Targeting MYC dependence in cancer by inhibiting BET bromodomains

Jennifer A. Mertz[1], Andrew R. Conery[1], Barbara M. Bryant, Peter Sandy, Srividya Balasubramanian, Deanna A. Mele, Louise Bergeron, and Robert J. Sims III[2]

Constellation Pharmaceuticals, Inc., Cambridge, MA 02142

Edited* by Robert N. Eisenman, Fred Hutchinson Cancer Research Center, Seattle, WA, and approved August 30, 2011 (received for review May 23, 2011)

The MYC transcription factor is a master regulator of diverse cellular functions and has been long considered a compelling therapeutic target because of its role in a range of human malignancies. However, pharmacologic inhibition of MYC function has proven challenging because of both the diverse mechanisms driving its aberrant expression and the challenge of disrupting protein–DNA interactions. Here, we demonstrate the rapid and potent abrogation of *MYC* gene transcription by representative small molecule inhibitors of the BET family of chromatin adaptors. *MYC* transcriptional suppression was observed in the context of the natural, chromosomally translocated, and amplified gene locus. Inhibition of BET bromodomain–promoter interactions and subsequent reduction of *MYC* transcript and protein levels resulted in G_1 arrest and extensive apoptosis in a variety of leukemia and lymphoma cell lines. Exogenous expression of *MYC* from an artificial promoter that is resistant to BET regulation significantly protected cells from cell cycle arrest and growth suppression by BET inhibitors. *MYC* suppression was accompanied by deregulation of the MYC transcriptome, including potent reactivation of the *p21* tumor suppressor. Treatment with a BET inhibitor resulted in significant antitumor activity in xenograft models of Burkitt's lymphoma and acute myeloid leukemia. These findings demonstrate that pharmacologic inhibition of *MYC* is achievable through targeting BET bromodomains. Such inhibitors may have clinical utility given the widespread pathogenetic role of *MYC* in cancer.

BRD2 | BRD3 | BRD4 | JQ1

The BET protein family employs tandem bromodomains to recognize specific acetylated lysine residues in the N-terminal tails of histone proteins (1). Members of the BET family, including BRD2, BRD3, BRD4, and BRDT, modulate gene expression by recruiting transcriptional regulators to specific genomic locations (1). BRD4 is a well-established regulator of P-TEFb, a complex consisting of cyclin-dependent kinase (CDK) 9 and cyclin T, among other polypeptides (2–4). Through its interaction with BRD4, P-TEFb is recruited to promoters to phosphorylate the carboxyl-terminal domain of the large subunit of RNA polymerase II (RNAPII). Functional studies have suggested that BRD4 plays an important role in the regulation of growth-associated genes at the M/G_1 boundary by retaining P-TEFb at the promoters of key regulatory genes throughout mitosis (3, 4). Consistent with this model, experimental reduction of BRD4 levels or activity has been shown to cause profound effects on cell cycle progression and cellular viability (3, 4). BRD2 and BRD3 also regulate the transcription of growth-promoting genes, suggesting that the BET family has reinforcing roles to enable proper cell growth (5).

The Mitsubishi Tanabe Pharma Corporation first described thienodiazepine analogs that potently inhibit the closely related bromodomains of the BET family (6). These and related small molecules competitively occupy the acetyl-binding pockets of BET bromodomains, resulting in the release of BET proteins from chromatin (7, 8). NF-κB–dependent gene expression is one canonical pathway suppressed by BET inhibitors (7). Accordingly, BET inhibitors were found to be therapeutically effective in preclinical animal models of endotoxic shock caused by activation of Toll-like receptors that trigger the up-regulation of cytokines controlled by NF-κB (7). Importantly, although RNAi-mediated depletion of any one individual BET protein has broad effects on transcription, pharmacologic inhibition of the BET bromodomains perturbs the transcription of a narrower range of genes (7). These findings suggest an opportunity to use BET-bromodomain inhibitors to fine-tune key regulatory pathways for therapeutic benefit.

BET inhibitors exhibit anticancer effects in both in vitro and in vivo models of midline carcinoma (8), a rare but lethal disease that frequently harbors a chromosomal translocation in which the bromodomains of BRD4 are fused to the NUT gene (9). Although other genetic abnormalities of BET family members in cancer have not been described, we speculated that BET-bromodomain inhibition may have broader utility as an anticancer agent because BRD2, BRD3, and BRD4 are functionally linked to pathways important for cellular viability and cancer signaling.

Results

Leukemia and Lymphoma Cell Lines Are Sensitive to BET-Bromodomain Inhibition. We treated a panel of cancer cell lines by using the recently described BET inhibitor (+)-JQ1 to assess its effects on cellular proliferation. This small molecule is closely related to the Mitsubishi Tanabe compounds and inhibits the individual BET family bromodomains to a similar extent, but does not bind bromodomains from proteins outside of this subfamily (8). Assessment of cellular viability revealed that (+)-JQ1 had antiproliferative activity in many, but not all, cell lines of various origins (Fig. 1*A*). Marked growth inhibition was observed consistently in cell lines derived from patients with leukemia or lymphoma. BET inhibitors have been shown, in stimulated macrophages, to suppress genes in the NF-κB transcriptional pathway (7) that contribute to the pathogenesis of multiple myeloma (10, 11). Hence, we tested the effects of (+)-JQ1 on a broader set of human myeloma cell lines (HMCL). Fifty percent inhibition of growth (GI_{50}) was seen in 14 of 15 HMCL at concentrations of (+)-JQ1 between 50 and 500 nM (Fig. 1 *A* and *B*). To determine whether the observed phenotypes were due to BET-bromodomain inhibition, we treated affected cells with an enantiomer, (-)-JQ1, that has no appreciable binding to BET

Author contributions: J.A.M., A.R.C., P.S., L.B., and R.J.S. designed research; J.A.M., A.R.C., P.S., S.B., and D.A.M. performed research; J.A.M., A.R.C., B.M.B., P.S., S.B., D.A.M., L.B., and R.J.S. analyzed data; and J.A.M., A.R.C., L.B., and R.J.S. wrote the paper.

