monocytic leukemias and that regulate monocytic differentiation—as primary drivers of BETi resistance (47–50). Our CRISPR resensitization screen of BETi-R cells revealed CDK4/6, BCL2, and NPM1 as top hits in resensitizing to BETi. Thus, our study identified both resistance and resensitization components of BETi sensitivities, tethered to blast differentiation state. Loss of function of genes expression enriched in monocytic AML, such as hematopoietic TFs SPI1, FOS, and JUNB as well as AHR...
Romine et al.
correlates HARTI LE, subtypes (51). Interestingly, SPI1 expression in AML has demonstrated the ability to bidirectionally transition between differentiated and undifferentiated states (57), which supports the notion that leukemia cells could acquire BETi resistance by selecting for cells in a repressed SPI1 state without acquiring de novo mutations. Both the AP-1 complex and AHR signaling, is revealed as a primary driver of resistance to BET inhibition, whereas inactivation of genes that are expression enriched in primitive AML, such as CDK6 and BCL2, resensitizes resistant cells to BETi.

**Dysregulation of Hematopoietic TFs in AML**

Hematopoietic TFs such as SPI1 and JUNB suppress myeloid leukemias by enabling proper differentiation (51-53). Not surprisingly, mutations that cause reduced function in these TFs are frequently found in AML and generally associate with a poor prognosis (3, 54-56). Despite the large body of evidence surrounding the biology of TFs in hematologic malignancies, the manner by which they can affect drug sensitivity is largely unknown.

In normal hematopoiesis, SPI1 drives monocytic differentiation by positively regulating the AP-1 TFs, JUN/FOS, and, as such, their expression highly correlates with AML M4 and M5 subtypes (51). Interestingly, SPI1 expression in AML has demonstrated the ability to bidirectionally transition between differentiated and undifferentiated states (57), which supports the notion that leukemia cells could acquire BETi resistance by selecting for cells in a repressed SPI1 state without acquiring de novo mutations. Both the AP-1 complex and AHR signaling regulate cellular proliferation by directly (indirectly for AHR via upregulation of CDKN1A/p21) repressing Cyclin D1 and regulating expression of CDK2 and CDK4 (52, 58). Together, these data may explain our findings that CDK4/6i and BETi synergize. We also noted increased sensitivity to BCL2i and resensitization to genetic knockdown in acquired BETi-R cells that overexpressed BCL family member proteins. Previous work has shown that sustained expression of NFkB-regulated antiapoptotic genes occurs in SPI1-depleted AML cells (59).

**Monocytic Surface Markers as Correlates of BETi Efficacy**

We identified monocytic markers CSF1R, VCAN, and LILRA1 as strongly correlating with BETi sensitivity in AML patient samples. Of particular interest is CSF1R, as there are highly specific flow-cytometry antibodies that could be integrated into clinical immunophenotyping panels. CSF1R is a surface receptor that binds CSF1 or IL34 to promote proliferation, survival, and differentiation of monocytes and macrophages. Upon activation, CSF1R activates proliferation and survival pathways that upregulate the SPI1 (60). VCAN is an extracellular matrix proteoglycan expressed across...
many human tissues. It increases activation and adhesion of monocytes through interactions with CD44. Interestingly, monocytes highly upregulate VCAN, allowing a stronger self-promoting response to inflammation (61–63). LILRs are highly expressed in monocytes/macrophages, neutrophils, and B cells, and interact with HLA class I molecules, which have both activating and inhibitory functions (64). Together, our findings suggest a possibility of predicting BETi sensitivity based on CSF1R/VCAN/LILR expression and further suggest the impetus for investigating the efficacy of combining CSF1R and BET inhibitors in monocyteic leukemias.

**Combined BETi-R and BETi-Naïve Genome-Wide Screening to Identify Novel Combinatorial Treatment Strategies to Overcome BETi Resistance**

Our study highlights the power of performing genome-wide CRISPR drug screens on both treatment-naïve and treatment-resistant AML cells to identify rational combinatorial treatment strategies to overcome resistance. We identified in both settings that targeting BCL2 or CDK4/6 may sensitize BETi-R AML cells. Several groups have published in vitro and in vivo efficacy of targeting BCL2 in combination with BETi, lending credence to these approaches (35, 65–68). However, we believe we are the first to rationally describe this phenomenon in the context of differentiation state and highlight a novel therapeutic vulnerability in differentiated AMLs. In addition, we describe a novel role for AHR signaling in mediating BETi response in AML and as a measure of leukemic differentiation state, a topic that we will be exploring further. Further, in silico predictions based on the Beat AML data set identified BETi and CDK4/6 as a synergistic combination strategy, indicating a potentially viable combination strategy for both treatment-naïve and BETi-R settings (69, 70). Future studies will investigate unreported combination strategies identified in the screens, such as AHR agonists with BETi.

