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BET bromodomain inhibition as a therapeutic strategy to target c-Myc

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SUMMARY

MYC contributes to the pathogenesis of a majority of human cancers, yet strategies to modulate the function of the c-Myc oncoprotein do not exist. Toward this objective, we have targeted *MYC*

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AUTHOR CONTRIBUTIONS

JEB and CSM designed the study, analyzed data and prepared the manuscript. JED, HMJ, and EK assayed MM drug sensitivity. GCI and JED assessed the effects of JQ1 on Myc expression. JQ performed scaling synthesis and purification of JQ1. PGR and KCA provided primary MM samples. PBR and TG conducted ChIP experiments, and PBR and RAY contributed to their interpretation. RMP, TPH and MRM performed RNA expression analysis. IMG and KCA provided support and interpreted cellular data. ACS and WCH designed and performed shRNA screens. MEL analyzed expression array data. JS and CRV performed Myc rescue experiments. ALK supervised *in vivo* efficacy and biostatistical studies. MC and PLB performed *in vivo* GEMM studies. JEB and CSM supervised the research. All authors edited the manuscript.

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SUPPLEMENTAL INFORMATION

Supplemental information includes Extended Experimental Procedures and seven figures

transcription by interfering with chromatin-dependent signal transduction to RNA polymerase, specifically by inhibiting the acetyl-lysine recognition domains (bromodomains) of putative co-activator proteins implicated in transcriptional initiation and elongation. Using a selective small-molecule bromodomain inhibitor, JQ1, we identify BET bromodomain proteins as regulatory factors for c-Myc. BET inhibition by JQ1 downregulates *MYC* transcription, followed by genome-wide downregulation of Myc-dependent target genes. In experimental models of multiple myeloma, a Myc-dependent hematologic malignancy, JQ1 produces a potent antiproliferative effect associated with cell cycle arrest and cellular senescence. Efficacy of JQ1 in three murine models of multiple myeloma establishes the therapeutic rationale for BET bromodomain inhibition in this disease and other malignancies characterized by pathologic activation of c-Myc.

INTRODUCTION

c-Myc is a master regulatory factor of cell proliferation (Dang et al., 2009). In cancer, pathologic activation of c-Myc plays a central role in disease pathogenesis, by the coordinated upregulation of a transcriptional program influencing cell division, metabolic adaptation and survival (Dang, 2009; Kim et al., 2008). Amplification of *MYC* is among the most common genetic alterations observed in cancer genomes (Beroukhim et al., 2010). Validation of c-Myc as a therapeutic target is supported by numerous lines of experimental evidence. Murine models of diverse malignancies have been devised by introducing genetic constructs overexpressing *MYC* (Harris et al., 1988; Leder et al., 1986; Stewart et al., 1984). In addition, conditional transgenic models featuring tunable transcriptional suppression have shown that even transient inactivation of *MYC* is capable of promoting tumor regression (Jain et al., 2002; Soucek et al., 1998; Soucek et al., 2002). Elegant studies of systemic induction of a dominant-negative Myc allele within an aggressive, *KRAS*-dependent murine model of lung adenocarcinoma have further suggested the putative therapeutic benefit of c-Myc inhibition (Fukazawa et al., 2010). Importantly these studies establish the feasibility of c-Myc inhibition within an acceptable therapeutic window of tolerability.

Nevertheless, a therapeutic approach to target c-Myc has remained elusive. The absence of a clear ligand-binding domain establishes a formidable obstacle toward direct inhibition, which is a challenging feature shared among many compelling transcriptional targets in cancer (Darnell, 2002). c-Myc functions as a DNA-binding transcriptional activator upon heterodimerization with another basic helix-loop-helix leucine zipper (bHLH-LZ) transcription factor, Max (Amati et al., 1993; Blackwood and Eisenman, 1991). High-resolution structures of the complex fail to identify a hydrophobic involution compatible with the positioning of an organic small molecule (Nair and Burley, 2003).

Therefore, we have targeted c-Myc transcriptional function by another means, namely the disruption of chromatin-dependent signal transduction (Schreiber and Bernstein, 2002). c-Myc transcription is associated locally and globally with increases in histone lysine side-chain acetylation, a covalent modification of chromatin regionally associated with transcriptional activation (Frank et al., 2003; Vervoorts et al., 2003). Histone acetylation templates the assembly of higher-ordered transcriptional complexes by recruiting proteins with one or more acetyl-lysine binding modules or bromodomains (Dhalluin et al., 1999;

Haynes et al., 1992). Members of the bromodomain and extra-terminal (BET) subfamily of human bromodomain proteins (BRD2, BRD3 and BRD4) associate with acetylated chromatin and facilitate transcriptional activation by increasing the effective molarity of recruited transcriptional activators (Rahman et al.). Notably, BRD4 has been shown to mark select M/G1 genes in mitotic chromatin as transcriptional memory and direct post-mitotic transcription (Dey et al., 2009), via direct interaction with the positive transcription elongation factor complex b (P-TEFb) (Bisgrove et al., 2007). The discovery that c-Myc regulates promoter-proximal pause release of Pol II, also through the recruitment of P-TEFb (Rahl et al., 2010), established a rationale for targeting BET bromodomains to inhibit c-Myc-dependent transcription.

Recently, we reported the development and biochemical characterization of a first potent, selective small-molecule inhibitor of BET bromodomains, JQ1 (Figure 1A) (Filippakopoulos et al., 2010). JQ1 is a thieno-triazolo-1,4-diazepine which displaces BET bromodomains from chromatin by competitively binding to the acetyl-lysine recognition pocket. In the present study, we leverage the properties of JQ1 as a chemical probe (Frye, 2011), to interrogate the role of BET bromodomains in Myc-dependent transcription and to explore the role of BET bromodomains as cancer dependencies.

Multiple myeloma (MM) represents an ideal model system for these mechanistic and translational questions given the known role of *MYC* in disease pathophysiology. MM is an incurable hematologic malignancy, typified by the accumulation of malignant plasma cells harboring diverse genetic lesions (Chapman et al., 2011). Dysregulation of transcription factors feature prominently in the biology of MM, including NF-κB (Keats et al., 2007), c-Maf (Hurt et al., 2004), XBP1 (Claudio et al., 2002), HSF1 (Mitsiades et al., 2002), GR (Gomi et al., 1990), IRF4 (Shaffer et al., 2008), Myb (Palumbo et al., 1989), and notably c-Myc (Dean et al., 1983). Rearrangement or translocation of *MYC* is among the most common somatic events in early and late stage MM (Shou et al., 2000), and transcriptional profiling identifies Myc pathway activation in more than 60% of patient-derived MM cells (Chng et al., 2011). Experimental support for the central role of c-Myc in the pathogenesis of MM is contributed by an informative, genetically-engineered murine model of MM. Lineage-specific and stochastic Activation-Induced Deaminase (AID)-dependent activation of a conditional *MYC* transgene in the late stages of B-cell differentiation establishes genetically-engineered mice with a plasma cell malignancy that shares clinically relevant features of MM (Chesi et al., 2008). Thus, *MYC* dysregulation represents a largely unifying molecular feature observed across the otherwise complex genetic landscape of MM.

