Highly Active Combination of BRD4 Antagonist and Histone Deacetylase Inhibitor against Human Acute Myelogenous Leukemia Cells

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Abstract

The bromodomain and extra-terminal (BET) protein family members, including BRD4, bind to acetylated lysines on histones and regulate the expression of important oncogenes, for example, c-MYC and BCL2. Here, we demonstrate the sensitizing effects of the histone hyperacetylation-inducing pan–histone deacetylase (HDAC) inhibitor panobinostat on human acute myelogenous leukemia (AML) blast progenitor cells (BPC) to the BET protein antagonist JQ1. Treatment with JQ1, but not its inactive enantiomer (R-JQ1), was highly lethal against AML BPCs expressing mutant NPM1c with or without coexpression of FLT3-ITD or AML expressing mixed lineage leukemia fusion oncoprotein. JQ1 treatment reduced binding of BRD4 and RNA polymerase II to the DNA of c-MYC and BCL2 and reduced their levels in the AML cells. Cotreatment with JQ1 and the HDAC inhibitor panobinostat synergistically induced apoptosis of the AML BPCs, but not of normal CD34⁺ hematopoietic progenitor cells. This was associated with greater attenuation of c-MYC and BCL2, while increasing p21, BIM, and cleaved PARP levels in the AML BPCs. Cotreatment with JQ1 and panobinostat significantly improved the survival of the NOD/SCID mice engrafted with OCI-AML3 or MOLM13 cells (P < 0.01). These findings highlight cotreatment with a BRD4 antagonist and an HDAC inhibitor as a potentially efficacious therapy of AML.

Introduction

Acetylation–deacetylation is among the several post-translational modifications of the histones involved in regulating gene expression (1). The resulting histone states or "marks" are recognized by the "reader" proteins, which assemble a complex of coregulatory proteins at the enhancers or promoters that initiate and regulate gene transcription (2, 3). Among these "reader" proteins is the family of bromodomain and extra-terminal (BET) proteins, including BRD2 (bromodomain 2), BRD3, and BRD4 (4). Structurally, BET proteins contain the N-terminal double, tandem, 110 amino acid-long bromodomains, which bind to the acetylated lysines on the nucleosomal histones (5). BET proteins also contain an extra terminal protein-interacting domain in the C-terminus, through which they interact and recruit coactivators and corepressor complexes containing chromatin modifying enzymes, chromatin remodeling factors, and the mediator elements to the chromatin for regulating gene transcription (4, 5). The C-terminal domain (CTD) of BRD4 also includes a proline- and glutamine-rich unstructured region, similar to the highly phosphorylatable CTD of the RNA polymerase II (5, 6). This region interacts with pTEFb (positive transcription elongation factor b), the heterodimer composed of CDK9 and cyclin T, which phosphorylates serine 2 on the CTD of RNA pol II for mRNA transcript elongation (5, 6). Thus, BRD4 and the other BET proteins have been shown to couple histone acetylation to transcript elongation, especially at the promoters and enhancers of important cell growth and survival genes such as c-MYC, cyclin D1, BCL2, and FOSL1 (7–9). The essential role of BRD4 in mammalian cells is further supported by the fact that null mutation of BRD4 is early embryonic lethal (4, 5). Recently, pertinent for therapy, an RNA interference screen identified BRD4 as an effective and promising target in human acute myelogenous leukemia (AML; ref. 10). In addition, prompted by this, several structure/activity-based BET protein small-molecule inhibitors have been developed, including JQ1 and I-BET151 (IB), which displace the BET proteins, along with the associated transcript initiation and elongation factors, from the chromatin (11–13). This results in transcriptional...
repression of BCL2, c-MYC, and CDK6 as well as induces growth arrest and apoptosis of AML cells (14, 15). BET protein inhibitors have been shown to be especially active against AML carrying the mixed lineage leukemia (MLL) containing fusion oncoproteins (16).