Conflict of interest statement: All authors are employees of Constellation Pharmaceuticals, Inc.

*This Direct Submission article had a prearranged editor.

Freely available online through the PNAS open access option.

Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE29449).

[1]J.A.M. and A.R.C. contributed equally to this work.

[2]To whom correspondence should be addressed. E-mail: robert.sims@constellationpharma.com.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1108190108/-/DCSupplemental.

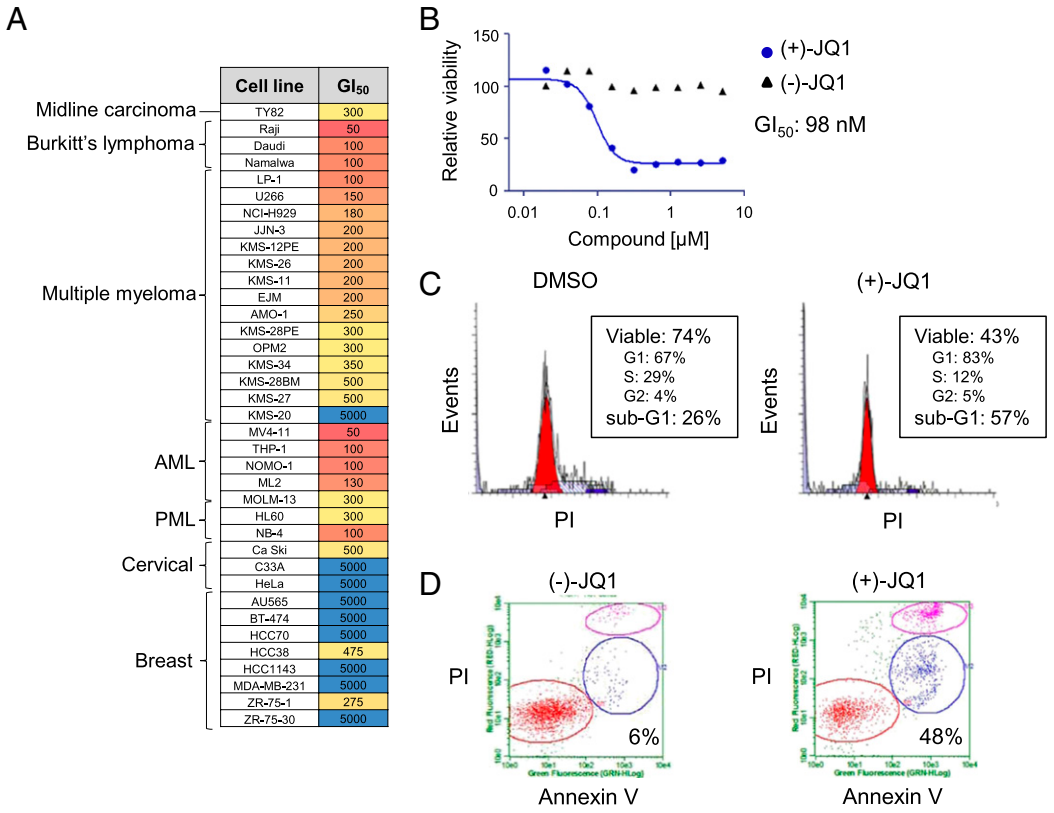

Fig. 1. Leukemia and lymphoma cell lines are broadly sensitive to BET-bromodomain inhibition. (*A*) GI_{50} values of cell lines treated with an active BET inhibitor for 72 h. (*B*) Dose–response curve of LP-1 cells treated with (+)-JQ1 or (-)-JQ1 for 72 h. (*C*) Cell cycle profile of LP-1 cells after treatment with DMSO or (+)-JQ1 [625 nM] for 72 h. Data are representative of three independent experiments. (*D*) Annexin V and PI staining of cells treated with (+)-JQ1 or (-)-JQ1 [5 μM] for 72 h. The percentage of cells that stained positive for annexin V is indicated (red, viable; blue, early apoptosis; pink, late apoptosis). Data are representative of two independent experiments.

bromodomains (8), and detected no effect at high concentrations (Fig. 1*B*).

Previous studies using RNAi showed that the BET family member, BRD4, is required for the transition from mitosis to G_1 (3). Consistent with this observation, the cell cycle profile of LP-1 (HMCL) and Raji (Burkitt's lymphoma; BL) cell lines revealed an increase in the percentage of cells in G_0/G_1 upon exposure to (+)-JQ1 (Fig. 1*C* and Fig. S1 *A* and *B*). To test whether BET inhibitors affect progression through S phase and entry into mitosis, we synchronized AMO-1 (HMCL) cells by using a double thymidine block and released the cells in the presence of DMSO, (+)-JQ1, or (-)-JQ1. We observed no gross defects in the timing of S phase progression or entry into mitosis (Fig. S1*C*). However, at 16 h after release from thymidine, cells grown in the presence of (+)-JQ1 accumulated in G_0/G_1, whereas cells grown in (-)-JQ1 or DMSO did not. These data suggest that BET-bromodomain inhibition perturbs cells either during mitosis or at the M/G_1 border, leading to a failure to enter the next cell cycle. Treatment with (+)-JQ1 increased the population of cells in sub-G_1 (Fig. 1*C* and Fig. S1 *A* and *B*) and induced a significant increase in the number of cells that were Annexin V-positive, suggesting that these cells were undergoing active apoptosis (Fig. 1*D*). Therefore, BET bromodomain inhibitors induce cell cycle arrest and apoptosis with a range of sensitivity across a panel of cancer cells.