In this study, we report that c-Myc transcriptional function can be modulated pharmacologically by BET bromodomain inhibition. Unexpectedly, we have discovered that *MYC*, itself, is transcriptionally regulated by BET bromodomains. Chromatin immunopreciptitation studies show that BRD4 is strongly enriched at immunoglobulin heavy chain (IgH) enhancers in MM cells bearing IgH rearrangement at the *MYC* locus. BET inhibition with JQ1 depletes enhancer-bound BRD4 and promptly inhibits *MYC* transcription in a dose- and time-dependent manner. In translational models of MM, JQ1 leads to depletion of the c-Myc oncoprotein and selective downregulation of the coordinated c-Myc transcriptional program, prompting cell cycle arrest and cellular senescence. These

results indicate that targeting protein-protein interactions within the c-Myc transcriptional signaling network can modulate the function of c-Myc in cancer.

RESULTS

BET bromodomains as therapeutic targets in MM

We first evaluated the expression of *BRD2, BRD3, BRD4* and *BRDT* transcripts in MM, by integrating publicly-available compendia of gene expression datasets. Among asymptomatic patients with pre-malignant disease (Zhan et al., 2007), we observed increasing expression of *BRD4* in monoclonal gammopathy of undetermined significance (MGUS) and smoldering MM (SMM) compared to normal bone marrow plasma cells (Figure 1B). In a second, independent dataset (Mattioli et al., 2005), we observed significantly higher expression of *BRD4* in plasma cell leukemia (PCL) compared to MM or MGUS samples (Figure 1C). Thus, *BRD4* expression correlates positively with disease progression. *BRD2* and *BRD3* are also expressed in MM, but expression does not clearly correlate with stage of disease (data not shown). *BRDT*, a testis-specific bromodomain-containing protein, is not expressed in MM.

Analysis of copy number polymorphism (CNP) data collected on 254 MM patients by the Multiple Myeloma Research Consortium (MMRC) revealed that the *BRD4* locus is frequently amplified in MM patient samples (Figure 1D). The majority of patient samples exhibit broad amplification of chromosome 19p, but focal amplification at the *BRD4* locus is observed (Figure S1). Among 45 established MM cell lines, expression of BRD4 was pronounced and did not correlate with amplification status (Figure 1E). Expression of BRD4 was pronounced across samples with or without *BRD4* amplification (Figure 1E). Human MM cells are highly osteotropic *in vivo*, and interaction with bone marrow stromal cells (BMSCs) induces proliferation and contributes to drug resistance (McMillin et al., 2010). Analysis of BET bromodomain expression as influenced by MM cell binding to BMSCs (McMillin et al., 2010), revealed marked upregulation of BRD4 in the INA-6 human MM cell line upon interaction with HS5 stromal cells (Figure 1F), suggesting a plausible role for BRD4 function in MM cells within the bone marrow microenvironment.

To explore the function of BET bromodomains in MM, we examined the effect on proliferation of small hairpin RNAs (shRNAs) targeting each of the four BET proteins in comparison to shRNAs targeting 1011 kinases, phosphatases and oncogenes in a lentivirally-delivered, arrayed shRNA screen in INA-6 cells. As illustrated in Figure 1G-H, shRNA constructs targeting each of the expressed BET bromodomains are identified as reducing INA-6 proliferation as shown by normalized B-scores (Malo et al., 2006). Together, these data establish a rationale for the study of BET bromodomains, and BRD4 in particular, as tumor dependencies in MM.

BET inhibition with JQ1 arrests c-Myc transcriptional programs

To test the hypothesis that BET inhibition will specifically abrogate Myc-dependent transcription, we utilized global transcriptional profiling and unbiased gene set enrichment analysis (GSEA). We first characterized the transcriptional consequences of BET inhibition

in three MM cell lines with genetically distinct activating lesions at the *MYC* locus (KMS11, MM.1S and OPM1) (Dib et al., 2008). Unsupervised hierarchical clustering segregated samples based on treatment assignment suggesting a common transcriptional consequence in response to JQ1 (Figure 2A). Acute JQ1 treatment did not prompt global, non-specific transcriptional silencing, but instead produced significant changes in a finite number of genes (88 down- and 25 up-regulated genes by two-fold or greater in all three MM lines).

To examine higher-order influences over biological networks regulated by c-Myc, we evaluated four canonical transcriptional signatures of *MYC*-dependent genes (Kim et al., 2006; Schlosser et al., 2005; Schuhmacher et al., 2001; Zeller et al., 2003). All four signatures were strongly correlated with downregulation of expression by JQ1 (Figure 2B). As a measure of the specificity of this effect, an open-ended enrichment analysis was performed on the entire set of transcription factor target gene signatures available from the Molecular Signatures Database (MSigDB). Gene sets defined by adjacency to Myc-binding motifs were in almost all cases significantly enriched in JQ1-suppressed genes (Figure 2C, D). In marked contrast, JQ1 treatment did not exert significant suppression of gene sets for other transcription factors linked to pathophysiology of MM, including NF-κB, AP-1, STAT3, GR and XBP-1 (Figure 2E, S2). Notably, 27 of the 28 significantly correlated gene sets are annotated as predicted targets of MYC or E2F (Figures 2C, S2). Consistent with Myc-specific inhibition, biological modules associated with Myc (e.g. ribosomal biogenesis and assembly, and glycolysis) were also anti-correlated with JQ1 treatment (Figure 2E). BET bromodomain inhibition by JQ1 confers a selective repression of transcriptional networks induced by c-Myc.

Regulation of *MYC* transcription by BET bromodomains

An unexpected finding was the observed, robust inhibition of *MYC* expression following treatment with JQ1 (Figure 2A). As *MYC* is commonly activated by upstream oncogenic signaling pathways, we studied the consequence of JQ1 treatment on the expression of 230 cancer-related genes in a human MM cell line (MM.1S) using a multiplexed transcript detection assay (Figure 3A). Excellent concordance was observed between replicate measurements of expressed genes (Figure S3A). Unsupervised hierarchical clustering segregated replicate data correctly into early and late treatment time-points. Surprisingly, we observed immediate, progressive and profound downregulation of *MYC* transcription, itself, a unique finding among all transcripts studied ($p < 0.05$).

Downregulation of *MYC* was further confirmed by RT-PCR and immunoblot (Figure 3B, C). This effect was BET bromodomain-specific, supported by the nearly comparable activity of an analogous BET inhibitor subsequently published by Glaxo SmithKline (iBET; Figure 1A) (Nicodeme et al., 2010)), and the lack of activity of the inactive (-)-JQ1 enantiomer, which we previously characterized as structurally incapable of inhibiting BET bromodomains (Filippakopoulos et al., 2010) (Figure 3C). Inhibition of *MYC* transcription by JQ1 was observed to be dose- and time-dependent, with peak inhibition at sub-micromolar concentrations (Figure 3D, E). Rapid depletion of chromatin-bound c-Myc was confirmed by nuclear ELISA transcription factor binding assays (Figure 3F). In contrast,

NF-κB and AP-1 chromatin binding assays failed to reveal any decrease in DNA binding within 8 hours of JQ1 treatment (Figure 3G, S4A).