**BETi Vulnerability as a Consequence of Leukemic Differentiation State**

Hematopoietic differentiation is accompanied by large changes to the chromatin landscape and requires HATs,
methytransferases, and others (71, 72). Monocytic differentiation in particular is associated with stark increases in histone acetylation and chromatin accessibility at hematopoietic TF binding sites (73). Hematopoietic TFs recruit BRD4 by directly stimulating activity of HATs, such as EP300, which is corroborated by studies demonstrating that EP300 inhibition phenocopies BRD4 inhibition (7, 13, 34, 74). In this context, BRD4 acts as a cofactor for hematopoietic TFs, offering a likely explanation of increased sensitivity to BETi in hematologic malignancies. Our findings suggest that BETi resistance is driven by differentiation state as a consequence of evolving histone acetylation status. This is consistent with studies demonstrating WNT-driven transcriptional plasticity as a driver for BETi resistance in AML (23, 26). Further, Sheng and colleagues have recently described a mechanism for WNT signaling blocking monocytic differentiation through inhibition of SPI1 (75). Intriguingly, studies have also shown that AHR activation induces SPI1 production and monocytic markers in human AML (40, 76). Thus, we propose a unified mechanism of resistance involving decreased expression of hematopoietic TFs as a consequence of differentiation state, which can be driven by loss of WNT signaling, that consequently decreases BRD4 dependence and BETi sensitivity. Differentiated leukemia cells upregulate hematopoietic TFs, which recruit histone-modifying machinery and serve to attract BRD4 to target sites, resulting in increased BETi sensitivity (Fig. 7D). Conversely, less differentiated cells have lower expression of these TFs and thus have reduced BRD4 recruitment and, subsequently, reduced sensitivity to a BETi. Further complexity to the proposed mechanisms could result from the transient and often unpredictable nature of differentiation/epigenetic dysregulation in leukemia, driven by differences in patient-specific mutations. These are important topics for investigation moving forward. However, we believe these data support more targeted clinical exploration of BETi in AML, such as an emphasis on recruiting M4 and M5 FAB subtypes or patients with high expression of CSF1R. In addition, our findings provide a further mechanistic understanding of increased sensitivity to BETi in hematologic malignancies.

**METHODS**

**Cell Lines**

OCI-AML2 (DSMZ cat. #AC-99, RRID: CVCL_1619), OCI-AML3 (DSMZ cat. #AC-582, RRID: CVCL_1844), and HL-60 (CLS cat. #300209/p671_HL-60, RRID: CVCL_0002) were obtained from ATCC. Cell lines were authenticated using the Oregon Health and Science DNA Serviced Core Facility and tested biweekly for Mycoplasma. All cell lines were maintained in RPMI, 20% FBS, L-Glutamine, penicillin/streptomycin, amphotericin-B, and normocin. BETi-R cells were generated by incubating OCI-AML2s at respective JQ1 IC_{50} or CPI-0610 IC_{50} concentration twice weekly, and viability was monitored three times per week by Guava easyCyte. Once viability returned to ~80%, cell sensitivity was remeasured and cells were incubated at their increased IC_{50} concentration. This was repeated until a 5x IC_{50} increase was achieved. Cells were maintained resistant by treating with 800 nmol/L JQ1 or 800 nmol/L CPI-0610 weekly.

**CRISPR/Cas9 Library Screen and CRISPR/Cas9 Gene Inactivation by Individual sgRNA**

Cas9-expressing cells were generated using Cas9Blast (Addgene, #52962). Loss-of-function screens were performed as described (9), using a human genome-wide sgRNA library (77), purchased from Addgene (#67989). High-titer lentivirus was generated using calcium phosphate precipitation procedures in HEK 293T cells (NCBI cat. #C498, RRID: CVCL_0063). Viral supernatant was concentrated and titered using a viral titration kit (ABMGood). Cells (100^6) were used for viral transduction at MOI (multiplicity of infection) 0.3 and selected with puromycin for 5 to 7 days. Cells were screened with 200 nmol/L JQ1, 500 nmol/L CPI-0610, or DMSO. DNA was harvested after puromycin selection on day 14 (JQ1) and day 21 (CPI-0610). PCR-amplified barcode libraries were generated as previously described (77) and deep sequenced using Illumina platform.