In previous reports, we demonstrated that treatment with hydroxamic acid analog pan–histone deacetylase (HDAC) inhibitors, such as panobinostat (LBH589), induces hyperacetylation of histones as well as mediates growth arrest and apoptosis of cultured and primary AML cells (17, 18). Concomitantly, panobinostat treatment attenuated the levels of progrowth and prosurvival proteins, for example, BCL2 and c-MYC, while simultaneously inducing the levels of proapoptotic protein BIM (17–19). On the basis of these observations, we hypothesized that the lysine hyperacetylation induced by treatment with panobinostat would increase the dependency of AML cells on BET protein–regulated transcription of the oncogenes c-MYC and BCL2, such that this would make the AML cells especially susceptible to the activity of the BET (BRD4/2) protein antagonist JQ1. Therefore, in the present studies, we determined whether cotreatment with panobinostat would augment the in vitro and in vivo effects of JQ1 against cultured and primary AML cells. Our findings demonstrate that combined treatment with panobinostat and JQ1 is synergistically active against human AML blast progenitor cells (BPC), including those with mutant NPM1c+ or MLL fusion oncoprotein with coexpression of FLT3-ITD. In addition, we discovered that cotreatment with panobinostat and JQ is more effective than each agent alone in significantly improving the survival of nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice engrafted with AML cells expressing mutant nucleophosmin 1 (NPM1c+) or MLL fusion oncoprotein with FLT3-ITD.

Materials and Methods

Reagents

(S)-JQ1 (active enantiomer, hereafter referred to as JQ1) and its inactive enantiomer (R)-JQ1 were developed as previously described (11). IBET-151 was obtained from Xcess Biologicals. Panobinostat was kindly provided by Novartis Pharmaceuticals Inc. Chemical structures for the molecules are provided as Supplementary Fig. S1. All compounds were prepared as 10 mmol/L stocks in 100% dimethyl sulfoxide (DMSO) and frozen at −20°C in 10 µL aliquots to allow for single use, thus avoiding multiple freeze-thaw cycles that could result in compound decomposition and loss of activity. Anti-BRD4 antibody for chromatin immunoprecipitation (ChIP) and Western blot analysis was obtained from Bethyl Laboratories. Anti-p-SER2 POL II antibody and RNA POL II antibody for ChIP were obtained from Millipore. Anti–cleaved PARP, anti–c-MYC, and anti-BIM antibodies were obtained from Cell Signaling Technology. Anti-BCL2 and anti-CDK6 antibodies were obtained from Santa Cruz Biotechnologies. Anti-p21WAF antibody was obtained from Neomarkers. Anti-p27KIP antibody and anti-FLT3-PE–conjugated antibody were obtained from BD Biosciences. Anti-NPM1, anti–β-actin antibody, and lentiviral short hairpin RNAs (shRNA) targeting BRD4 or nontargeting shRNA (sh-NT) were obtained from Sigma Aldrich.

Cell culture

OCI-AML2, OCI-AML3, and MOLM13 cells were obtained from the DSMZ. HL-60, U937, and MV4-11 cells were obtained from American Type Culture Collection (ATCC). All experiments with cell lines were performed within 6 months after thawing or obtaining from ATCC or DSMZ. Cell line authentication was performed by ATCC or DSMZ. The ATCC and DSMZ use short tandem repeat profiling for characterization and authentication of cell lines.

Primary normal progenitor and AML BPCs

Primary peripheral blood and/or bone marrow aspirate AML samples were obtained and prepared for the studies below, as previously described (17, 19, 20). Banked, delinked, and deidentified, normal or AML CD34+ or AML CD34+CD38-LIN− bone marrow progenitor/stem cells were purified, as previously described (18). The clinical presentation of the patient and mutation status of the primary AML samples used in these studies is provided in Supplementary Table S1.

Cell-cycle analysis

Following the designated treatments with JQ1, cell cycle status was analyzed on a BD Accuri C6 flow cytometer (BD Biosciences), as previously described (19).

Assessment of apoptosis by Annexin-V staining

Untreated or drug-treated cells were stained with Annexin-V (Pharmingen) and TO-PRO-3 iodide and the percentages of apoptotic cells were determined by flow cytometry, as previously described (18, 19). The combination index (CI) for each drug combination and the evaluation of the synergistic interactions were calculated by median dose effect analyses (assuming mutual exclusivity) using the commercially available software CalcuSyn (Biosoft; ref. 21). CI values of less than 1.0 represent a synergistic interaction of the two drugs in the combination.

Assessment of percentage nonviable cells

Following designated treatments, cells were washed with 1× PBS, stained with propidium iodide, and analyzed by flow cytometry, as previously described (19, 20).

