Highly active combination of BRD4 antagonist and histone deacetylase inhibitor against human acute myeloid leukemia (AML) cells

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ABSTRACT:
The BET (bromodomain and extra terminal) protein family members including BRD4 bind to acetylated lysines on histones and regulate the expression of important oncogenes, e.g., c-MYC and BCL2. Here we demonstrate the sensitizing effects of the histone hyperacetylation inducing pan-histone deacetylase inhibitor (HDI) panobinostat (PS) on human AML blast progenitor cells (BPCs) 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 co-expression of FLT3-ITD, or AML expressing MLL 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. Co-treatment with JQ1 and the HDAC inhibitor panobinostat (PS) 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. Co-treatment with JQ1 and PS significantly improved the survival of the NOD/SCID mice engrafted with OCI-AML3 or MOLM13 cells (p < 0.01). These findings highlight co-treatment with a BRD4 antagonist and an HDI 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 co-regulatory proteins at the enhancers or promoters that initiate and regulate gene transcription (2,3). Among these ‘reader’ proteins is the family of BET (bromodomain and extra terminal) proteins, including BRD2 (bromodomain 2), BRD3 and BRD4 (4). Structurally, BET proteins contain the N-terminal double, tandem, 110 amino acids-long bromodomains, which bind to the acetylated lysines on the nucleosomal histones (5). BET proteins also contain an extra terminal (ET) protein-interacting domain in the C-terminus, through which they interact and recruit co-activators and co-repressor 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, BCL-2 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 RNAi screen identified BRD4 as an effective and promising target in human AML (10). Additionally, 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 BCL-2, 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 analogue pan-histone deacetylase inhibitors, such as panobinostat (LBH589, PS), induce hyperacetylation of histones, as well as mediate growth arrest and apoptosis of cultured and primary AML cells (17,18). Concomitantly, PS treatment attenuated the levels of pro-growth and pro-survival proteins e.g., BCL2 and c-MYC, while simultaneously inducing the levels of pro-apoptotic protein BIM (17-19). Based on these observations, we hypothesized that the lysine hyperacetylation induced by treatment with PS 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 co-treatment with PS would augment the in vitro and in vivo effects of JQ1 against cultured and primary AML cells. Our findings demonstrate that combined treatment with PS and JQ1 is synergistically active against human AML blast progenitor cells (BPCs), including those with mutant NPM1c+ or MLL fusion oncoprotein with co-expression of FLT3-ITD. Additionally, we discovered that co-treatment with PS and JQ is more effective than each agent alone in significantly improving the survival of NOD/SCID mice.
engrafted with AML cells expressing mutant nucleophosmin 1 (NPM1c+) or MLL fusion oncoprotein with FLT3-ITD.

METHODS AND MATERIALS

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 (PS) was kindly provided by Novartis Pharmaceuticals Inc. (East Hanover, NJ). Chemical structures for the molecules are provided as Supplemental Figure 1. All compounds were prepared as 10 mM stocks in 100% 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 ChIP and Western blot was obtained from Bethyl Labs (Montgomery, TX). Anti p-SER2 POL II antibody and RNA POL II antibody for chIP were obtained from Millipore (Billirica, MA). Anti-cleaved PARP, anti-c-MYC and anti-BIM antibodies were obtained from Cell Signaling. Anti-BCL2 and anti-CDK6 antibodies were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). Anti-p21WAF antibody was obtained from Neomarkers (Fremont, CA). Anti-p27KIP antibody and anti-FLT3-PE conjugated antibody were obtained from BD Biosciences (San Jose, CA). Anti-NPM1, anti-β-actin antibody and lentiviral short hairpin RNAs targeting BRD4 or non-targeting shRNA (sh-NT) were obtained from Sigma Aldrich (St. Louis, MO).

Cell Culture: OCI-AML2, OCI-AML3 and MOLM13 cells were obtained from the DSMZ (Braunschweig, Germany). HL-60, U937 and MV4-11 cells were obtained from ATCC (Manassas, VA). 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 utilize short tandem repeat (STR) 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, de-linked and de-identified, normal or AML CD34+ or AML CD34+CD38-LIN- bone marrow progenitor/stem cells were purified, as previously described.
The clinical presentation of the patient and mutation status of the primary AML samples utilized in these studies is provided in Supplemental Table 1.

**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, San Diego, CA) 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) utilizing the commercially available software CalcuSyn (Biosoft, Ferguson, MO) (21). CI values of less than 1.0 represent a synergistic interaction of the two drugs in the combination.

**Assessment of percentage non-viable cells:** Following designated treatments, cells were washed with 1X 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 PS 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 7-10 days after plating (19,20).