To assess the breadth of these findings in MM, we expanded gene expression studies to three MM cells with distinct lesions at the *MYC* locus. MM.1S cells have a complex *MYC* rearrangement involving an IgH insertion at the breakpoint of a derivative chromosome der3t(3;8), KMS-11 cells have both *MYC* duplication and inversion, and OPM1 cells feature a der(8)t(1;8) (Dib et al., 2008). Among 230 genes studied, *MYC* was one of only four genes downregulated by treatment with JQ1, along with *MYB, TYRO3* and *TERT* (Figure 3H and S4B). Immunoblotting analyses confirmed the JQ1 suppression of c-Myc protein expression in a further expanded panel of Myc-dependent MM cell lines (Figure 3I). Despite the intriguing potential effect on E2F transcriptional function and *MYB* gene expression, JQ1 did not influence E2F or MYB protein abundance through 24 hours of drug exposure (Figure S4C, D). Together, these data support the general observation that BET inhibition specifically suppresses *MYC* transcription across MM cells with different genetic lesions affecting the *MYC* locus, and with striking selectivity in comparison to other oncogenic transcriptions factors with established roles in MM pathophysiology.

BRD4 binds IgH enhancers, regulating *MYC* expression and function

Based on the integrated, functional genomic analysis of BET bromodomains in MM (Figure 1), we pursued further mechanistic studies of BRD4. Silencing of *BRD4* using directed shRNAs validated by RT-PCR analysis (Figure 4A), elicited a marked decrease in *MYC* transcription (Figure 4B) accompanied by G1 cell cycle arrest in JQ1-sensitive MM cells (OPM-1; Figure 4B, S5A). We reasoned that early and sustained JQ1-induced suppression of *MYC* transcription may be mechanistically explained by physical interaction of BRD4 with regulatory elements influencing *MYC* expression. Indeed, avid binding of BRD4 to established IgH enhancers was observed by chromatin immunoprecipitation (ChIP) in MM. 1S cells (Figure 4C, S6), which harbor an IgH insertion proximal to the *MYC* transcriptional start site (TSS). BRD4 binding was not observed at five characterized enhancer regions adjacent to the *MYC* gene (Pomerantz et al., 2009a; Pomerantz et al., 2009b). JQ1 treatment (500 nM) for 24 hours significantly depleted BRD4 binding to IgH enhancers and the TSS, supporting direct regulation of *MYC* transcription by BET bromodomains, and a model whereby BRD4 acts as a co-activator of *MYC* transcription potentially through long-range interactions with distal enhancer elements. Forced overexpression of c-Myc in MM cells (OPM1) by retroviral infection rescues, in part, the cell cycle arrest observed with JQ1 treatment (Figure 4D, E), arguing that *MYC* down-regulation by JQ1 contributes functionally to cell physiology in MM.

Therapeutic implications of BET inhibition in MM

Based on this mechanistic rationale, we evaluated the therapeutic opportunity of *MYC* transcriptional inhibition using established translational models of MM. Antiproliferative activity of JQ1 was assessed using a panel of 25 MM cell lines or isogenic derivative lines (Figure 5A). MM cell proliferation was uniformly inhibited by JQ1 (Figure 5A), including several MM cell lines selected for resistance to FDA-approved agents (dexamethasone-resistant MM.1R and melaphalan-resistant LR5). As expected, MM cells possessing diverse

genetic lesions involving *MYC* (Dib et al., 2008), were comparably sensitive to JQ1 (Figure 5B).

As interaction of MM cells with BMSCs is widely recognized to confer resistance to numerous therapeutic agents (Hideshima et al., 2007; McMillin et al., 2010), we sought to characterize the effect of BMSCs on MM cell sensitivity to BET inhibition. Using compartment specific bioluminescence imaging assays (CS-BLI), we observed that the sensitivity of MM cell lines to JQ1 is largely unchanged by the presence of HS-5 bone marrow stroma cells (Figure 5C). This pattern of broad activity in MM without evident stroma-mediated chemoresistance has been associated with efficacy of FDA-approved agents bortezomib and lenalidomide.

MM cells were then further phenotyped for Myc-specific biological effects of BET inhibition. Flow cytometry of JQ1-treated MM.1S cells revealed a pronounced decrease in the proportion of cells in S-phase, with a concomitant increase in cells arrested in G0/G1 (Figure 6A). Only a modest induction of apoptosis was observed after 48 hours of JQ1 treatment (Figure 6B), in contrast to the non-selective cytotoxic kinase inhibitor, staurosporine (Figure S5B). Transcripts previously associated with induction of cellular senescence were enriched following treatment with JQ1, by GSEA (Figure 6C). Experimentally, treatment with JQ1 resulted in pronounced cellular senescence by beta-galactosidase staining (Figure 6D). Overall, these phenotypes of arrested proliferation, G1 cell cycle arrest and cellular senescence are highly specific to anticipated effects of inhibiting cellular c-Myc function (Wu et al., 2007).

We next extended the study of JQ1 in MM cells to primary MM samples. JQ1 exposure led to a significant reduction in cell viability among the majority of CD138+ patient-derived MM samples tested (Figure 7A). In primary cells isolated from a patient with relapsed/refractory MM, JQ1 treatment *ex vivo* conferred a time-dependent suppression of c-Myc expression (Figure 7B). In contrast, JQ1 treatment of phytohemaglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMCs) suppressed PHA-induced proliferation, but did not adversely influence cell viability, indicating that the anti-MM effect of JQ1 is not accompanied by a non-specific, toxic effect on all hematopoietic cells (Figure S7A). To model the therapeutic effect of JQ1 *in vivo*, we evaluated anti-MM efficacy in multiple orthotopic models of advanced disease. First, JQ1 was studied using an established, bioluminescent MM model (MM.1S-luc), which recapitulates the clinical sequelae, anatomic distribution of MM lesions, and hallmark bone pathophysiology observed in MM patients (Mitsiades et al., 2004). Tumor-bearing mice were treated with JQ1 administered by intraperitoneal injection (50 mg/kg daily) or vehicle control. JQ1 treatment significantly decreased the burden of disease measured by serial, whole-body, non-invasive bioluminescence imaging (Figure 7C-D). Importantly, treatment with JQ1 resulted in a significant prolongation in overall survival compared to vehicle-treated animals (Figure 7E). In a second, plasmacytoma xenograft which more accurately models extramedullary disease, JQ1 also exhibited a significant disease-modifying response (Figure S7B). Finally, the effect of JQ1 was explored in an aggressive, genetically-engineered model of Myc-dependent MM (Chesi et al., 2008). To date, two animals with advanced disease have completed 14 days of JQ1 treatment (25 mg/kg daily, adjusted to tolerability). Both animals reveal objective

evidence of response by measurement of monotypic serum immunoglobulins, including a complete response (CR) in the second animal (Figure 7F, S7C). In this orthotopic model, only the FDA-approved agent, bortezomib, has previously prompted a CR (M.C. and P.L.B., unpublished data). These results establish *in vivo* proof-of-concept supporting the investigational study of BET bromodomain inhibitors in MM.