**Single sgRNA Knockouts**

Single sgRNA sequences were designed using Synthego design tool (https://design.synthego.com/#/) and converted into DNA sequences (Supplementary Table S2). Individual sgRNAs were cloned into plentiCRISPRV2 (Addgene, #52961, RRID: Addgene_127644). Phosphorolyated complementary oligonucleotides were annealed and ligated into BsmBI-digested plentiCRISPRV2 backbone, which contains sequences for Cas9 and puromycin resistance, and then validated by Sanger sequencing. Lipofectamine 2000 (Invitrogen, #11668019) was used to transfect HEK 293T cells with single transfer vectors with packaging plasmids psPax2 (Addgene, #12260, RRID: Addgene_12260) and VSVG (Invitrogen) to generate virus. Viral supernatants were collected, filtered through 0.45-μm filters, and used for transduction of AML cells using spinoculation method as described (77). Cells were selected with 2 μg/mL of puromycin for 5 to 7 days and outgrown for 14 days in culture before testing for BETi resistance.

**Biostatistical Analysis of CRISPR Screens**

Pipeline for executing analyses of CRISPR library sequences was performed using modified Mageck analyses. Adaptor sequences were removed using cutadapt, and reads were aligned to the K. Yusa library using bowtie2 (RRID: SCR_016368). Sequences that aligned to more than one region were discarded. An overall alignment rate of 92% was achieved. The overall set of sgRNAs was first filtered to remove any that had zero reads in all samples or in the plasmid. For each contrast, sgRNAs that did not achieve 100 reads (adjusting for library size) in at least half the samples were also removed. Significance of each sgRNA was determined via edgeR (RRID: SCR_012802) after trimmed mean of M values (TMM; ref. 78) normalization for the following contrasts: JQ1 screen at day 14 to DMSO screen at day 14, and CPI-0610 screen at day 21 to DMSO screen day 21. Sequencing data are deposited at Gene Expression Omnibus (GEO; GSE19689). Hits were prioritized according to a previously described tiering structure (32). Briefly, considering only significant sgRNAs (FDR < 0.05), genes were classified into five ordered groups. Tier 1 genes had more than one significant sgRNA, a minimum log2 fold change ≥ 22, 75% of sgRNAs per gene present, and concordance among sgRNAs per gene ≥75%; tier 2 hits had log2 fold change ≥ 22 and 100% concordance among sgRNAs per gene; and tier 3 hits had log2 fold change ≥ 21 and 100% concordance among sgRNAs per gene. Singleton hits represent significantly enriched genes with log2 fold change ≥ 22 but only a single significant sgRNA. Enriched hits not satisfying these criteria were classified into the unassigned group.

**Biostatistical Analysis of H3K27Ac ChIP-seq Data Set**

Processing was performed using ENCODE’s protocol for unreplicated H3K27Ac ChIP-seq experiments. Differential binding was determined using DiffBind (RRID: SCR_012918) from Bioconductor (RRID: SCR_006442; ref. 79). The Annotatr Package from Bioconductor (RRID: SCR_006442) was then used to annotate regions 1 to 5 kb from gene
BETi Response Is Tethered to Leukemic Differentiation State

Drug Viability Testing and ZIP Synergy Scores

Drug viability testing was performed as previously described (3, 69) using MTS-based assays. Absorbance values were normalized to a kill control (10 μmol/L FSV) and media/cells only. AML cells were plated for 72 hours in replicates of six at 1,250 cells/well in a 96-well plate and titrated using a log scale (0–10 μmol/L) against JQ1 and CPI-0610. Viability of the cells was measured using Guava easyCyto prior to plating to ensure >90% viability. P values for individual sgRNAs versus nontargeting were calculated using a two-stage linear step-up procedure of Benjamini–Krieger–Yekutieli. Combinations of CPI-0610 with CH-223191 or FICZ were tested for efficacy using the MTS assay and a matrix dose layout wherein each drug/ligand in the combination was titrated over eight concentrations. Dose-specific normalized cell viability percentages were averaged across replicates, and ZIP synergy scores were calculated as previously described (81, 82) using the “synergyfinder” R package.