**Chromatin immunoprecipitation and Real Time Polymerase Chain Reaction:** OCI-AML3, OCI-AML2, MOLM13 and primary AML cells were treated with JQ1 for 16 hours. Following drug exposure, cross-linking, cell lysis, sonication and chromatin immunoprecipitation for BRD4 or POL II was performed according to the manufacturer’s protocol (Millipore). For quantitative assessment of binding of BRD4 or RNA POL II to the c-MYC, BCL-2 and CDK6 promoter in the chromatin immunoprecipitates, a SYBR Green PCR Mastermix from Applied Biosystems was used (Foster City, CA). Relative enrichment of the promoter DNA in the chromatin immunoprecipitates was normalized against the amount of c-MYC, BCL-2 and CDK6 promoter DNA in the input samples (19).
Short hairpin RNA to BRD4: Lentiviral short hairpin (sh) RNAs targeting BRD4 or non-targeting shRNA (sh-NT) were transduced into OCI-AML3 cells, as previously described (20). Forty-eight hours post transduction, the cells were washed with complete media and plated with or without PS for 48 hours for assessing apoptosis.

RNA isolation and quantitative polymerase chain reaction: Following the designated treatments with JQ1, total RNA was isolated from cultured and primary AML cells with a High Pure RNA isolation kit (Roche Diagnostics, Indianapolis, IN) and reverse transcribed. Quantitative real time PCR analysis for the expression of c-MYC, BCL-2, CDK6 and p21 was performed on cDNA using TaqMan probes from Applied Biosystems (Foster City, CA) (19,20). Relative mRNA expression was normalized to the expression of 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, Louisville, KY). 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 cRNA were profiled using the Affymetrix Human Genome-U133-Plus 2.0 microarray (14). Array data were imported into Affymetrix Expression Console (Affymetrix, CA), and normalized with RMA method. Relative fold change analysis was calculated utilizing Partek Genomic Suite. Genes were selected for p <.01 and 2 fold-changes of treated versus untreated. A one-way heatmap for the selected genes was generated from JMP 8 (SAS Inc., NC). The microarray data are from the analyses of data from an experiment performed in duplicate and is representative of two separate experiments. All microarray data used in this manuscript are deposited in the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under the Accession number (GSE51950).

Ingenuity pathway analysis (IPA): 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., Redwood City, CA, USA; http://www.ingenuity.com) for assignment of biologic function and identification of differentially altered genetic networks. Up and down regulated 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 biological 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 over-represented in the gene set, as well as the signaling pathways and biological networks to which they belong was tested by Fisher’s exact test p-value. The created biological 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, Piscataway, NJ).

**In vivo model of acute myeloid leukemia:** All in vivo studies were approved by, and conducted in accordance with the guidelines of the IACUC at Houston Methodist Research Institute. 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 4-7 days. Following treatments were administered in cohorts of 8 mice for each treatment: vehicle alone, 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 administered daily for 5 days per week (M-F) intraperitoneally (IP) for 3 weeks, and then discontinued. Panobinostat (formulated in 5% DMSO/95% normal saline) was administered by IP injection 3 days per week (M-W-F) 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 PS utilized 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 utilizing OCI-AML3 cells. Following engraftment of the AML cells, mice were treated with JQ1 and/or PS, as described above, for one week. Six hours after the last dose of panobinostat, the mice were humanely euathanized 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, Inc., CA). P values of less than 0.05 were assigned significance. Statistical differences in the survival of the mice treated with JQ1, PS or JQ + PS 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 blast progenitor cells. 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 oncoprotein with or without FLT3-ITD (MV4-11 and MOLM13) (17, 20). Treatment with JQ1 dose-dependently increased the % of cells in the G1 phase while reducing the % of S phase cells, as well as concomitantly induced apoptosis in the AML cell types, as has been previously reported (Figure 1A and 1B) (10, 15, 16). Among these, OCI-AML3 cells exhibited greater sensitivity to JQ1 than MOLM13 and MV4-11 cells (Figure 1A). The IC50 values for inducing apoptosis after 48-hour exposure to JQ1 were 165 nM for OCI-AML3 versus 280 nM and 1480 nM 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 (Figure 1C). Since cultured AML cells co-expressing endogenous mutant NPM1c+ and FLT3-ITD, a commonly encountered normal karyotype (NK) 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 cells) for determining the activity of JQ1 against these cells. As compared to the control OCI-AML3, OCI-AML3/FI cells exhibit higher expression of FLT3 protein, as determined both by flow cytometry and immunoblot analysis (Figure 1D and 1E). However, JQ1 was equally effective in inducing apoptosis of OCI-AML3 and OCI-AML3/FI cells (Figure 1E). Treatment with the inactive enantiomer of JQ1, i.e., R-JQ1, neither perturbed the cell cycle nor induced apoptosis in OCI-AML3 or MOLM13 cells (Supplemental Figure 2A, 2B and 2C) (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 anti-leukemia effects against primary CD34+ AML cells (Figure 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 co-expressed FLT3-ITD (p >0.05) (Figure 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 (Figure 2A and Supplemental Figure 3A). 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 Figure 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 (Figure 2C and 2D). Data in Figure 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 (Supplemental Figure 3B and 3C). 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+ (Figure 3A and 3B; Supplemental Figure 4A, 4B and 5A). In contrast, in these cell types, JQ1 treatment concomitantly up regulated the mRNA and protein expression of p21 (Supplemental Figure 4C, 5B and 5C). 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 heat map of the greatest gene expression changes following treatment with JQ1 for 8 hours. As shown, JQ1 treatment down regulated the mRNA expression of more genes, as compared to the number of genes whose mRNA expression was up regulated (Figure 3C). The fold changes in the most altered mRNA gene expressions are shown
in Supplemental Table 2. Data sets of genes with altered expression profile derived from microarray analyses were imported into the Ingenuity Pathway Analysis (IPA) Tool (Ingenuity H Systems, Redwood City, CA; http://www.ingenuity.com). Within the gene list, IPA identified the top five most perturbed networks in OCI-AML3 cells following treatment with JQ1 and assigned a score for these associated network functions (Supplemental Table 3). 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 quantitative PCR analysis utilizing 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 (Figure 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. As shown in the immunoblot analyses in Figure 4A, while it had no effect on BRD4 and NPM1 (not shown), JQ1 treatment dose-dependently depleted the protein levels of c-MYC, BCL2, CDK6 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 (Figure 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 exerted no effect on NPM1 levels (Figure 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 (Figure 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 co-expressed FLT3-ITD.