DISCUSSION

Despite the centrality of Myc in the pathogenesis of cancer, conventional approaches toward direct Myc inhibition have not proven successful. To date, efforts to target c-Myc have identified only a small number of molecules with low biochemical potency and limited biological characterization (Bidwell et al., 2009; Hammoudeh et al., 2009; Jeong et al., 2010), underscoring both the challenge of targeting c-Myc as well as the enduring need for chemical probes of c-Myc transcriptional function. Considering chromatin as a platform for signal transduction (Schreiber and Bernstein, 2002), we have undertaken to inhibit Myc transcription and function through displacement of chromatin-bound, co-activator proteins using competitive small molecules. Using a first-in-class, small-molecule bromodomain inhibitor developed by our laboratories, JQ1, we validate BET bromodomains as determinants of c-Myc transcription and as therapeutic targets in MM, an ideal model system for the mechanistic and translational study of Myc pathway inhibitors.

Most importantly, we illustrate the feasibility of selectively downregulating transcription of *MYC*, itself, via the molecular action of a selective, small molecule. The ensuing suppression of c-Myc protein levels, depletion of chromatin-bound c-Myc and concomitant downregulation of the Myc-dependent transcriptional network, lead to growth-inhibitory effects sharing the specificity of phenotypes associated with prior genetic models of Myc inhibition. This is a notable observation, which distinguishes the transcriptional consequences of BET inhibition from other, non-selective transcriptional inhibitors, such as actinomycin D, alpha-amanitin and flavopiridol.

A compelling finding is the observed, direct interaction of BRD4 with IgH enhancers in MM cells possessing IgH rearrangement into the *MYC* locus, and the depletion of BRD4 binding by JQ1. This suggests BET inhibition as a putative strategy for targeting other structural rearrangements in cancer involving IgH or other strong enhancers, and has potential implications for the modulation of immunoglobulin gene expression in autoimmune diseases. An unexpected finding was the pronounced and concordant suppression of multiple E2F-dependent transcriptional signatures. In this instance, *E2F1* protein and transcript levels were not affected by BET inhibition, suggesting either an unrecognized function of BET bromodomains in E2F transcriptional complexes or a dominant effect of Myc downregulation causing cell cycle arrest in G1 leading to silencing of E2F. These observations are also compatible with the known role of Myc and E2F1 as transcriptional collaborators in cell cycle progression and tumor cell survival (Matsumura et al., 2003; Trimarchi and Lees, 2002).

Insights provided by our study identify rational strategies for combination therapeutic approaches warranting exploration in MM. *MYC* activation is commonly accompanied by

anti-apoptotic signaling in human cancer. In MM, constitutive or microenvironment-inducible activation of anti-apoptotic Bcl-2 proteins has been reported (Harada et al., 1998; Legartova et al., 2009). Thus, Myc pathway inhibition by JQ1 may demonstrate synergism with targeted pro-apoptotic agents (e.g. ABT-737) (Oltersdorf et al., 2005; Trudel et al., 2007). Additionally, the selective effect of JQ1 on Myc and E2F1 transcriptional programs provides an opportunity to combine BET inhibitors with pathway-directed antagonists of the NF-κB, STAT3, XBP1 or HSF1 transcriptional programs.

Direct inhibition of *MYC* remains a central challenge in the discipline of ligand discovery. Inhibition of *MYC* expression and function, demonstrated herein, presents an immediate opportunity to study and translate the concept of *MYC* inhibition more broadly in human cancer. During the course of this research, a collaborative effort with the laboratories of Christopher Vakoc and Scott Lowe revealed *BRD4* as a tumor dependency in acute myeloid leukemia. Consistent with our observations described here in MM, leukemia cells similarly require BRD4 to sustain *MYC* expression to enforce aberrant self-renewal (Zuber et al.). Collectively, these findings highlight a broad role for BRD4 in maintaining *MYC* expression in diverse hematopoietic malignancies and suggest the utility of drug-like BET bromodomain inhibitors as novel therapeutic agents in these diseases.

EXPERIMENTAL PROCEDURES

Gene expression analysis

MM cells treated with JQ1 (500 nM, 24 h) were processed for transcriptional profiling using Affymetrix Human Gene 1.0 ST microarrays. Expression of individual genes was assessed in the context of dose- and time-ranging experiments by real-time quantitative polymerase chain reaction, multiplexed direct detection (Nanostring) and immunoblotting using antibodies as described in Extended Experimental Procedures.

Chromatin immunoprecipitation

ChIP was performed on MM.1S cells cultured in the presence or absence of JQ1 (500 nM, 24 hours). Specific antibodies, detailed methods, primer sequences for MYC and IgH enhancers, as well as the MYC TSS, are described in Extended Experimental Procedures.

In vitro and *in vivo* MM studies

The impact of JQ1 on cell viability, proliferation and cell cycle was assessed in human MM cells as documented in Extended Experimental Procedures. *In vivo* efficacy studies were performed with protocols approved by Institutional Animal Care and Use Committees at the DFCI or Mayo Clinic Arizona. JQ1 was administered by intraperitoneal injection into SCID-beige mice with MM lesions established after subcutaneous or intravenous injections; and in non-immunocompromised tumor-bearing Vk*myc mice. Tumor burden in these models was quantified by caliper measurement, whole-body bioluminescence imaging and serum protein electrophoresis, respectively, as detailed in Extended Experimental Procedures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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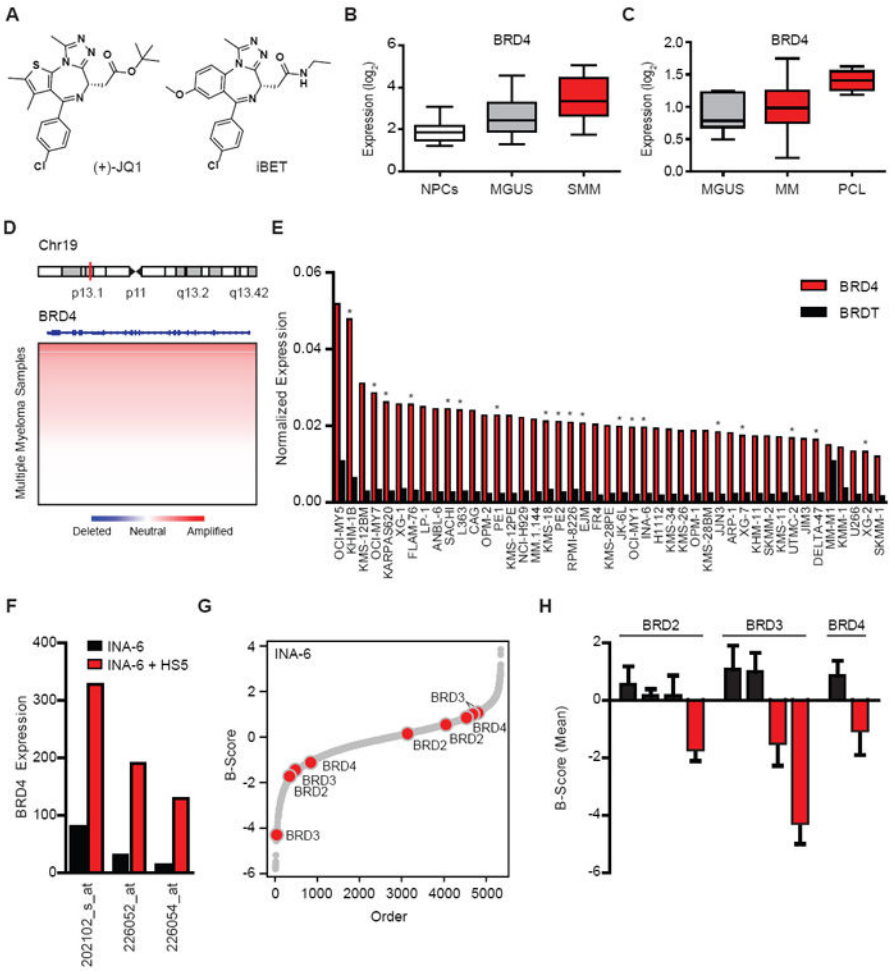


Figure 1. Integrated genomic rationale for BET bromodomains as therapeutic targets in MM

(A) Structures of the BET bromodomain inhibitors JQ1 and iBET.