BET-Bromodomain Inhibition Potently Suppresses *MYC* Gene Expression. Given the broad activity of the inhibitor in leukemia and lymphoma cells, we sought to define potential mechanisms that could explain the phenotypic responses. Thus, we performed gene expression profiling in LP-1 and Raji cells lines treated with the active and inactive BET inhibitor. We treated cells for 4 and 8 h to identify early, and potentially direct, transcriptional targets of (+)-JQ1. Upon sorting by an average differential expression score representing data across cell lines and time points, the most down-regulated gene was *MYC* (Fig. 2*A*). *MYC* ranked at or near the top in each of the individual cell lines and at each time point. Interestingly, LP-1 and Raji cells overexpress *MYC* by virtue of a chromosomal translocation that fuses the Ig heavy chain locus (IgH) to the promoter proximal region of *MYC* (12). The top 20 down- and up-regulated genes in LP-1 and Raji cells according to the differential expression score are shown in Fig. 2*A*, and the fold changes are reported in Table S1. At these time points, the transcriptional effects of the inactive BET inhibitor were minor, highlighting the linkage of these effects to the specific binding of (+)-JQ1 (Fig. 2*A*). We used the GSEA program to identify curated gene signatures and transcription factor target genes whose expression changed significantly with BET inhibition (13, 14). Among the top gene signatures that overlapped with our down-regulated BET signature were gene sets shown to be up-regulated upon over-expression of MYC (Fig. 2*B* and Table S2). Consistent with this observation, the MYC-MAX DNA binding motif was the top motif gene set repressed by BET inhibition (Fig. 2*C* and Table S3). Collectively, these unbiased expression and computational analyses suggest that suppression of *MYC* transcription by BET inhibitors results in the deregulation of the MYC transcriptome. A number of HDAC inhibitory gene signatures also overlapped with the BET signature (Table S2). Given that HDAC inhibition blocked the transcriptional affects of BET bromodomain inhibition in the context of NF-κB signaling (7), it will be interesting to test the relationship between BET bromodomain inhibition and HDAC inhibition with respect to *MYC* suppression. A more comprehensive list of the top gene sets that correlate with BET-bromodomain inhibition is presented in Table S2 and Table S3.

To define the specificity of *MYC* suppression in further detail, we treated LP-1 cells with a range of compound concentrations. We observed a dose-dependent suppression of *MYC* by the active BET inhibitor (Fig. 3*A*). Importantly, we observed that the concentrations of (+)-JQ1 required to suppress *MYC* strongly

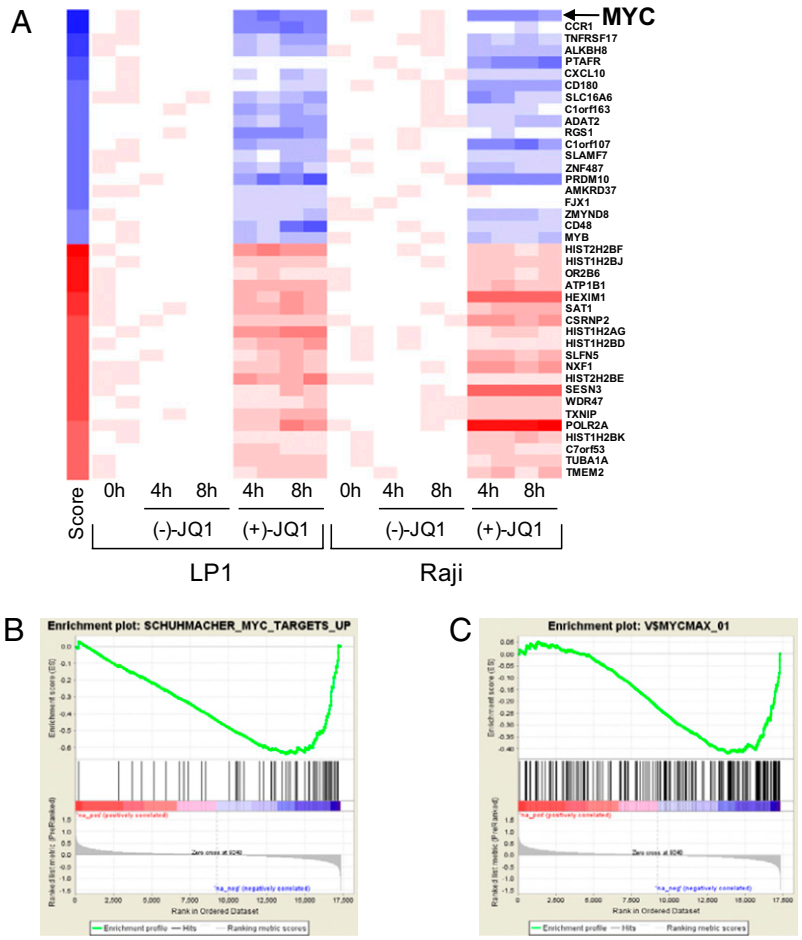

Fig. 2. Gene expression profiling of LP-1 and Raji cells treated with active or inactive BET inhibitors. (*A*) Top 20 genes down-regulated (blue) and up-regulated (red) by BET-bromodomain inhibition. LP-1 and Raji cells were treated for 4 or 8 h with (+)-JQ1 or (-)-JQ1 at 500 nM. Expression changes for individual time points and cell lines were used to calculate the final expression change score shown in the left column. Detailed data analysis methods, expression data, and differential expression scores can be found in *SI Methods*. (*B* and *C*) GSEA enrichment plots showing the down-regulation of MYC targets in BET-treated cell lines. Genes are ordered by differential expression score. Gene sets are from MSIGDB collections: genes up-regulated in P493-6 (BL) cells induced to express MYC (*B*); genes with a MYC-MAX binding motif (*C*). See *SI Methods* for details.