Colony growth assay

Cultured AML cells were treated with JQ1 and/or panobinostat for 48 hours. At the end of treatment, cells were washed free of the drugs and 500 cells per condition were plated in methylcellulose and incubated at 37°C.
Colony formation was measured 7 to 10 days after plating (19, 20).

**ChIP and real-time PCR**

OCI-AML3, OCI-AML2, MOLM13, and primary AML cells were treated with JQ1 for 16 hours. Following drug exposure, crosslinking, cell lysis, sonication, and ChIP for BRD4 or POL II were performed according to the manufacturer’s protocol (Millipore). For quantitative assessment of binding of BRD4 or RNA POL II to the c-MYC, BCL2, and CDK6 promoter in the chromatin immunoprecipitates, a SYBR Green PCR Mastermix from Applied Biosystems was used. Relative enrichment of the promoter DNA in the chromatin immunoprecipitates was normalized against the amount of c-MYC, BCL2, and CDK6 promoter DNA in the input samples (19).

**shRNA to BRD4**

Lentiviral shRNAs targeting BRD4 or nontargeting shRNA (sh-NT) were transduced into OCI-AML3 cells, as previously described (20). Forty-eight hours after transduction, the cells were washed with complete media and plated with or without panobinostat for 48 hours for assessing apoptosis.

**RNA isolation and quantitative PCR**

After the designated treatments with JQ1, total RNA was isolated from cultured and primary AML cells with a High Pure RNA Isolation Kit (Roche Diagnostics) and reverse transcribed. Quantitative real-time PCR analysis for the expression of c-MYC, BCL2, CDK6, and p21 was performed on cDNA using TaqMan probes from Applied Biosystems (19, 20). Relative mRNA expression was normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

**Gene expression microarray analysis**

Total RNA from OCI-AML3 cells treated with JQ1 for 8 hours was extracted using the RNeasy Plus Mini Kit (Qiagen). One microgram of RNA was used for the generation of labeled cRNA and hybridization of the labeled cRNA fragments, washing, staining, and scanning of the arrays were performed according to the manufacturer’s instructions. Labeled cRNAs were profiled using the Affymetrix Human Genome U133-Plus 2.0 microarray (14). Array data were imported into Affymetrix Expression Console (Affymetrix), and normalized with robust multiarray average (RMA) method. Relative fold change analysis was calculated using Partek Genomic Suite. Genes were selected for P < 0.01 and 2 fold changes of treated versus untreated. A one-way heatmap for the selected genes was generated from JMP 8 (SAS Inc.). The microarray data are from the analyses of data from an experiment performed in duplicate and are representative of two separate experiments. All microarray data used in this article are deposited in the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under the accession number GSE51950.

**Ingenuity pathway analysis**

Significantly perturbed gene lists acquired from the microarray analysis of JQ1-treated OCI-AML3 cells were imported into the Ingenuity Pathway Analysis Tool (IPA Tool; Ingenuity Systems Inc.; http://www.ingenuity.com) for assignment of biologic function and identification of differentially altered genetic networks. Up- and downregulated identifiers were defined as value parameters for the analysis. Within the IPA software, a core analysis was performed to identify the signaling and metabolic pathways as well as the molecular networks and biologic processes that were significantly perturbed in the differentially expressed gene (DEG) dataset. The DEGs were mapped to genetic networks in the database and ranked by score. The significance of the molecular and cellular functions overrepresented in the gene set, as well as the signaling pathways and biologic networks to which they belong was tested by Fisher exact test P value. The created biologic networks were ranked according to the number of significantly differentially expressed genes that they contained.

**Cell lysis, protein quantitation, and immunoblot analyses**

Untreated or drug-treated cells were centrifuged and the cell pellets were lysed and the protein quantitation and immunoblot analyses were performed, as previously described (19, 20). Immunoblot analyses were performed at least twice. Representative immunoblots were subjected to densitometry analysis. Densitometry was performed using ImageQuant 5.2 (GE Healthcare).