(B,C) Expression levels (log2 transformed, median-centered values) for *BRD4* transcripts were evaluated in oligonucleotide microarray data from normal plasma cells (NPCs) from healthy donors, individuals with MGUS or SMM patients (panel B, dataset GSE5900, (Zhan et al., 2007)); and in plasma cells from MGUS, MM and PCL patients (panel C, dataset GSE2113 (Mattioli et al., 2005)). Increased *BRD4* expression is observed in SMM (or MGUS) compared to NPCs (panel B) and in PCL compared to MM (panel C) (non-parametric Kruskal-Wallis one-way analysis of variance $p < 0.001$ and $p=0.0123$, respectively; Dunn's Multiple Comparison post-hoc tests $p<0.05$, in both cases).

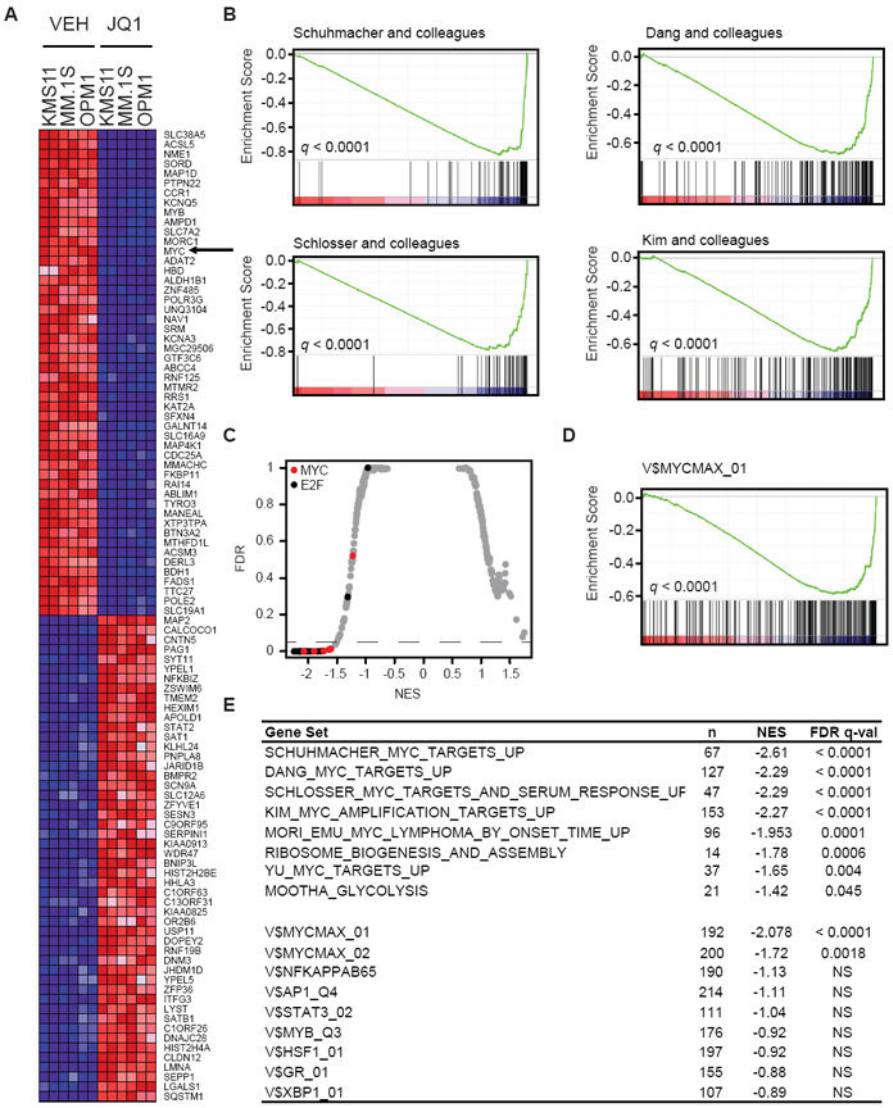
(D) Copy number analysis of the *BRD4* locus at human chromosome 19p13.1 in primary samples from 254 MM patients. Chromosome 19p amplifications are common in MM (Figure S1).

(E) Expression levels of *BRD4* (compared to *BRDT*) in human MM cell lines. Asterisks denote cell lines with amplification of the *BRD4* locus (19p13.1).

(F) *BRD4* expression (depicted on a linear scale for three different oligonucleotide microarray probes) in INA-6 MM cells cultured *in vitro* in the presence or absence of HS-5 bone marrow stromal cells.

(G) Silencing of BET bromodomains impairs proliferation in MM cells. Results of an arrayed lentiviral screen using a diverse shRNA library in INA-6 MM cells are presented in rank order of ascending B-Scores. The effect of shRNAs targeting BET bromodomains on INA-6 cell viability is highlighted by red circles and annotated by gene. Gray dots represent results for non-BET bromodomain shRNAs.

(H) Silencing of BET bromodomain family members in MM cells. Viability of INA-6 MM cells exposed to shRNAs directed against BRD2, BRD3 and BRD4 are reported as mean B-scores (±SD of the two normalized replicates).



Gene Set	n	NES	FDR q-val
SCHUHMACHER_MYC_TARGETS_UP	67	-2.61	< 0.0001
DANG_MYC_TARGETS_UP	127	-2.29	< 0.0001
SCHLOSSER_MYC_TARGETS_AND_SERUM_RESPONSE_UP	47	-2.29	< 0.0001
KIM_MYC_AMPLIFICATION_TARGETS_UP	153	-2.27	< 0.0001
MORI_EMU_MYC_LYMPHOMA_BY_ONSET_TIME_UP	96	-1.953	0.0001
RIBOSOME_BIOGENESIS_AND_ASSEMBLY	14	-1.78	0.0006
YU_MYC_TARGETS_UP	37	-1.65	0.004
MOOTHA_GLYCOLYSIS	21	-1.42	0.045
V$MYCMAX_01	192	-2.078	< 0.0001
V$MYCMAX_02	200	-1.72	0.0018
V$NFKAPPAB65	190	-1.13	NS
V$AP1_Q4	214	-1.11	NS
V$STAT3_02	111	-1.04	NS
V$MYB_Q3	176	-0.92	NS
V$HSF1_01	197	-0.92	NS
V$GR_01	155	-0.88	NS
V$XBP1_01	107	-0.89	NS

Figure 2. Inhibition of Myc-dependent transcription by the JQ1 BET bromodomain inhibitor

(A) Heat map representation of the top 50 down- and top 50 up-regulated genes (P < 0.001) following JQ1 treatment in MM cell lines. Data are presented row-normalized (range from -3- to +3-fold change in expression). *MYC* (arrow) is downregulated by JQ1 treatment.