correlate with the cellular affects on cell cycle arrest and the induction of apoptosis (Fig. S2*A*). Exposure of LP-1 cells to a related, but chemically distinct BET inhibitor (7), also resulted in transcriptional suppression of *MYC* (Fig. S2*B*). To determine whether the suppression of *MYC* by BET-bromodomain inhibition was reversible, we treated LP-1 cells for 2 h with (+)-JQ1 and then removed the inhibitor from the media. We observed a time-dependent restoration of *MYC* expression to untreated levels by 2 h after washout (Fig. 3*B*). We confirmed that *MYC* was silenced by BET inhibitors in Raji cells, as well as an additional cell line that also contains a chromosomal translocation driving *MYC* expression, AMO-1 (HMCL) (Fig. S2*C*). Thus, *MYC* expression was markedly suppressed after treatment with (+)-JQ1 in three independent cell lines harboring *MYC* translocations, suggesting that BET proteins play a key role in *IgH-MYC* regulation.

Expression of *MYC* was reduced by BET inhibitors within 1 h after treatment (Fig. 4*A*), suggesting that BET proteins might be exerting direct effects on the *MYC* locus. To determine whether BET proteins bind directly to the *MYC* locus, we performed chromatin immunoprecipitation (ChIP) studies by using antibodies against BRD3 and BRD4. We detected specific enrichment of both BRD3 and BRD4 upstream of the P1 promoter of *MYC* in Raji cells (Fig. S2*D*), which is the active promoter in this translocated allele (15). In the presence of (+)-JQ1, we observed the release of BRD3 and BRD4 from this promoter (Fig. S2*D*). In addition, we monitored BRD4 binding at several distinct regions of the *MYC* locus in LP-1 cells to track where BRD4 may be functioning to regulate *MYC* gene expression. Enrichment of BRD4 was observed at several locations including both up- and downstream of the transcription start site, although BRD4 binding appeared to peak within the P1 promoter of *MYC* in LP-1 cells (Fig. 3*C*). Treatment of these cells with (+)-JQ1 induced the release of BRD4 from the promoter and downstream of the transcription start site (Fig. 3*C*). The levels of histone H3 and acetylated H4 were unaffected upon treatment with (+)-JQ1 (Fig. 3*D*). Consistent with a role of BRD4 in p-TEFb recruitment, we observed release of CDK9 from the *MYC* locus upon BET bromodomain inhibition (Fig. 3*D*). The specificity of the BRD4 antibody used in these studies was corroborated by (+)-JQ1 mediated release of BRD4 on three additional genes, whereas a gene desert region on chromosome 12 displayed no BRD4 enrichment (Fig. S2*E*). These findings collectively indicate that release of BET proteins from chromatin suppresses the transcription of *MYC* in LP-1 and Raji cells.

***MYC* Is Suppressed in Diverse Genetic Contexts.** To determine whether BET inhibitors can suppress *MYC* in different genetic contexts, we assessed a panel of cell lines that overexpress *MYC* by amplification, viral insertion, or in the absence of recognized genetic changes. Reduced *MYC* transcript levels were observed within 4 h of (+)-JQ1 treatment in cell lines with either *MYC* amplification or an unamplified, wild-type locus (Fig. S3*A*). *MYC* mRNA was suppressed by 99.8% in the AML cell line MV4-11, which harbors a wild-type locus (Fig. S3*A*). Reflecting this reduction in *MYC* transcript levels in cell lines treated with BET inhibitor, the levels of MYC protein were also decreased (Fig. S3*B*). AU565 and HeLa cells, which overexpress *MYC* by amplification or viral insertion, respectively, displayed relative resistance to *MYC* suppression after 4 h of treatment, compared