**In vivo model of AML**

All in vivo studies were approved by and conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee at Houston Methodist Research Institute (Houston, TX). Female NOD/SCID mice were exposed to 2.5 Gy of radiation. The following day, 5 million OCI-AML3 or MOLM13 cells were injected into the lateral tail vein of the mice and the mice were monitored for 4 to 7 days. Following treatments were administered in cohorts of 8 mice for each treatment: vehicle control, 50 mg/kg JQ1, 5 mg/kg panobinostat, and JQ1 plus panobinostat. Treatments were initiated on day 7 for OCI-AML3 and on day 4 for MOLM13 cells. JQ1 (formulated in 10% 2-hydroxypropyl-β-cyclodextrin (CAS 128446-35-5)) was administrated daily for 5 days per week (Monday–Friday) intraperitoneally for 3 weeks, and then discontinued. Panobinostat (formulated in 5% DMSO/95% normal saline) was administrated by intraperitoneal injection 3 days per week (Monday, Wednesday, and Friday) for 3 weeks and discontinued. The survival of mice from both in vivo models is represented by a Kaplan–Meier survival plot. The doses of JQ1 and panobinostat used in these studies were determined to be safe and effective through previously reported studies (11, 19). A separate in vivo experiment was conducted for analysis of biomarkers using OCI-AML3 cells. Following engraftment of the...
AML cells, mice were treated with JQ1 and/or panobinostat, as described above, for 1 week. Six hours after the last dose of panobinostat, the mice were humanely euthanized and bone marrow was collected from both femurs for immunoblot analyses.

Statistical analysis

Significant differences between values obtained in a population of AML cells treated with different experimental conditions were determined using a two-tailed, paired t-test or a one-way ANOVA analysis within an analysis package of Microsoft Excel 2010 software or using GraphPad Prism (GraphPad Software). P values of less than 0.05 were assigned significance. Statistical differences in the survival of the mice treated with JQ1, panobinostat, or JQ + panobinostat were determined by log-rank (Mantel–Cox) test. P values of less than 0.05 were assigned significance.

Results

JQ1 exerts growth inhibitory and lethal effects in cultured and primary AML BPCs

We first determined the lethal activity of the BET protein inhibitor JQ1 against several cultured human AML cell lines, including those that express mutant NPM1c+ (OCI-AML3) or expressed MLL fusion oncprotein with or without FLT3-ITD (MV4-11 and MOLM13; refs. 17, 20). Treatment with JQ1 dose dependently increased the percentage of cells in the G1 phase while reducing the percentage of S-phase cells, as well as concomitantly induced apoptosis in the AML cell types, as has been previously reported (Fig. 1A and B; refs. 10, 15, 16). Among these, OCI-AML3 cells exhibited greater sensitivity to JQ1 than MOLM13 and MV4-11 cells (Fig. 1A). The IC50 values for inducing apoptosis after 48-hour exposure to JQ1 were 165 nmol/L for OCI-AML3 versus 280 nmol/L and 1,480 nmol/L for MV4-11 and MOLM13 cells, respectively. JQ1 treatment also inhibited the clonogenic survival of OCI-AML3 cells more than of MOLM13, OCI-AML2, or HL-60 cells (Fig. 1C). Because cultured AML cells coexpressing endogenous mutant NPM1c+ and FLT3-ITD, a commonly encountered normal karyotype AML cell type in the clinic (22), have not been isolated and hence are unavailable, we created OCI-AML3 cells with ectopic expression of FLT3-ITD (OCI-AML3/FI) for determining the activity of JQ1 against these cells. As compared with the control OCI-AML3, OCI-AML3/FI cells exhibit higher expression of FLT3 protein, as determined both by flow cytometry and immunoblot analysis (Fig. 1D and E). However, JQ1 was equally effective in inducing apoptosis of OCI-AML3 and OCI-AML3/FI cells (Fig. 1E). Treatment with the inactive enantiomer of JQ1, that is, R-JQ1, neither perturbed the cell cycle nor induced apoptosis in OCI-AML3 or MOLM13 cells (Supplementary Fig. S2A–S2C; ref. 11). We also determined the lethal effects of JQ1 against 10 separate samples of patient-derived primary AML cells with normal karyotype expressing mutant NPM1 and/or FLT3-ITD. Similar to the effects observed in the cultured AML cells lines, treatment with JQ1 also dose dependently exerted lethal antileukemia effects against primary CD34+ AML cells (Fig. 1F). Although, the loss of viability in the primary AML cells was lower than in the cultured AML cell types, we observed no significant difference in JQ1-induced loss of viability in AML cells with mutant NPM1c+ alone versus those that coexpressed FLT3-ITD (P > 0.05; Fig. 1F).