(B) GSEA of four Myc-dependent gene sets (Kim et al., 2006; Schlosser et al., 2005; Schuhmacher et al., 2001; Zeller et al., 2003), in transcriptional profiles of MM cells treated (left) or untreated (right) with JQ1.

(C) Quantitative comparison of all transcription factor target gene sets available from the MSigDB by GSEA for reduced expression in JQ1-treated MM cell lines. Data are presented as scatterplot of false discovery rate (FDR) versus normalized enrichment score (NES) for each evaluated gene set. Colored dots indicate gene sets for MYC (red), E2F (black), or other (gray) transcription factors.

(D) GSEA showing downregulation, in JQ1-treated MM cells, of a representative set of genes with proximal promoter regions containing Myc-Max binding sites.

(E) Table of gene sets enriched among genes downregulated by JQ1 in MM cells (top group), highlighting the number of genes in each set (n), the normalized enrichment score (NES) and test of statistical significance (FDR q-value). The bottom group represents comparisons of top-ranking transcription factor target gene sets of MM master regulatory proteins, enriched among genes downregulated by JQ1 in MM cells.

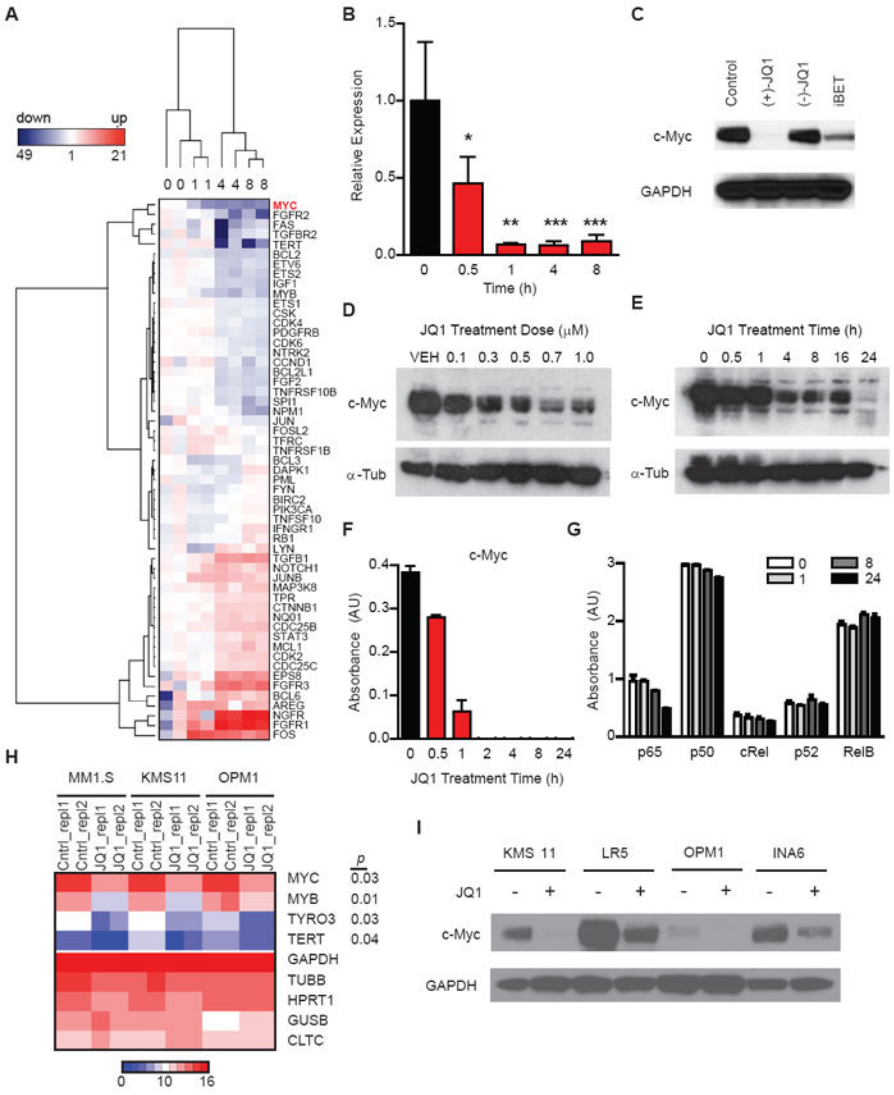


Figure 3. BET inhibition suppresses *MYC* transcription in MM

(A) Heatmap of cancer-related genes expressed in MM cells (MM.1S), treated with JQ1 (500 nM over 1, 4 and 8 h). *MYC* (red) is downregulated by JQ1 in a time-dependent manner, uniquely among all oncogenes studied (230 total). MYC was identified as the only statistically significant decrease in transcription at all four time-points analyzed ($p < 0.05$).

(B) Quantitative RT-PCR analysis for *MYC* levels in JQ1-treated MM.1S cells (500 nM, 0 - 8 h). Data are presented as ratio of *MYC* expression at each time-point compared to baseline *MYC* expression. Asterisks denote the level of statistical significance (* $p < 0.01$, ** $p < 0.0002$, *** $p < 0.006$; paired Student's t-test each relative to t = 0 h).

(C) The active JQ1 enantiomer and the structurally analogous BET inhibitor, iBET (Nicodeme et al., 2010), but not the inactive (-)-JQ1 enantiomer, downregulate c-Myc expression, as determined by immunoblotting of MM.1S cells treated with compounds (500 nM) or vehicle control for 24 h.

(D-E) Immunoblotting analyses of the (D) dose- and (E) time-dependent effects of JQ1 treatment on c-Myc expression in MM.1S cells.

(F-G) Selective depletion of nuclear c-Myc following JQ1 treatment (500 nM) as measured by ELISA-based DNA binding assays for the activity of (F) c-Myc (depleted after 1-2 h) and (G) NF-κB family members (unaffected). Data represent mean ± SEM.

(H) Heatmap of clustered gene expression data from multiplexed measurement (Nanostring) of cancer-associated genes in three human MM cell lines treated with JQ1 or vehicle control. Among 230 genes studied (Figure S4), four genes (*MYC, TERT, TYRO3* and *MYB*) exhibited statistically significant ($p<0.05$) downregulation. Replicate expression measurements exhibited high concordance among low and highly expressed genes (Figure S3B).

(I) Immunoblotting study of four MM lines (KMS11, LR5, OPM1, and INA-6) identifies a JQ1-induced decrease in c-Myc expression (500 nM, 24 h).