Co-treatment with JQ1 and panobinostat is synergistically active against cultured AML cells expressing mutant NPM1c+ or MLL fusion oncoprotein. We next determined whether co-treatment with the potent pan-HDAC inhibitor such as panobinostat (PS), known to induce in
vitro and in vivo lysine acetylation of histones, would increase the dependency on BRD4-regulated pro-growth and pro-survival gene expressions and thereby sensitize AML BPCs to JQ1-induced apoptosis (19,24). Figure 5A and Supplemental Figures 6A, 6B and 6C demonstrate that co-treatment with PS 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 co-treatment with (R)-JQ1 did not enhance PS-induced apoptosis of OCI-AML3 and MOLM13 cells (Figure 5B). Co-treatment with another BET protein antagonist, I-BET151, and PS also synergistically induced apoptosis of AML cells (Supplemental Figure 6D). As compared to each agent alone, combined treatment with JQ1 and PS also induced greater loss of the clonogenic survival of OCI-AML3 and MOLM13, as well as of the other AML cell types (Figure 5C and Supplemental Figure 6E). This was associated with marked depletion in the protein levels of p-Ser2 RNA POL II and c-MYC, with concomitant up regulation of the levels of BIM protein isoforms (Figure 5D and Supplemental Figure 6F). PS and JQ1 combination did not alter the total NPM1 levels in OCI-AML3 cells (Figure 5D). Taken together, these findings show that co-treatment with PS sensitizes cultured AML cells to the anti-AML activity of a BRD4 antagonist. We next determined whether specific depletion of BRD4 by shRNA would phenocopy the effects of JQ1 in increasing the anti-AML activity of PS. Figure 5E demonstrates that, as compared to treatment with the non-targeted control shRNA, treatment with BRD4 shRNA reduced the mRNA levels of BRD4, c-MYC and BCL-2, while simultaneously increasing the mRNA levels of p21. In the cells treated with BRD4 shRNA, but not the non-targeted shRNA, PS treatment induced significantly more apoptosis of OCI-AML3 cells (p < 0.05) (Figure 5F). The IC_{50} values for PS were significantly lower in OCI-AML3 cells treated with BRD4 shRNA versus those treated with non-targeted shRNA (p=0.0165) (Figure 5G).

Co-treatment 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 PS and JQ1 against 9 samples of primary AML versus CD34+ normal hematopoietic progenitor cells. Figure 6A (and the inset) demonstrates that, as compared to the treatment with each agent alone, co-treatment with PS and JQ1 induced significantly more apoptosis with increased PARP cleavage in primary AML BPCs that expressed mutant NPM1c+ with or without the co-expression of FLT3-ITD. While treatment with JQ1 alone (500 nM)
depleted the levels of c-MYC and BCL-2 and induced p21 levels, co-treatment with PS and JQ1 caused more down regulation of c-MYC and BCL-2, as well as, induced more p21 and BIM levels in the AML BPCs (Figure 6B). Importantly, the combination of PS and JQ1 exerted synergistic lethal activity in the sub-population of CD34+CD38-Lin- BPCs (Figure 6C). In this combination, the levels of PS employed are clinically achievable and safe, and have been demonstrated to induce in vivo histone acetylation in cells of patients with AML (24). Notably, co-treatment with PS and JQ1 did not exert significantly greater lethal activity against normal CD34+ hematopoietic progenitor cells (Figure 6D). These findings highlight the anti-AML selectivity of the co-treatment with PS and JQ1 against AML BPCs.