JQ1 inhibits the binding of BRD4 and RNA POL II and attenuates the mRNA expression of c-MYC and BCL2 in AML cells

Disruption of binding of the bromodomain of BET protein to acetylated histones has been shown to deplete the BET protein occupancy on the chromatin associated with the promoters of BET protein target genes (14, 15). Consistent with this, ChIP analyses showed that treatment with JQ1 reduced the BRD4 occupancy at the promoters of c-MYC, BCL2, and CDK6 in OCI-AML3 and MOLM13 cells (Fig. 2A and Supplementary Fig. S3A). We also observed that JQ1 reduced the occupancy of BRD4 at the same promoters in NPM1c+–expressing primary AML cells, as presented in the data in Fig. 2B, representative of two primary AML samples. BRD4 is known to regulate the transcriptional elongation of these genes through recruitment of P-TEFb, which phosphorylates and activates RNA POL II (5, 6). Accordingly, we also found that treatment with JQ1 reduced the binding of RNA POL II to the promoters of c-MYC, BCL2, and CDK6 genes in the OCI-AML3 as well as in the primary AML cells (Fig. 2C and D). Data in Fig. 2D are also representative of two primary AML samples. Similar effects were also observed in JQ1–treated cultured AML OCI-AML2 cells that express wild-type NPM1 (Supplementary Fig. S3B and S3C). Quantitative PCR (qPCR) analyses of the gene expressions showed that JQ1 treatment attenuated the mRNA expression of c-MYC, BCL2, and CDK6 in the OCI-AML3, MOLM13, and MV4-11, as well as in primary AML cells expressing NPM1c+ (Fig. 3A and B; Supplementary Figs. S4A, S4B, and S5A). In contrast, in these cell types, JQ1 treatment concomitantly upregulated the mRNA and protein expression of p21 (Supplementary Figs. S4C, S5B, and S5C). In tumor cells, high levels of c-MYC cause transcriptional amplification of the gene expression program involving a larger number of genes (23). Therefore, we also determined the effects of JQ1 treatment on gene expression microarray profile in OCI-AML3 cells. Figure 3C shows a heatmap of the greatest gene expression changes following treatment with JQ1 for 8 hours. As shown, JQ1 treatment downregulated the mRNA expression of more genes, as compared with the number of genes whose mRNA expression was upregulated (Fig. 3C). The fold changes in the most altered mRNA gene expressions are shown in Supplementary Table S2. Data sets of genes with altered expression profile derived from microarray analyses were imported into the Ingenuity Pathway Analysis (IPA) Tool (Ingenuity H Systems; http://www.ingenuity.com). Within the gene list, IPA identified the top
The five most perturbed networks in OCI-AML3 cells following treatment with JQ1 and assigned a score for these associated network functions (Supplementary Table S3). The score (i.e., a score of 36) assigned by IPA indicates the probability (1 in 10^36) that the focus genes in the dataset are grouped together in a perturbed network due to random chance alone. Next, total RNA from the untreated and JQ1-treated cells used for the microarray analysis was also reverse transcribed and the resulting cDNA was used for qPCR analysis using c-MYC and BCL2-specific TaqMan real-time PCR probes. This confirmed that JQ1 treatment markedly decreased the mRNA expression of the c-MYC and BCL2 genes (Fig. 3D).

**JQ1 treatment depletes p-Serine 2 RNA POL II, c-MYC, and BCL2 but induces p21 and BIM protein levels**

We next compared the effects of JQ1 treatment on the protein expression of BRD4, c-MYC, BCL2, CDK6, and pSer2 RNA POL II in OCI-AML3 versus MOLM13 cells.
synergistically active against cultured AML

Cotreatment with JQ1 and panobinostat is tant

NPM1c against cultured and primary AML cells expressing mu-

that, in addition to its known activity in AML cells expres-

sion of c-MYC, BCL2, and CDK6 in human AML cells. A and B, OCI-AML3 and primary AML cells were treated with the indicated concentration of JQ1 for 16 hours. Following this, ChIP was conducted with BRD4-specific antibody. The chromatin immunoprecipitated DNA was subjected to quantitative real-time PCR with primers for the promoter of c-MYC, BCL2, and CDK6. The fold enrichment was calculated using the Cv value of the ChIP DNA compared with the Ct value of the input DNA. C and D, OCI-AML3 and primary AML cells were treated with the indicated concentration of JQ1 for 16 hours. Following this, ChIP was conducted with RNA Pol II antibody. The chromatin immunoprecipitated DNA was subjected to quantitative real-time PCR with primers for the promoter of c-MYC, BCL2, and CDK6. The fold enrichment was calculated using the Ct value of the ChIP DNA compared with the Ct value of the input DNA.