Beat AML Patient Sample Surface Marker Analyses

A simple linear regression model was fit separately for each inhibitor and gene with the inhibitor area under the curve (AUC) values as the outcome. The T-statistic and P value test whether the slope is nonzero and corrected for multiple comparisons. Corresponding correlation coefficients were computed using the relationship to the linear model: slope*(sd(expr)/sd(auc)), where sd indicates standard deviation. Log, reads per kilobase per million mapped (RPKM) values are available in Tyner and colleagues (3).

HL-60 Differentiation Assay and Drug Sensitivity

In triplicate, 250,000 HL-60 cells (>95% viability) were plated in a 6-well dish in 3 mL R20 and treated with either vehicle (100% EtOH), 300 nmol/L JQ1, 1 μmol/L ATRA (Sigma, #R2625) suspended in 100% EtOH, or 1 μmol/L ATRA and 300 nmol/L BETi. After 72 hours, cells were resuspended in fresh R20. A portion of the vehicle- and ATRA-alone–treated cells were taken and assessed for BETi sensitivity by MTS as previously described. The rest of the vehicle, BETi alone, ATRA alone, or BETi + ATRA cells were stained for viability and differentiation marker CD38 as described below.

HL-60 Morphologic Assessment

HL-60 cells were subjected to vehicle, 300 nmol/L JQ1, 1 μmol/L ATRA, or 300 nmol/L JQ1 + 1 μmol/L ATRA for 72 hours as described previously for flow-cytometric assessment. Cells were then spun onto glass slides via cytospin (800 rpm; 3 min), fixed in methanol, and stained with 1:20 Giemsia (Sigma-Aldrich) for 20 minutes prior to brightfield imaging (Leica).

Doxycycline-Inducible SPI1-Expressing HL-60s

Doxycycline 2000 was used to transfect HEK 293T cells with pINDUCER21-SPI1 (Addgene #97039) with packaging plasmids psPax2 (Addgene, #12260, RRID: Addgene_12260) and VSVG (Invitrogen) to generate virus. Viral supernatants were collected, filtered by 0.45-μm filters and used for transduction of AML cells using an 80%2 dry transfer system, blocked with BSA, and incubated with 1:1,000 in TBST primary antibody overnight at 4°C. The membrane was then washed with TBST before incubation in appropriate HRP-conjugated secondary for 3 hours and activated for imaging. Antibodies used were BCL2 (Cell Signaling Technologies, #4233), JUNB (Cell Signaling Technologies, #9803S), Tubulin (Cell Signaling Technologies, #2146S), and MCL1 (Cell Signaling Technologies, #3746S).

Statistical Analyses

Specific statistical analyses are described in figure legends. In all figures, “ns” denotes not significant (P > 0.05), and *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001, respectively.

Authors’ Disclosures

R. Majeti is on the board of directors for BeyondSpring Inc.; on the scientific advisory boards for Kodakz Therapeutic Solutions Inc. and Coherus Biosciences; a consultant for Acuta Capital Partners; and an inventor on a number of patents related to CD47 cancer immuno-therapy licensed to Gilead Sciences, Inc. J.W. Tyner reports other support from Constellation during the conduct of the study, as well as other support from Agios, Apotype, Array, AstraZeneca, Genentech, Gilead, Incyte, Janssen, Petra, Seattle Genetics, Syros, Takeda, and Tolero outside the submitted work. No disclosures were reported by the other authors.

Authors’ Contributions

K.A. Romine: Conceptualization, formal analysis, validation, visualization, methodology, writing—original draft, writing—review and editing. T. Nechiporuk: Conceptualization, methodology. D. Bottomly: Conceptualization, formal analysis, visualization, methodology. S. Jeng: Formal analysis, visualization. S.K. McWeeny: Conceptualization, resources, formal analysis, visualization. A. Kaempf: Formal analysis. M.R. Corces: Resources, data curation. R. Majeti: Resources, data curation. J.W. Tyner: Conceptualization, resources, formal analysis, supervision, funding acquisition, investigation, visualization, methodology, writing—original draft, project administration, writing—review and editing.

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