**Combined treatment with PS 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 PS 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 IP JQ1 and/or IP PS (M, W and F) for 3 weeks was compared to the effects of the treatment with the vehicle alone. The Kaplan Meier plot depicting the survival of mice demonstrated that, as compared to treatment with the vehicle alone, treatment with either JQ1 or PS significantly improved the survival of the mice infused with OCI-AML3 cells (p < 0.05) (Figure 7A). Notably, combined treatment with PS and JQ1 further significantly improved survival of the mice, as compared to treatment with JQ1 or PS alone (p < 0.001). In cohorts of three mice treated with the vehicle control versus treatment with JQ1 and/or PS for 5 days, bone marrow was harvested, and the cell lysates were analyzed for protein expression. Figure 7B demonstrates that as compared to treatment with each agent alone, co-treatment with PS and JQ1 was associated with the most reduction in the levels of c-MYC, BCL-2 and CDK6 proteins. We also determined the effects of JQ1 and/or panobinostat on the expression levels of BIM. As shown in Figure 7B, while treatment with each drug alone increased the levels of BIM, co-treatment 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 PS 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 Figure 7C, the combination regimen of JQ1 and PS for 3 weeks was superior to the treatment with JQ1 or PS 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 (Figure 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 in vitro and in vivo activity against AML BPCs expressing the mutant NPM1c+, with or without the co-expression 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, where 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 down regulate 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 pTEFb to the enhancers and, in the case of some oncogenes such as c-MYC and BCL2 to the clusters of enhancers called the super enhancers, thereby depleting the transcript levels of c-MYC and BCL2 (7,14). Accordingly, in our studies, treatment with JQ1 clearly inhibited the binding of BRD4 and RNA POL II to the c-MYC, BCL2 and CDK6 promoters, accompanied with the attenuation of mRNA levels of these genes in the cultured and primary AML cells expressing NPM1c+. In contrast, since c-MYC is known to repress p21, JQ1 treatment increased p21 levels in the AML cells (25). Thus, JQ1 perturbed the levels of not only c-MYC but also of the c-MYC-targeted genes. We also found that shRNA to BRD4 reduced c-MYC and BCL2 mRNA levels, while inducing the p21 levels, along with inhibition of growth and induction of apoptosis of OCI-AML3 cells. In the gene expression microarray analysis in the OCI-AML3 cells, more genes were suppressed than induced, following 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 (TKIs) alone or in conjunction with standard induction chemotherapy has exposed novel mechanisms by which AML BPCs acquire resistance to FLT3 kinase inhibitors (28, 29). Since these are type II FLT3 TKIs that bind and inactivate the ‘DFG-out’ inactive conformation of FLT3, the clinically relevant resistance mechanisms 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 co-expressing FLT3-ITD and NPM1c+ or MLL fusion proteins. This highlights the promise of including a BET protein antagonist in combination with standard chemotherapy and/or a FLT3 kinase inhibitor in the treatment of high risk AML expressing FLT3-ITD.

Additionally, our findings also support the rationale that, by inducing hyperacetylation of lysine residues on the histone proteins, PS could be inducing greater dependency on the BRD4-regulated transcription of oncoproteins, such that co-treatment with PS and JQ1 synergistically leads to growth inhibition and apoptosis of the cultured and primary AML cells regardless of the co-expression of FLT3-ITD. A molecular schema demonstrating the enhanced activity of BET protein antagonist and PS is shown in Figure 8. Indeed, consistent with this, the synergistic activity of PS and JQ1 was associated with marked depletion of the protein levels of c-MYC, BCL2 and CDK6, as was previously reported (22). Findings presented here also demonstrate that this synergy extended in vitro to the immune-phenotypically defined leukemia stem cells (LSCs) that have been previously demonstrated to exhibit in vivo leukemia initiating potential (30). Targeting LSCs is an important goal in achieving deeper remissions and overcoming treatment refractoriness in minimal residual AML, since LSC gene expression program confers a poor prognosis and influences the clinical outcome in AML (30,31). Co-treatment with PS 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-AF9 and FLT3-ITD, respectively. The improvement in survival due to PS 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 PS 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 anti-leukemia 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 NFκB and regulate its transcriptional activity (32,33). By inducing RelA acetylation, PS treatment may also increase the BRD4 dependency of the NFκB activity in AML cells. Therefore, suppression of this activity by co-treatment with PS 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 histone deacetylase inhibitor against AML.