As shown in the immunoblot analyses in Fig. 4A, although it had no effect on BRD4 and NPM1 (not shown), JQ1 treatment dose dependently depleted the protein levels of c-MYC, BCL2, and pSer2 RNA POL II, as well as induced the levels of p21, p27, BIM, and cleaved PARP to a similar extent in both OCI-AML3 and MOLM13 cells. Exposure to (R)-JQ1 did not alter the levels of these proteins in either the OCI-AML3 or MOLM13 cells (Fig. 4B and data not shown). In a representative primary AML BPC sample that expressed mutant NPM1c+ and FLT3-ITD, JQ1 treatment also caused a marked decline in c-MYC, BCL2, and p-Ser2 RNA POL II levels, with a concomitant increase in the levels of p21, p27, and BIM, as well as no exerted no effect on NPM1 levels (Fig. 4C). Figure 4D shows the mean ± SEM for the decline in c-MYC, and induction of BIM and p21 protein levels in three primary AML samples (Fig. 4D). Collectively, these data show that, in addition to its known activity in AML cells expressing MLL fusion oncoprotein, JQ1 treatment is effective against cultured and primary AML cells expressing mutant NPM1c+ irrespective of coexpressed FLT3-ITD.

Cotreatment with JQ1 and panobinostat is synergistically active against cultured AML cells expressing mutant NPM1c- or MLL fusion oncoprotein

We next determined whether cotreatment with the potent pan-HDAC inhibitor such as panobinostat, known to induce in vitro and in vivo lysine acetylation of histones, would increase the dependency on BRD4-regulated pro-growth and prosurvival gene expressions and thereby sensitize AML BPCs to JQ1-induced apoptosis (19, 24). Figure 5A and Supplementary Fig. S6A to S6C demonstrate that cotreatment with panobinostat and JQ1 synergistically induced apoptosis of OCI-AML3, MOLM13, MV4-11, and HL-60 cells, with combination indices of less than 1.0 by the isobologram analyses. The specific activity of JQ1 in this synergistic interaction is underscored by the fact that cotreatment with (R)-JQ1 did not enhance panobinostat-induced apoptosis of OCI-AML3 and MOLM13 cells (Fig. 5B). Cotreatment with another BET protein antagonist, I-BET151, and panobinostat also synergistically induced apoptosis of AML cells (Supplementary Fig. S6D). As compared with each agent alone, combined treatment with JQ1 and panobinostat also induced greater loss of the clonogenic survival of OCI-AML3 and MOLM13, as well as of the other AML cell types (Fig. 5C and Supplementary Fig. S6E). This was associated with marked depletion in the protein levels of p-Ser2 RNA POL II and c-MYC, with concomitant upregulation of the levels of BIM protein isomers (Fig. 5D and Supplementary Fig. S6F). Panobinostat and JQ1 combination did not alter the total NPM1 levels in OCI-AML3 cells (Fig. 5D). Taken together, these findings show that cotreatment with panobinostat sensitizes cultured AML cells to the anti-AML activity of a BRD4 antagonist. We next determined whether specific depletion of BRD4 by siRNA would phenocopy the effects of JQ1 in increasing the anti-AML activity...
of panobinostat. Figure 5E demonstrates that, compared with treatment with the nontargeted control shRNA, treatment with BRD4 shRNA reduced the mRNA levels of BRD4, c-MYC, and BCL2, while simultaneously increasing the mRNA levels of p21. In the cells treated with BRD4 shRNA, but not the nontargeted shRNA, panobinostat treatment induced significantly more apoptosis of OCI-AML3 cells ($P < 0.05$; Fig. 5F). The IC$50$ values for panobinostat were significantly lower in OCI-AML3 cells treated with BRD4 shRNA versus those treated with nontargeted shRNA ($P = 0.0165$; Fig. 5G).