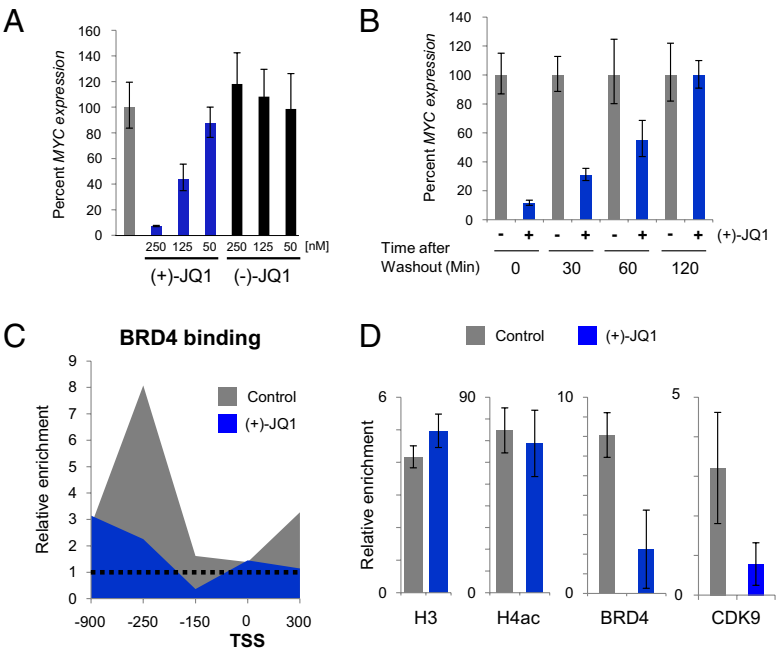

Fig. 3. Small molecule BET-bromodomain inhibition suppresses *MYC* transcription. (*A*) Quantitative PCR (qPCR) of *MYC* expression in LP-1 cells treated with DMSO (gray bar) or increasing doses of (+)-JQ1 (blue bars) or (-)-JQ1 for 4 h (black bars). (*B*) Raji cells were treated with (+)-JQ1 at a final concentration of 1 μM for 2 h. Compound was then washed out of the medium, and samples were taken at indicated time points for qPCR analysis of *MYC* expression levels. For each time point, *MYC* expression from DMSO treated sample (gray bar) was set to 100%. (*C*) Chromatin immunoprecipitation from LP-1 cells treated with DMSO (-) or (+)-JQ1 [500 nM] for 4 h using an antibody against BRD4. qPCR was performed by using primer pairs corresponding to the regions indicated on the *x* axis. Enrichment relative to no antibody control is indicated. (*D*) qPCR ChIP from LP-1 cells treated with DMSO (-) or (+)-JQ1 [500 nM] for 4 h using the indicated antibodies. Enrichment relative to no antibody control is indicated. The primer sets used are the following, H3, H4ac, BRD4:-250, and CDK9:TSS.

with other cell lines (Fig. S3). Thus, whereas BET inhibitors measurably suppress *MYC* transcription in the context of translocation, amplification, or with an unaltered, wild-type *MYC* locus, cells exhibit a range of sensitivity to BET-inhibitor–dependent *MYC* suppression.

***MYC* Is Linked to the Growth Suppression Mediated by BET Inhibitors.** Given the known proliferative activity of MYC, transcriptional reprogramming via *MYC* suppression may significantly contribute to the growth inhibitory effects mediated by BET bromodomain inhibition. Consistent with the observed defects in cell cycle progression, a number of key G_1-associated cell cycle genes were deregulated in LP-1 cells after 4 and 8 h of treatment with (+)-JQ1. The *p21* tumor suppressor, a potent CDK inhibitor, was dramatically reactivated in LP-1 cells upon (+)-JQ1 treatment (Fig. 4*A*). Numerous studies have established that MYC represses *p21* transcription through its physical association with MIZ1 (16, 17). We therefore performed a time-course experiment to evaluate whether *MYC* suppression was coincident with *p21* reactivation. Within 1 h of (+)-JQ1 treatment, *MYC* mRNA was diminished and a concomitant 10-fold induction of *p21* was observed (Fig. 4*A*). By 4 h, *MYC* was suppressed by 98% and *p21* was reactivated by nearly 400-fold (Fig. 4*A*).

To test more directly whether MYC mediates the cell cycle arrest facilitated by BET inhibitors, we made use of a tetracycline-inducible MYC expression construct that is resistant to the effects of BET-bromodomain inhibition (Fig. 4*B* and Fig. S4*A*). This construct was stably transduced into LP-1 cells, which were then grown in the presence or absence of doxycycline to induce MYC expression for 3 d. (+)-JQ1 or (-)-JQ1 was then added to the cells, and various phenotypes were assessed. After 4 h of treatment with (+)-JQ1, we observed that the robust induction of *p21* was strongly attenuated in the presence of exogenous MYC (Fig. 4*B*). Similar neutralization of (+)-JQ1–induced transcriptional deregulation was observed for several other genes known to be downstream targets of MYC (Fig. S4 *B* and *C*). Consistent with these transcriptional changes, after 24 h of treatment we noticed a striking rescue of (+)-JQ1–induced G_1 arrest in the presence of exogenous MYC (Fig. 4 *C* and *D*). Although our observations at longer time points were somewhat confounded by the induction of apoptosis with extended periods of MYC overexpression (Fig. S4*D*), we observed a significant protection ($P < 0.001$) from BET inhibitor-mediated growth suppression by exogenous expression of MYC after 4 d (Fig. 4*E*). Taken together, these observations strongly support the conclusion that MYC down-regulation is a primary cause of the growth inhibition observed with BET inhibitors in tumor cells driven by deregulated MYC.

BET Inhibitors Display Antiproliferative Effects in Animal Models of BL and Acute Myeloid Leukemia (AML). To further investigate the antitumor effects of BET inhibition, we explored xenograft models of BL and AML. Raji (BL) or MV4-11 (AML) cells were grown s.c. in immunocompromised mice, and (+)-JQ1 was administered i.p. either twice a day at 30 mg/kg, or once a day at 50 mg/kg. As shown in Fig. 5*A*, Raji BL tumors grew significantly slower in compound-treated mice compared with vehicle-treated controls. In this model, the average tumor volume was 45% smaller in the compound-treated group at day 14 (Fig. 5*A*). After this time, survival in the vehicle group was significantly decreased and, therefore, tumor size could not be adequately compared because of the increasingly unequal number of animals between the groups resulting from high tumor-related mortality in the control group. Tumor growth to an average of 1,000 mm^3 was delayed by 7 d in the treatment group compared with the vehicle control group. Consistently, pharmacodynamic analysis indicated that *MYC* mRNA expression was reduced in Raji tumors by 80% at 4 h and 60% at 8 h after dosing at 25 mg/kg (Fig. 5*B*). (+)-JQ1 treatment led to a highly significant increase in survival in the treatment group (Fig. 5*C*). One of 15 animals in the treatment group was euthanized because of a large tumor volume. Treatment with (+)-JQ1 in a disseminated model of Raji BL (18) also resulted in significant antitumor activity without weight loss or any other obvious adverse systemic effects (Fig. S5 *A* and *B*).