References:


Figure Legends

Figure 1. Treatment with the BET protein antagonist, JQ1, induces cell cycle growth arrest, and lethal effects in cultured and primary AML blast progenitor cells. A. HL-60, U937, OCI-AML2, MOLM13, MV4-11 and OCI-AML3 cells were treated with the indicated concentrations of JQ1 for 48 hours. Following this, the percentages of annexin V-positive, apoptotic cells were determined by flow cytometry. Columns, mean of three independent experiments; Bars, standard error of the mean. B. OCI-AML3 and MOLM13 cells were treated with the indicated concentrations of JQ1 for 24 hours. Cell cycle status was determined by flow cytometry. Columns, mean of three independent experiments; Bars, standard error of the mean. C. HL-60, OCI-AML2, MOLM13, OCI-AML3 and MV4-11 cells were treated with the indicated concentrations of JQ1 for 48 hours. At the end of treatment, cells were washed free of the drug and plated in methylcellulose for 7-10 days. The
number of colonies in each condition was counted and the percentage colony growth was determined relative to the untreated control cells. Columns, mean of three independent experiments; Bars, standard error of the mean. D. OCI-AML3 cells were transduced with lentivirus expressing FLT3-ITD. The expression of FLT3 in the cells was detected by flow cytometry utilizing anti-FLT3 antibody. E. OCI-AML3 cells with or without ectopic expression of FLT3-ITD were treated with the indicated concentrations of JQ1 for 48 hours. At the end of treatment, cells were stained with annexin V and TO-PRO-3 iodide, and the percentages of annexin V-positive, apoptotic cells were determined by flow cytometry. Columns, mean of three experiments; Bars, standard error of the mean. IC_{50} values for JQ1 were calculated for both cell lines. The IC_{50} value for OCI-AML3 and OCI-AML3/FI is 165 nM and 188.4 nM, respectively. Inset shows the FLT3 protein expression in the two cell lines. Expression of β-actin in the lysates served as the loading control. F. Primary CD34+ AML cells (n=10) were exposed to the indicated concentrations of JQ1 for 48 hours. Then, cells were stained with propidium iodide and the percentages of PI-positive, non-viable cells were determined by flow cytometry. The plot shows the individual values for each sample. The horizontal line indicates the median loss of viability for all of the samples. The bar indicates the standard error of the mean.

Figure 2. Treatment with JQ1 reduces BRD4 and Pol II occupancy on the promoters of c-MYC, BCL2, and CDK6 in human AML cells. A-B. OCI-AML3 and primary AML cells were treated with the indicated concentration of JQ1 for 16 hours. Following this, chromatin immunoprecipitation was conducted with BRD4-specific antibody. The ChIP’ed 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 to the Ct value of the input DNA. C-D. OCI-AML3 and primary AML cells were treated with the indicated concentration of JQ1 for 16 hours. Following this, chromatin immunoprecipitation was conducted with RNA Pol II antibody. The ChIP’ed 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 to the Ct value of the input DNA.

Figure 3. Treatment with JQ1 depletes the mRNA expression of c-MYC and BCL2 in AML cells. A-B. OCI-AML3, MOLM13 and primary AML cells were treated with the indicated concentrations of JQ1 for 16 hours. At the end of treatment, RNA was isolated and reverse transcribed. The resulting cDNA was used for real-time, quantitative PCR analysis of c-MYC and BCL-2. The relative mRNA expression was normalized to GAPDH and compared to the untreated cells. C. Heat map showing the 50 most up and down regulated mRNA expression changes in OCI-AML3 cells treated with 500 nM of JQ1 for 8 hours. D. Relative mRNA expression changes of BCL2 and c-MYC in the mRNA used for expression analyses were quantified by quantitative RT-PCR. Relative expression changes were normalized to GAPDH and compared to the untreated cells.
Figure 4. Treatment with JQ1 depletes p-Serine 2 RNA POL II, c-MYC and BCL2 but induces p21, p27, and BIM protein levels in AML cells. A. OCI-AML3 and MOLM13 cells were treated with the indicated concentrations of JQ1 for 24 hours. Following this, total cell lysates were prepared and immunoblot analyses were conducted for the expression levels of BRD4, c-MYC, BCL2, CDK6, pSer2 POL II, p21, p27, BIM, cleaved PARP and β-actin in the lysates. B. OCI-AML3 and MOLM13 cells were treated with the indicated concentrations of the inactive enantiomer of JQ1, (R)-JQ1 for 24 hours. At the end of treatment, cell lysates were prepared and immunoblot analyses were conducted for the expression levels of c-MYC, BCL2, CDK6, pSer2 POL II, and β-actin in the lysates. C. Primary AML cells were treated with the indicated concentrations of JQ1 for 24 hours. Then, total cell lysates were prepared and immunoblot analyses were conducted for the expression levels of c-MYC, BCL2, CDK6, pSer2 POL II, and β-actin in the lysates. D. Primary AML cells (n=3) were treated with the indicated concentrations of JQ1 for 24 hours. After this, total cell lysates were prepared and immunoblot analyses were conducted for the expression levels c-MYC, p21, and BIM in the lysates. Densitometry analysis was performed and the percent depletion of c-MYC (left panel) or the induction of BIM and p21 (right panel) were calculated. Columns represent the mean depletion of c-MYC or induction of p21 and BIM in the three AML samples; Bars represent the standard error of the mean.