Cotreatment with JQ1 and panobinostat exerts synergistic lethal activity against primary AML BPCs but not normal hematopoietic progenitor cells

We next compared the lethal activity of the combination of panobinostat and JQ1 against 9 samples of primary AML versus CD34$^+$ normal hematopoietic progenitor cells. Figure 6A (and the inset) demonstrates that, compared with the treatment with each agent alone, cotreatment with panobinostat and JQ1 induced significantly more apoptosis with increased PARP cleavage in primary AML BPCs that expressed mutant NPM1c$^+$ with or without the coexpression of FLT3-ITD. Although treatment with JQ1 alone (500 nmol/L) depleted the levels of c-MYC and BCL2 and induced p21 levels, cotreatment with panobinostat and JQ1 caused more downregulation of c-MYC and BCL2 as well as induced more p21 and BIM levels in the AML BPCs (Fig. 6B). Importantly, the combination of panobinostat and JQ1 exerted synergistic lethal activity in the subpopulation of CD34$^+$CD38-Lin$^{--}$ BPCs (Fig. 6C). In this combination, the levels of panobinostat used are clinically achievable and safe and have been demonstrated to induce in vivo histone acetylation in cells of patients with AML (24). Notably, cotreatment with panobinostat and JQ1 did not exert significantly greater
lethal activity against normal CD34<sup>+</sup> hematopoietic progenitor cells (Fig. 6D). These findings highlight the anti-AML selectivity of the cotreatment with panobinostat and JQ1 against AML BPCs.

Combined treatment with panobinostat and JQ1 exerts superior in vivo activity against the established AML xenografts in NOD/SCID mice

We next determined the in vivo anti-AML activity of panobinostat and/or JQ1 against the OCI-AML3 AML xenografts engrafted in the bone marrow of the NOD/SCID mice. Following the tail vein infusion and engraftment of OCI-AML3 cells in the bone marrow of the NOD/SCID mice, the anti-AML effects and the survival improvement due to treatment with daily intraperitoneal JQ1 and/or intraperitoneal panobinostat (Monday, Wednesday, and Friday) for 3 weeks were compared with the effects of the treatment with the vehicle alone. The Kaplan–Meier plot depicting the survival of mice demonstrated that, as compared with treatment with the vehicle alone, treatment with either JQ1 or panobinostat significantly improved the survival of the mice infused with OCI-AML3 cells (P < 0.05; Fig. 7A). Notably, combined treatment with panobinostat and JQ1 further significantly improved survival of the mice, as compared with treatment with JQ1 or panobinostat alone.
alone (P < 0.001). In cohorts of three mice treated with the vehicle control versus treatment with JQ1 and/or panobinostat for 5 days, bone marrow was harvested, and the cell lysates were analyzed for protein expression. Figure 7B demonstrates that as compared with treatment with each agent alone, cotreatment with panobinostat and JQ1 was associated with the most reduction in the levels of c-MYC, BCL2, and CDK6 proteins. We also determined the effects of JQ1 and/or panobinostat on the expression levels of BIM. As shown in Fig. 7B, although treatment with each drug alone increased the levels of BIM, cotreatment with JQ1 and panobinostat did not exhibit further increase in the levels of BIM in the mice. We also determined the in vivo anti-AML activity of panobinostat and/or JQ1 against the more aggressive MOLM13 xenograft model, in
which all mice treated with vehicle control succumbed to AML in less than 25 days. As shown in Fig. 7C, the combination regimen of JQ1 and panobinostat for 3 weeks was superior to the treatment with JQ1 or panobinostat alone in improving the median survival and overall survival of the mice \((P < 0.0001)\), which translated into a plateau in the survival curve. This suggests a potentially curative impact of the combination on the survival of the mice (Fig. 7C).