In addition to BL, MV4-11 AML xenografts were observed to be particularly sensitive to (+)-JQ1 treatment. In the AML xenograft model, (+)-JQ1 dosed twice daily at 30 mg/kg resulted in a 20% tumor regression when the study was terminated after 32 d of treatment (Fig. 5*D*). A 70% decrease in tumor growth was observed with once-a-day dosing of (+)-JQ1 at 50 mg/kg (Fig. 5*D*). The higher effectiveness seen with more frequent dosing of a lower concentration of (+)-JQ1 is consistent with the expected

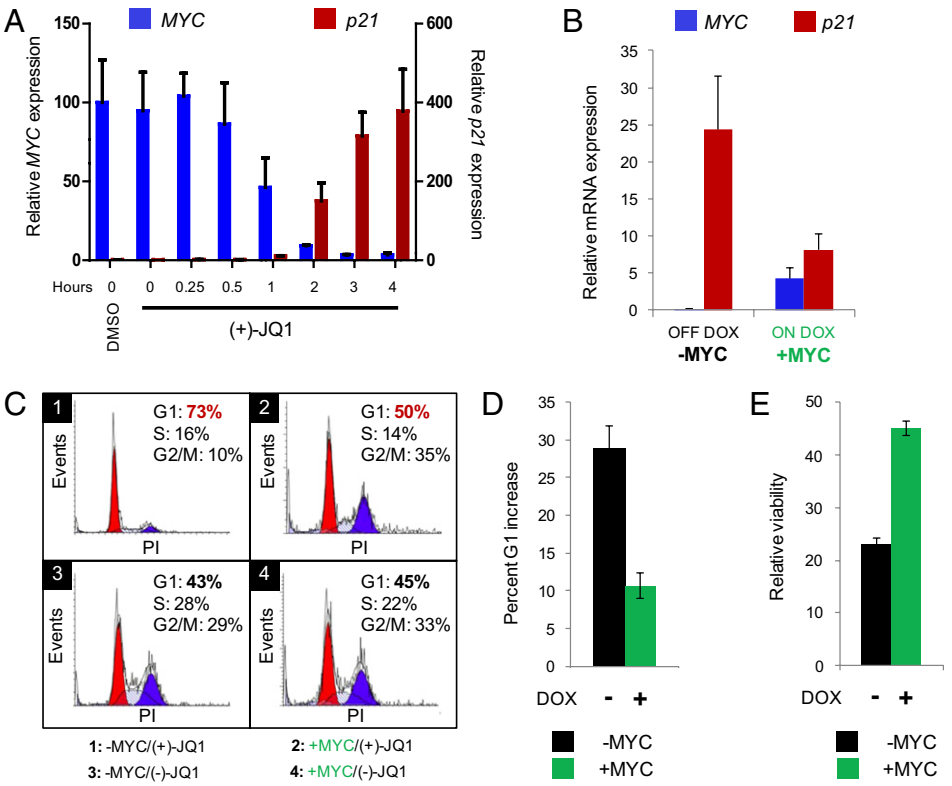

Fig. 4. MYC reconstitution significantly protects cells from BET-mediated effects. (*A*) mRNA expression of *MYC* and *p21* by qPCR upon treatment with (+)-JQ1 in LP-1 cells for the indicated times. mRNA expression is shown relative to DMSO control, where *MYC* is set to 100% and *p21* is set to 1. (*B*) mRNA expression of *MYC* or *p21* upon (+)-JQ1 treatment for 4 h in LP-1 cells in the absence (MYC off) or the presence (MYC on) of doxycycline. mRNA expression was determined relative to mRNA expression after (-)-JQ1 treatment. (*C*) LP-1 cells stably transduced with tetracycline-inducible MYC were grown in the absence or presence of doxycycline [250 nM] for 3 d. Cells were then treated with (+)-JQ1 or (-)-JQ1 at 500 nM for 24 h and fixed and stained for FACS analysis of DNA content. The percentage of cells in G_1, S, and G_2/M are indicated. Data are representative of three independent experiments. (*D*) Quantification of the increase in the percentage of cells in G_1 after (+)-JQ1 treatment in the absence or presence of doxycycline (mean ± SD, n = 3). Values were calculated by subtracting the $\%G_1$ after (-)-JQ1 treatment from the $\%G_1$ after (+)-JQ1 treatment. (*E*) The relative viability of cells upon treatment of (+)-JQ1 after 4 d in the absence or presence of doxycycline (mean ± SD, n = 3). Values were calculated by determining the ratio of the viability of cells exposed to (+)-JQ1 to the viability of cells exposed to (-)-JQ1 in the absence or presence of doxycycline.

dependence on continuous down-regulation of *MYC* for efficacy in the context of the pharmacokinetic/pharmacodynamic relationship observed in the Raji BL tumors. Standard-of-care agent cytarabine was modestly effective with a 33% decrease in tumor growth (Fig. 5*D*). (+)-JQ1 was generally well tolerated throughout the course of all three studies (Fig. S5 *C* and *D*). Lack of systemic effects was not attributable to an inability of (+)-JQ1 to repress murine *MYC,* as treatment of the mouse cell lines EL4 and RAW264.7 with (+)-JQ1 resulted in substantial *MYC* suppression (Fig. S5*E*). These data demonstrate significant in vivo antitumor activity linked to BET inhibition and suggest a potential therapeutic use for small molecule inhibitors of BET for the treatment of hematological malignancies.