Figure 5. Co-treatment with JQ1 or shRNA-mediated knockdown of BRD4 synergistically enhances panobinostat-induced apoptosis of AML cells. A. OCI-AML3 and MOLM13 cells were treated with JQ1 and PS for 48 hours. Then, the percentages of annexin V-positive, apoptotic cells were determined by flow cytometry. Median dose effect and isobologram analyses were performed utilizing the commercially available software, CalcuSyn, assuming mutual exclusivity. Combination index (CI) values less than 1.0 indicate a synergistic interaction of the two agents in the combination. B. OCI-AML3 and MOLM13 cells were treated with the indicated concentrations of JQ1 or (R)-JQ1 and/or PS for 48 hours. At the end of treatment, the percentages of annexin V-positive, apoptotic cells were determined by flow cytometry. Columns, mean of three independent experiments; Bars, standard error of the mean. * indicates values significantly greater in the combination than treatment with JQ1 or PS alone (p< 0.05). C. OCI-AML3 and MOLM13 cells were treated with JQ1 and/or PS for 48 hours. Following this, cells were washed free of the drug, plated in methylcellulose and cultured for 7-10 days. Colonies were counted and the colony growth for each cell line is reported as a percentage of the untreated cells. Columns, mean of three experiments; Bars, standard error of the mean. D. OCI-AML3 cells were treated with the indicated concentrations of JQ1 and/or PS for 24 hours. Following this, cells were harvested and total cell lysates were prepared. Immunoblot analyses were conducted for the expression levels of BRD4, p-Ser2 POL II, c-MYC, BIM, NPM1, and β-actin in the lysates. E. OCI-AML3 cells were transduced with non-targeting (NT) or BRD4-specific shRNA-containing lentivirus for 48 hours. Then, total RNA were harvested, reverse transcribed and q-PCR was conducted for the indicated targets. The relative expression of each
mRNA was normalized to GAPDH. **F.** OCI-AML3 cells transfected with NT shRNA or BRD4 shRNA for 48 hours were treated with the indicated concentrations of PS for 48 hours. At the end of treatment, cells were stained with annexin V and TO-PRO-3 iodide and the percentages of annexin V positive, apoptotic cells were determined by flow cytometry. * indicates values significantly greater in BRD4 shRNA-transfected cells compared to NT shRNA-transfected cells. **G.** IC50 values for PS in OCI-AML3 cells transduced with NT-shRNA or BRD4 shRNA for 48 hours, then treated with PS for 48 hours.

**Figure 6. Co-treatment with JQ1 and PS exerts synergistic lethal activity against primary AML BPCs but not normal hematopoietic progenitor cells.** **A.** Primary CD34 + AML cells (n=9) were treated with the indicated concentrations of JQ1 and/or PS for 48 hours. Following this, cells were stained with propidium iodide and the percentages of non-viable cells were determined by flow cytometry. Columns, mean loss of viability of the samples; Bars, standard error of the mean. * indicates values significantly greater in the combination than treatment with either agent alone (p<0.01). Inset shows a representative Western blot of cleaved PARP following treatment with the indicated concentrations of JQ1 and/or PS in primary AML cells. **B.** Primary AML cells were treated with the indicated concentrations of JQ1 and/or PS for 24 hours. Then, cells were harvested and total cell lysates were prepared. Immunoblot analyses were conducted for the expression levels of p-Ser2 POL II, c-MYC, BCL2, BIM, p21, NPM1 and β-actin in the lysates. **C.** Primary CD34+CD38-Lin-AML cells were treated with JQ1 and/or PS for 48 hours and the percentages of non-viable cells were determined by flow cytometry. Median dose effect and isobologram analysis were performed utilizing CalcuSyn. CI values less than 1.0 indicate a synergistic interaction of the two agents in the combination. **D.** Primary CD34+ normal cells (n=2) were treated with the indicated concentrations of JQ1 and/or PS for 48 hours. At the end of treatment, cells were stained with propidium iodide and the percentages of non-viable cells were determined by flow cytometry.