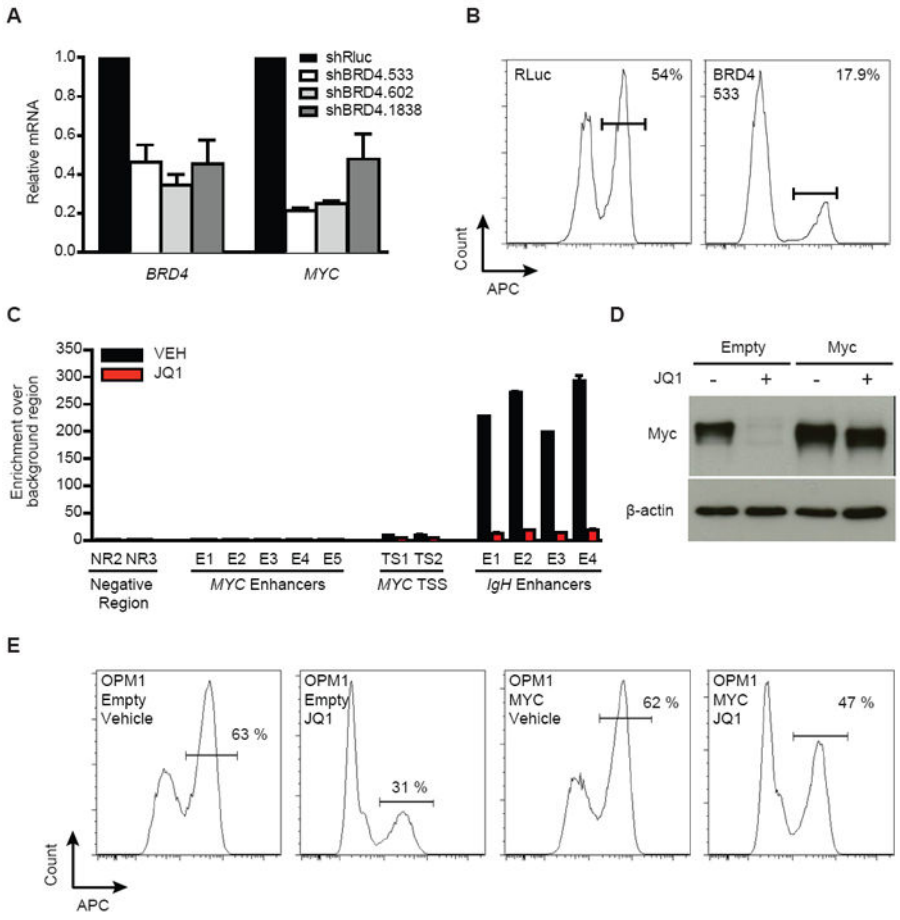


Figure 4. Regulation of *MYC* transcription by BET bromodomains

(A) *BRD4* and *MYC* expression in OPM1 cells transduced with either shBRD4 (three different hairpins) or a Renilla control hairpin (shRluc). shRNAs were induced for five days before analysis by qRT-PCR. Data were normalized to GAPDH.

(B). Cell cycle analysis of OPM1 cells transduced with the indicated shRNA for six days. BrdU staining (APC) identifies the fraction of cells in S-phase.

(C) ChIP studiesof BRD4 (anti-BRD4; Bethyl) binding to *MYC* TSS or proximal enhancers in MM.1S cells. Competitive displacement of BRD4 from IgH enhancers is observed upon treatment with JQ1 (500nM for 24 h; red bars) compared to vehicle control (black bars).

(D) Immunolotting of whole-cell lysates from empty MSCV vector- or Myc overexpression vector-transduced OPM1 cells after treating with JQ1 (500nM, 24h) or DMSO control.

(E) Cell cycle analysis of either empty or Myc over-expressing OPM1 cells treated with JQ1 (500nM, 24h). BrdU staining (APC) identifies the fraction of cells in S-phase.

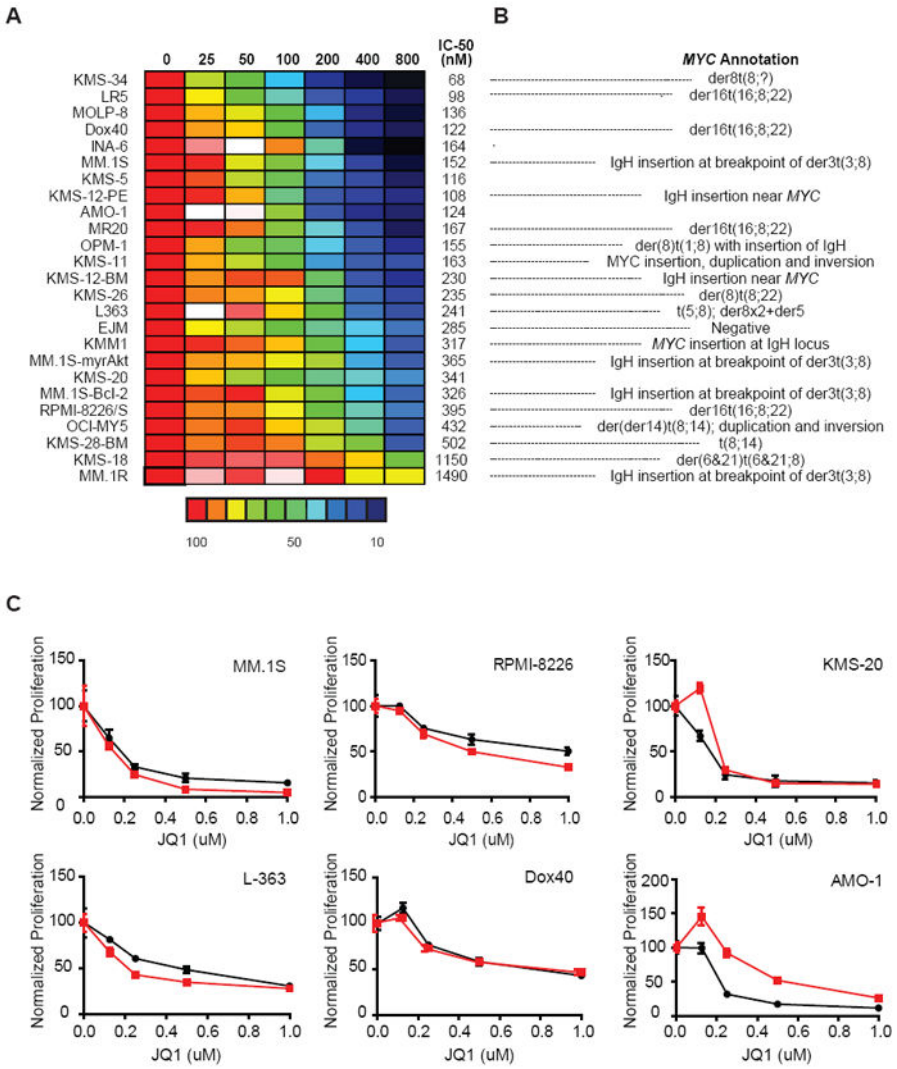


Figure 5. Anti-myeloma activity of JQ1 *in vitro*

(A) A panel of MM cell lines was tested for *in vitro* sensitivity to JQ1 (12.5 - 800 nM, 72h), by measurement of ATP levels (Cell TiterGlo; Promega).

(B) *MYC* genomic status of selected MM cell lines from panel A, as annotated (Dib et al., 2008).

(C) Activity of JQ1 against MM cell lines cultured in the presence (red lines) or absence (black lines) of the HS-5 stromal cell line, assessed by CS-BLI (McMillin et al., 2010).