Discussion

We provide evidence that *MYC* gene expression can be potently and reversibly abrogated by BET bromodomains inhibitors. Transcriptional silencing of *MYC* is coincident with release of BET proteins from the *MYC* locus, indicating that BET proteins directly regulate *MYC* gene expression. Although BET inhibitors influence the expression of an assortment of genes, the MYC transcriptome heavily dominates the gene expression changes observed upon BET-bromodomain inhibition. The *MYC* gene is translocated in all patients with BL and a significant fraction of patients with multiple myeloma or diffuse large B-cell lymphoma (19). BL is well established to depend on the functions of MYC (15, 19). We observed that several cell lines harboring *MYC* translocations are sensitive to BET inhibitors as measured by both transcriptional effects and apoptotic responses (Fig. 1, Fig. S2, and Fig. S3), perhaps reflecting their degree of MYC addiction (20). Apart from the special case of midline carcinoma, we have yet to observe a cell type that is sensitive to BET inhibitors without down-regulation of *MYC* (Fig. S6*A*). However, *MYC* reduction did not always correlate with a cell proliferation phenotype in vitro. For example, MYC protein was significantly reduced in the breast cancer cell line MDA-MB-231, which nevertheless grew normally upon administration of (+)-JQ1 (Fig. 1*A* and Fig. S3*B*). Previous studies have indicated that the in vitro proliferation of the MDA-MB-231 cell line is only mildly affected by a severe reduction of MYC protein by shRNA (21). To corroborate this observation, we infected MDA-MB-231 cells with four different shRNAs that knockdown MYC mRNA and assessed their effect on proliferation. As shown in Fig. S6*B*, MDA-MB-231 cells are not phenotypically sensitive to MYC knockdown by shRNA at comparable mRNA knockdown levels to (+)-JQ1–mediated suppression of *MYC*. No gross toxicity was observed upon high-dose treatment of BET inhibitors in this MYC-insensitive cell line, revealing no marked, general MYC-independent effects on cellular viability in this setting. Suppression of *MYC* by BET inhibitors was not observed in every cell line tested, and the degree of *MYC* silencing also varied at the time points tested. It is possible that variation in the permeability and efflux of BET inhibitors across cell lines contributes to some of these observations. Conceptually, it will be important to elucidate in detail the chromatin or regulatory contexts that define *MYC* sensitivity to BET inhibitors. These regulatory features may ultimately identify the patients who will respond best to this class of small molecule inhibitors.

MYC is a member of a related family of transcription factors that also includes *MYCN* and *MYCL* (22, 23). *MYCN* and *MYCL* are both intimately linked to the oncogenic phenotype by means of their genomic translocation, amplification, and overexpression in human tumors. Notably, *MYCN* is frequently amplified and overexpressed in human neuroblastoma and is functionally believed to be a driver of this disease (23). High-dose, transient treatment of various cell lines harboring *MYCN* amplification and overexpression with (+)-JQ1 resulted in the transcriptional suppression of *MYCN,* although the responses were less pronounced than previously observed for *MYC* in some contexts (Fig. S6*C*). Although the range of responses varies, multiple members of the MYC family appear to be vulnerable to BET-bromodomain inhibition, perhaps reflecting a common tran-

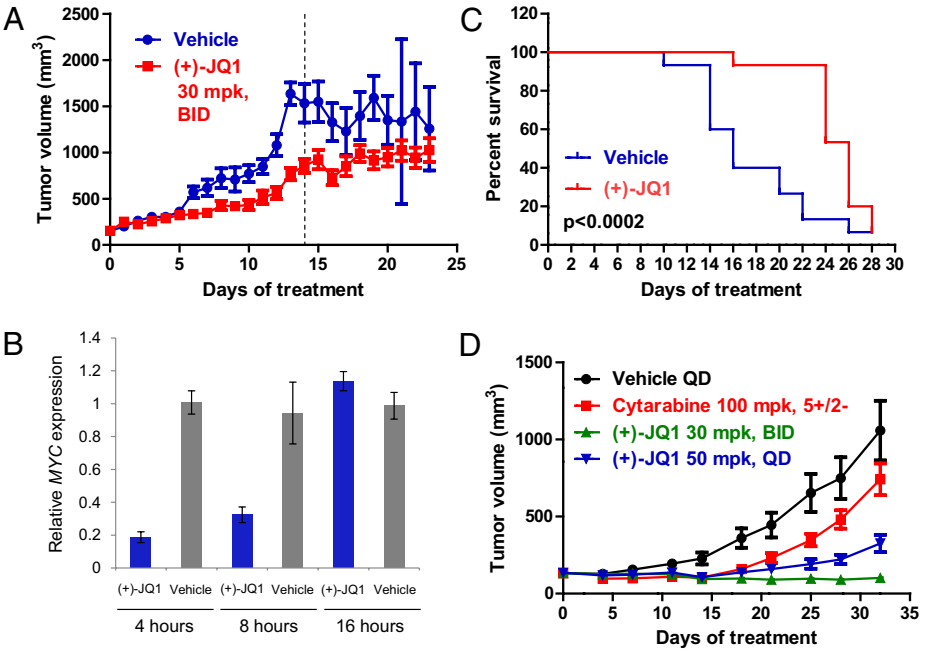

Fig. 5. BET-bromodomain inhibition decreases tumor load in vivo. (*A–C*) Mice bearing Raji xenografts were treated with vehicle or with (+)-JQ1 [30 mg/kg (mpk), twice daily, i.p.] for the indicated times. (*A*) The relative survival of mice in the vehicle group at specific times is indicated under the graph. The dashed line indicates the point in time after which statistical significance cannot be measured accurately because of decreased survival of animals in the control group. Statistical significance for differences in tumor growth rate was determined by using the TTEST method. *P* value at day 14 = 0.0015. (*C*) *MYC* mRNA expression measured by qPCR in s.c. Raji xenografts at the indicated time following a single dose of (+)-JQ1 at 25 mg/kg (mpk). Error bars represent SEM. (*B*) Kaplan-Meier survival plot. Mice (n = 15 in each group) were killed when tumor volume reached 2,000 mm^3. The shown *P* value was calculated by using the Gehan–Breslow–Wilcoxon test. (*D*) Mice bearing MV4-11 xenografts were treated with vehicle or with (+)-JQ1 [30 mg/kg (mpk), twice daily, i.p. or 50 mg/kg (mpk) daily, i.p.] for the indicated time. Statistical significance for difference in tumor growth rate was determined by using the TTEST. *P* value at day 32 were 0.0065 for (+)-JQ1 dosed at 50 mg/kg daily and 0.0008 dosed at 30 mg/kg (mpk) twice a day.

scriptional regulatory mechanism. Considering the importance of *MYCN* in neuroblastoma, the study of BET inhibitors in this cancer subtype warrants further exploration.