**Figure 7. Compared to treatment with either agent alone, combined treatment with JQ1 and PS significantly improves the survival of NOD/SCID mice bearing OCI-AML3 or MOLM13 xenografts.** **A.** After a preconditioning dose of gamma irradiation (2.5 Gy), mice were injected with 5 million OCI-AML3 cells by lateral tail vein. Seven days after implantation, mice were treated with vehicle (10% 2-hydroxypropyl-β-cyclodextrin), JQ1 (50 mg/kg formulated in 10% 2-hydroxypropyl-β-cyclodextrin by IP injection daily x 5 days), panobinostat (5 mg/kg by IP injection 3 times per week) or the combination of JQ1 and PS for 3 weeks, then treatment was stopped. Survival of the mice is represented by Kaplan-Meier plot. In panel A, treatment with JQ1 significantly improved the survival of the mice compared to vehicle treated mice (p=0.009). In addition, mice treated with the combination of JQ1 and PS exhibited significantly improved survival compared to the mice treated with JQ1 or PS alone (p= 0.0002). **B.** NOD/SCID mice were radiated and injected with OCI-AML3 cells as in (A) and monitored for 2 weeks. Then, mice were treated
with vehicle (10% 2-hydroxypropyl-β-cyclodextrin), JQ1 (50 mg/kg by IP injection daily x 5 days), panobinostat (5 mg/kg by IP injection 3 times per week) or the combination of JQ1 and PS for 1 week, then treatment was stopped. Mice were sacrificed and bone marrow was collected from the femurs of mice in each treatment group. Total cell lysates were prepared and immunoblot analyses were conducted for the expression levels of c-MYC, BCL2, CDK6, BIM and β-actin in the lysates. Representative immunoblots are shown. C. After a preconditioning dose of gamma irradiation (2.5 Gy), mice were injected with 5 million MOLM13 cells by lateral tail vein. Four days after implantation, mice were treated with vehicle (10% 2-hydroxypropyl-β-cyclodextrin), JQ1 (50 mg/kg formulated in 10% 2-hydroxypropyl-β-cyclodextrin by IP injection daily x 5 days), panobinostat (5 mg/kg by IP injection 3 times per week) or the combination of JQ1 and PS (JQ1 was administered on alternating days with PS for this combination) for 3 weeks, then treatment was stopped. Survival of the mice is represented by Kaplan-Meier plot. Treatment with JQ1 significantly improved the survival of the mice compared to vehicle treated mice (p=0.0133). In addition, mice treated with the combination of JQ1 and PS had significantly improved survival compared to the mice treated with JQ1 or PS alone (p< 0.0001).

Figure 8. Molecular schema demonstrating the superior activity of BET protein antagonist and pan-HDAC inhibitor against AML cells. A. In AML cells, histone acetyltransferases (HATs) are bound to Transcription Factor Complexes (TFC) (e.g. c-MYC) at the promoters of target genes, which acetylates the lysine (K) residues on histone H3 and H4 and creates a permissive environment for transcription. Treatment with low doses of pan-HDAC inhibitors, such as panobinostat (PS), inhibits HDAC activity which results in hyper-acetylation of N-terminal tails on histone H3 and H4 and greater dependency on BET protein function. BRD4 binds to acetylated histones and recruits pTEFb to the enhancer/promoter of target genes such as c-MYC, BCL2 and CDK6. Upon its recruitment by BRD4, pTEFb phosphorylates the C-terminal domain of RNA POL II resulting in transcription of target genes. Treatment with BET protein inhibitors, JQ1 or IBET151, inhibits BRD4 activity causing depletion of RNA POL II phosphorylation at Ser 2, reduced binding of BRD4 and RNA POL II at target gene promoters and decreased transcription of target genes. In addition, treatment with JQ1 inhibits cell growth and induces apoptosis of AML cells. Combined treatment with BET protein inhibitor and PS results in greater depletion of target genes (e.g. c-MYC, BCL2, Bcl-xL, and PIM1). This leads to a greater loss of cell growth and further induction of apoptosis than treatment with either agent alone in the AML cells.
Figure 1

A

% apoptosis

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<thead>
<tr>
<th>HL-60</th>
<th>U937</th>
<th>OCI-AML2</th>
<th>MOLM13</th>
<th>MV4-11</th>
<th>OCI-AML3</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% of cells</td>
<td>Control</td>
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<td></td>
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<td></td>
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<tr>
<td></td>
<td>250 nM, JQ1, 48 h</td>
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<tr>
<td></td>
<td>500 nM, JQ1, 48 h</td>
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B

% of cells

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<thead>
<tr>
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<tr>
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</tr>
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<td>90</td>
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</tr>
<tr>
<td></td>
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C

% colony growth (relative to control)

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<tr>
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<th>MOLM13</th>
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<tr>
<td>% of cells</td>
<td>Control</td>
<td>100 nM, JQ1, 48 h</td>
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D

% apoptosis

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<th>Isotype control</th>
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<tr>
<td>Count</td>
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<td>FL-2 (FLT3 surface expression)</td>
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E

% of non-viable cells

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<tr>
<td>% of cells</td>
<td>Control</td>
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<td></td>
<td>250 nM, JQ1, 48 h</td>
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F

% of non-viable cells

<table>
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<th>CD34+ primary AML cells (n=10)</th>
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<tbody>
<tr>
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<tr>
<td>100 nM, JQ1, 48 h</td>
</tr>
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<td>250 nM, JQ1, 48 h</td>
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<tr>
<td>500 nM, JQ1, 48 h</td>
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Figure 2

A. 