Discussion

Findings presented here demonstrate for the first time that the BET protein antagonist (S)-JQ1, but not its inactive enantiomer (R)-JQ1, exerts a high level of \textit{in vitro} and \textit{in vivo} activity against AML BPCs expressing the mutant NPM1c+, with or without the coexpression of FLT3-ITD. This was observed not only in the cultured OCI-AML3 cells with ectopic expression of FLT3-ITD, but also in the primary AML BPCs. The BET protein BRD4 binds and recruits pTEFb to the promoters of transcriptionally active genes to phosphorylate CTD of RNA POL II, which is necessary to cause the pause release of RNA POL II for mRNA transcript elongation \((7, 23)\). This is especially true for the MYC-regulated transcriptome, in which c-MYC binds and recruits pTEFb to the core promoters of the actively transcribed genes, causing overall transcriptional amplification which may attenuate the rate-limiting constraints on tumor growth and proliferation \((7, 9, 23)\). Inhibition of BRD4 by JQ1 has been shown to downregulate c-MYC–dependent target genes in AML and other hematologic malignancies \((14, 15)\). Recently, JQ1 has also been demonstrated to deplete the binding of BRD4, mediator, and...
inducing the p21 levels, along with inhibition of growth 
BRD4 reduced c-MYC and BCL2 mRNA levels, while 
the c-MYC–targeted genes. We also found that shRNA to 
JQ1 perturbed the levels of not only c-MYC but also of 
targeted genes, such as c-MYC and BCL2, to the clusters of 
gene expression microarray analysis in the OCI-AML3 
cells, more genes were suppressed than induced after 
treatment with JQ1. Again, among the genes transcriptionally 
attenuated was c-MYC.

Gain-of-function mutations in FLT3 are associated with 
a poor prognosis following the standard induction therapy 
of AML (22, 26, 27). Although exhibiting a promising 
preliminary activity, the use of FLT3 tyrosine kinase 
inhibitors (TKI) alone or in conjunction with standard 
induction chemotherapy has exposed novel mechanisms 
by which AML BPCs acquire resistance to FLT3 kinase 
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identified so far have involved point substitutions in 
amino acid residue that disrupt the conformation and
binding of the TKI to FLT3. Our findings show that JQ1, due to its unique mechanism, which is disparate from the mechanism of activity of the TKIs, retains potent activity against cultured and primary AML BPCs coexpressing FLT3-ITD and NPM1c against cultured and primary AML BPCs coexpressing FLT3-ITD and NPM1c. Our findings show that JQ1, a FLT3 kinase inhibitor in the treatment of high-risk AML, as LSC gene expression program confers a poor prognosis and influences the clinical outcome in AML (30, 31). Cotreatment with panobinostat and JQ1 was also significantly superior to treatment with each agent alone in improving the survival of the mice engrafted with OCI-AML3 or MOLM13 cells, which express NPM1c+ or MLL-AP9 and FLT3-ITD, respectively. The improvement in survival due to panobinostat and JQ1 treatment of the mice engrafted with the aggressive MOLM13 AML cells was associated with a significantly higher plateau in the survival curve, which suggests the possibility of a prolonged disease-free survival and cure in the mice treated with the combination. The regimen of panobinostat and JQ1, as used here for 3 weeks, did not induce any discernible toxicity in the mice. The superior in vivo anti-AML selectivity was also associated with a marked and collective depletion of c-MYC, BCL2, and CDK6 in the engrafted bone marrow–derived OCI-AML3 cells, following only 5 days of treatment with the combination. Attenuation of these proteins could very well be the explanation for the superior antileukemia activity in the treated mice. However, other possible mechanisms, not probed here, may also be contributing toward the superior outcome. BRD4 has also been shown to bind to the acetylated lysine-310 of the RelA subunit of NF-kB and regulate its transcriptional activity (32, 33). By inducing RelA acetylation, panobinostat treatment may also increase the BRD4 dependency of the NF-kB activity in AML cells. Therefore, suppression of this activity by cotreatment with panobinostat and JQ1 may contribute toward the superior anti-AML activity of the combination. Collectively, the findings presented here provide a strong rationale for further in vivo testing of combined therapy with BET protein antagonists and HDAC inhibitor against AML.
Disclosure of Potential Conflicts of Interest

S. Sharma has ownership interest (including patents) in Convergene, Beta Cat Pharmaceuticals, and Saliarius Pharma. J.E. Bradner has ownership interest (including patents) and is a consultant/advisory board member in Tenopha Therapeutics. No potential conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics): K. Peth, M. Rodriguez, J.A. Valenta, M. Rodriguez, M. Zhian, S.P. Iyer, J.E. Bradner
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