Finally, at doses of (+)-JQ1 that suppressed *MYC* in tumor cells, repeated treatment in vivo did not result in weight loss or gross adverse effects in the animals used in these studies. These results are consistent with previous findings (8), perhaps suggesting that normal tissues may be relatively resistant to *MYC* suppression or loss of MYC function, at least during this time course. Future studies examining *MYC* suppression by BET inhibitors in normal regenerative tissues will be critical for defining the therapeutic applicability of these types of small molecule inhibitors, as will the development of compounds optimized for in vivo applications including medical uses. Inhibitors of BET bromodomains open a new avenue for probing the complex MYC-dependent and MYC-independent regulatory pathways in cancer cells and hold promise as a novel therapeutic for human cancer.

Methods

A detailed description of the reagents and protocols used in this study can be found in the *SI Methods*. Specific methods include cell cycle analysis, viability determination, annexin V/PI staining, gene expression profiling, rescue experiments, cell lysate preparation, Western blotting, chromatin immunoprecipitation, quantitative PCR, compound synthesis, and xenograft efficacy studies.

ACKNOWLEDGMENTS. We thank the many Constellation employees in their support of these studies. Special thanks to Kerry Spillane, Alex Taylor, Victor Gehling, Adrianne Neiss, Ted Peters, Christina Lee, and Sara Little for technical support and Mark Goldsmith, Jim Audia, Danny Reinberg, Michael Cooper, Rick Klausner, Jose Lora, Dave Goeddel, Patrick Trojer, Ed Harlow, Sara Little, Hon-Ren Huang, Rich Cummings, Steve Bellon, Brian Albrecht, and J. C. Harmange for helpful discussions and comments on the manuscript.

1. Wu SY, Chiang CM (2007) The double bromodomain-containing chromatin adaptor Brd4 and transcriptional regulation. *J Biol Chem* 282:13141–13145.
2. Sims RJ, 3rd, Belotserkovskaya R, Reinberg D (2004) Elongation by RNA polymerase II: The short and long of it. *Genes Dev* 18:2437–2468.
3. Yang Z, He N, Zhou Q (2008) Brd4 recruits P-TEFb to chromosomes at late mitosis to promote G1 gene expression and cell cycle progression. *Mol Cell Biol* 28:967–976.
4. Dey A, Nishiyama A, Karpova T, McNally J, Ozato K (2009) Brd4 marks select genes on mitotic chromatin and directs postmitotic transcription. *Mol Biol Cell* 20:4899–4909.
5. LeRoy G, Rickards B, Flint SJ (2008) The double bromodomain proteins Brd2 and Brd3 couple histone acetylation to transcription. *Mol Cell* 30:51–60.
6. Miyoshi S, Ooike S, Iwata K, Hikawa H, Sugaraha K (2009) International Patent No. PCT/JP2008/073864 (WO/2009/084693).
7. Nicodeme E, et al. (2010) Suppression of inflammation by a synthetic histone mimic. *Nature* 468:1119–1123.
8. Filippakopoulos P, et al. (2010) Selective inhibition of BET bromodomains. *Nature* 468: 1067–1073.
9. French CA (2010) NUT midline carcinoma. *Cancer Genet Cytogenet* 203:16–20.
10. Baud V, Karin M (2009) Is NF-kappaB a good target for cancer therapy? Hopes and pitfalls. *Nat Rev Drug Discov* 8:33–40.
11. Shaffer AL, et al. (2008) IRF4 addiction in multiple myeloma. *Nature* 454:226–231.
12. Dib A, Gabrea A, Glebov OK, Bergsagel PL, Kuehl WM (2008) Characterization of MYC translocations in multiple myeloma cell lines. *J Natl Cancer Inst Monogr* 25–31.
13. Subramanian A, et al. (2005) Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci USA* 102: 15545–15550.
14. Mootha VK, et al. (2003) PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nat Genet* 34:267–273.
15. Boxer LM, Dang CV (2001) Translocations involving c-myc and c-myc function. *Oncogene* 20:5595–5610.
16. Herold S, et al. (2002) Negative regulation of the mammalian UV response by Myc through association with Miz-1. *Mol Cell* 10:509–521.
17. Seoane J, Le HV, Massagué J (2002) Myc suppression of the p21(Cip1) Cdk inhibitor influences the outcome of the p53 response to DNA damage. *Nature* 419:729–734.
18. Lapalombella R, et al. (2008) A novel Raji-Burkitt's lymphoma model for preclinical and mechanistic evaluation of CD52-targeted immunotherapeutic agents. *Clin Cancer Res* 14:569–578.
19. Vita M, Henriksson M (2006) The Myc oncoprotein as a therapeutic target for human cancer. *Semin Cancer Biol* 16:318–330.
20. Weinstein IB, Joe A (2008) Oncogene addiction. *Cancer Res* 68:3077–3080, discussion 3080.
21. Wolfer A, et al. (2010) MYC regulation of a "poor-prognosis" metastatic cancer cell state. *Proc Natl Acad Sci USA* 107:3698–3703.
22. Eilers M, Eisenman RN (2008) Myc's broad reach. *Genes Dev* 22:2755–2766.
23. Albihn A, Johnsen JI, Henriksson MA (2010) MYC in oncogenesis and as a target for cancer therapies. *Adv Cancer Res* 107:163–224.