OCI-AML3 BRD4 ChIP

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<th>CDK6 promoter</th>
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<tr>
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<td>0.8</td>
<td>0.6</td>
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</table>

B. 

Primary AML BRD4 ChIP

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<th>BCL2 Promoter</th>
<th>CDK6 Promoter</th>
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<td>0.8</td>
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C. 

OCI-AML3 POL II ChIP

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D. 

Primary AML POL II ChIP

<table>
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<tr>
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<td>0.6</td>
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</tbody>
</table>
Figure 3

A

**OCI-AML3**
- **c-MYC**
- **BCL2**

Relative mRNA expression

- 0 nM, JQ1, 16 h
- 500 nM, JQ1, 16 h

B

**Primary AML**
- **c-MYC**
- **BCL2**

Relative mRNA expression

- 0 nM, JQ1, 16 h
- 500 nM, JQ1, 16 h

C

**MOLM13**
- **c-MYC**
- **BCL2**

Relative mRNA expression

- 0 nM, JQ1, 16 h
- 500 nM, JQ1, 16 h

D

**OCI-AML3**
- **BCL2**
- **c-MYC**

Relative mRNA expression

- Control
- 500 nM, JQ1, 16 h
Figure 5

A. OCI-AML3 and MOLM13 cells were treated with JQ1 and PS at various concentrations. The fractional effect of JQ1 and PS on cell viability was assessed.

B. The percentage of apoptosis was measured in OCI-AML3 and MOLM13 cells treated with JQ1 and PS alone or in combination.

C. The colony formation assay was performed to evaluate the effect of JQ1, PS, and their combination on OCI-AML3 and MOLM13 cells.

D. OCI-AML3 cells were treated with JQ1 and PS to assess the expression of BRD4, c-MYC, BCL2, p21, BIMEL, BIML, pSer2 POL II, NPM1, and β-actin.

E. The mRNA expression levels of BRD4, c-MYC, BCL2, p21, BIMEL, BIML, pSer2 POL II, NPM1, and β-actin were determined in OCI-AML3 cells treated with JQ1 and PS.

F. The percentage of apoptosis was measured in OCI-AML3 cells treated with BRD4 shRNA and JQ1/PS.

G. The IC50 values for JQ1 and PS were calculated in OCI-AML3 cells treated with BRD4 shRNA.

* indicates statistical significance.
Figure 6

A

Primary CD34+ AML cells (n=9)

% non-viable cells

- - + +
- - + +
500 nM JQ1
20 nM PS
- - + +
JQ1 + PS

Control
500 nM JQ1
20 nM PS
JQ1 + PS

B

Primary AML

+ 20 nM PS

0 500 0 500 nM JQ1, 24 h

pSer2 POL II

- c-MYC

- BCL2

- BIMEL

- p21

- NPM1

- β-actin

C

Primary CD34+CD38-LIN- AML

Cl

JQ1 and PS

D

Primary Normal CD34+ cells (n=2)

% non-viable cells

Control
250 nM JQ1
10 nM PS
JQ1 + PS
Figure 7

A

OCI-AML3 xenografts

Percent survival

150

100

50

0

0

10

20

30

40

50

60

70

80

Days Post Implantation

Median Survival: Days

Vehicle: 25
50 mg/kg JQ1: 34
5 mg/kg PS: 39.5
JQ1 + PS: 51.5

B

OCI-AML3 xenograft

- + - + 50 mg/kg JQ1
- - + + 5.0 mg/kg PS

c-MYC
BCL2
CDK6

BIML
BIMEL
BIMS

β-actin

C

MOLM13 xenografts

Percent survival

100

50

0

0

20

40

60

80

100

Days Post Implantation

Median Survival: Days

Vehicle: 18.5
50 mg/kg JQ1: 24.5
5 mg/kg PS: 26.5
JQ1 + PS: 39.5

P=0.0001, JQ1 vs vehicle
P=0.0133, JQ1 vs vehicle
P<0.0001, PS vs vehicle
P=0.0005, PS vs vehicle

P=0.0002
Figure 8

Transcription Factor Complex (e.g. MYC)

HAT

H4

H3

K5 ac
K8 ac
K12 ac
K16 ac

pTEFb

BRD4

Mediator

RNA Pol II

BET protein antagonist

K9 ac
K14 ac

Low Dose PS

K9 ac
K14 ac

pTEFb

BRD4

Mediator

RNA Pol II

c-MYC

PIM1

BCL2

BCL-xL

Cell growth

Apoptosis

Low Dose PS

pTEFb

BRD4

Mediator

RNA Pol II

BET protein antagonist

K9 ac
K14 ac

Low Dose PS
Molecular Cancer Therapeutics

Highly active combination of BRD4 antagonist and histone deacetylase inhibitor against human acute myeloid leukemia (AML) cells

Warren Fiskus, Sunil Sharma, Jun Qi, et al.

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