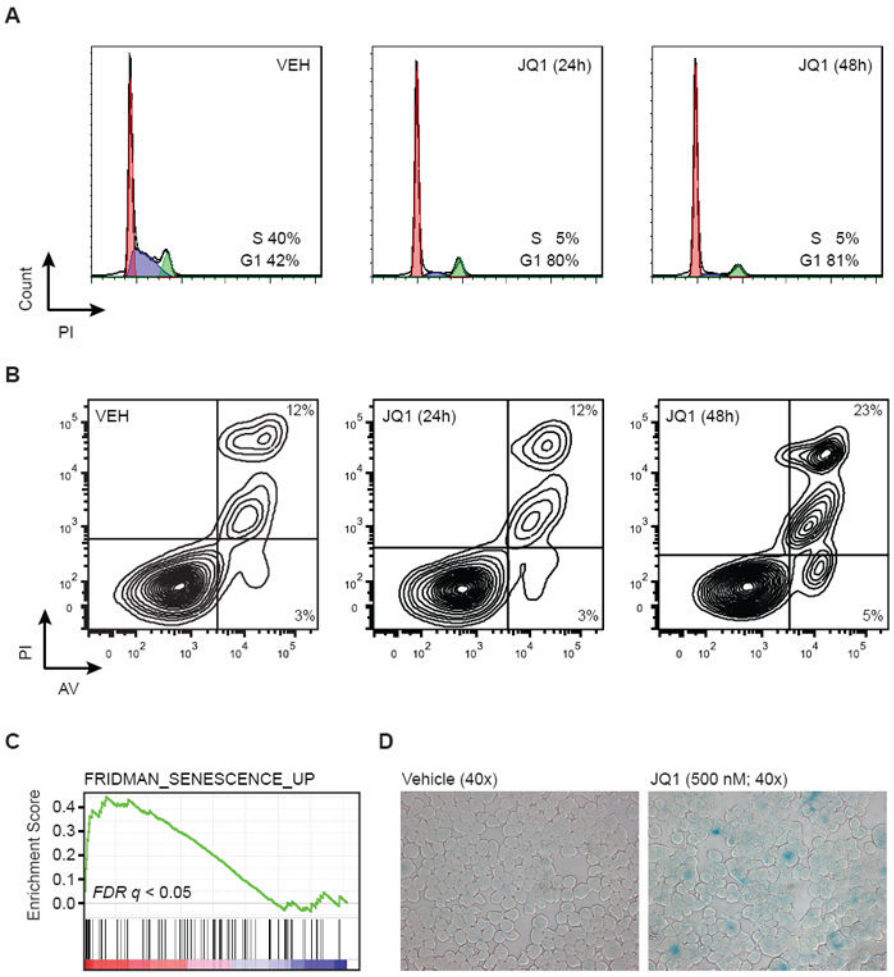


Figure 6. JQ1 induces cell cycle arrest and cellular senescence in MM cells

(A-B) Flow cytometric evaluation of propidium iodide (PI) staining for cell cycle analysis (panel A) and detection of Annexin V-positive apoptotic cells (panel B) in JQ1-treated MM.1S cells (0 - 48hrs, 500 nM).

(C) Enrichment of senescence-associated genes among JQ1-suppressed genes in MM.1S cells.

(D) Induction of cellular senescence in JQ1 treated MM.1S cells (500 nM, 72 h), as detected by ß-galactosidase staining.

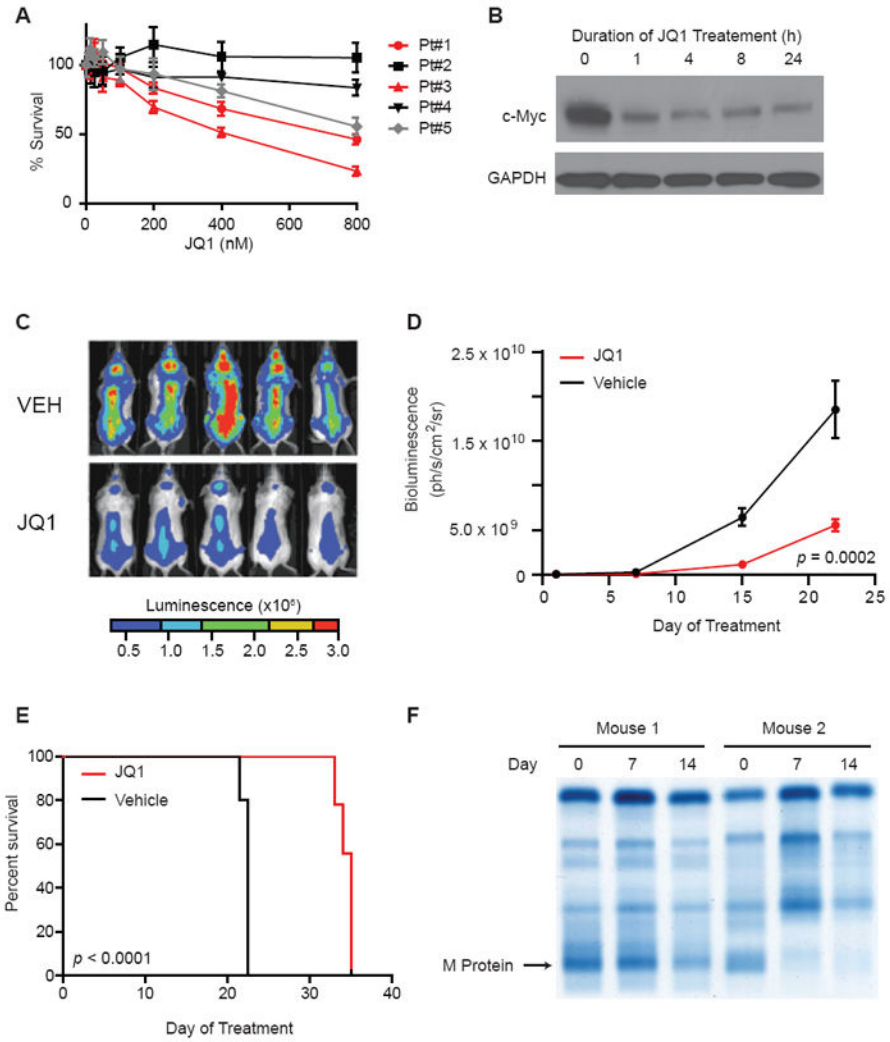


Figure 7. Translational implications of BET bromodomain inhibition in MM

(A) JQ1 arrests the proliferation of primary, patient-derived CD138+ MM cells (Cell TiterGlo; Promega).

(B) c-Myc immunoblot shows JQ1-induced downregulation in short-term culture of primary, patient-derived MM cells (500 nM, duration as indicated).

(C) Representative whole-body bioluminescence images of SCID-beige mice orthotopically xenografted after intravenous injection with MM.1S-luc+ cells and treated with JQ1 (50 mg/kg IP daily) or vehicle control.

(D) Tumor burden of SCID-beige mice orthotopically xenografted after intravenous injection with MM.1S-luc+ cells. Upon detection of MM lesions diffusely engrafted in the skeleton, mice were randomly assigned to receive JQ1 (50 mg/kg IP daily) or vehicle control. Data are presented as mean +/- SEM (n = 10/group).

(E) Survival curves (Kaplan-Meier) of mice with orthotopic diffuse MM lesions show prolongation of overall survival with JQ1 treatment compared to vehicle control (log-rank test, $p < 0.0001$).

(F) Serum protein electrophoresis to detect monoclonal, tumor-derived, immunoglobulin (M-protein) in two MM-bearing Vk*myc mice before or after 7 and 14 days of JQ1 treatment. JQ1 treatment induced partial and complete responses, respectively, in mouse 1 and mouse 2.