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## Inhibition of BET Bromodomain Targets Genetically Diverse Glioblastoma

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### Abstract

**Purpose**—Glioblastoma is refractory to conventional therapies. The bromodomain and extraterminal domain (BET) proteins are epigenetic readers that selectively bind to acetylated lysine residues on histone tails. These proteins recently emerged as important therapeutic targets in NUT midline carcinoma and several types of hematopoietic cancers. In this study, the therapeutic potential of a novel BET bromodomain inhibitor, JQ1, was assessed in a panel of genetically heterogeneous glioblastoma samples.

**Experimental Design**—The antineoplastic effects of JQ1 were shown using *ex vivo* cultures derived from primary glioblastoma xenograft lines and surgical specimens of different genetic background. The *in vivo* efficacy was assessed in orthotopic glioblastoma tumors.

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**Results**—We showed that JQ1 induced marked G<sub>1</sub> cell-cycle arrest and apoptosis, which was phenocopied by knockdown of individual BET family members. JQ1 treatment resulted in significant changes in expression of genes that play important roles in glioblastoma such as c-Myc, p21<sup>CIP1/WAF1</sup>, hTERT, Bcl-2, and Bcl-xL. Unlike the observations in some hematopoietic cancer cell lines, exogenous c-Myc did not significantly protect glioblastoma cells against JQ1. In contrast, ectopically expressed Bcl-xL partially rescued cells from JQ1-induced apoptosis, and knockdown of p21<sup>CIP1/WAF1</sup> attenuated JQ1-induced cell-cycle arrest. Cells genetically engineered for Akt hyperactivation or p53/Rb inactivation did not compromise JQ1 efficacy, suggesting that these frequently mutated signaling pathways may not confer resistance to JQ1. Furthermore, JQ1 significantly repressed growth of orthotopic glioblastoma tumors.

**Conclusion**—Our results suggest potentially broad therapeutic use of BET bromodomain inhibitors for treating genetically diverse glioblastoma tumors.

## Introduction

Glioblastoma is the most common and aggressive primary malignant brain tumor. Despite advances in the multimodality treatments including surgery and chemoradiotherapy, the overall survival of patients with glioblastoma has remained largely unchanged for decades (1). The dismal prognosis makes this disease an upfront challenge for development of novel therapeutic strategy. Over the past decade, a variety of molecular-targeted agents has been clinically tested for treating glioblastoma. The majority are kinase inhibitors that target signaling pathways recurrently activated in glioblastoma (2). These first-generation targeted agents, however, show at best modest and infrequent efficacy (2). While various mechanisms can be implicated, the widespread and heterogeneous genetic aberrations found amongst glioblastoma cases represent a major challenge for improving glioblastoma response to therapies targeting those heavily altered signaling pathways.

Bromodomains are protein motifs that primarily bind to acetylated lysine residues, including those on histone tails (3). Through this interaction, bromodomain-containing proteins direct the assembly of nuclear macromolecular complexes to specific sites on chromatin that regulate key biologic processes including DNA replication, DNA damage repair, chromatin remodeling, and transcription regulation (3, 4). The BET family proteins (Brd2, Brd3, BRD4, and Brdt) contain 2 amino-terminal bromodomains and are best known for their roles in transcriptional regulation (5). Recently, these proteins emerged as attractive therapeutic targets in the treatment of inflammation and cancer following development of several small-molecular inhibitors that selectively bound to BET bromodomains including benzodiazepine derivatives, I-BETs, and JQ1 (6–9). The oncogenic functions of BET proteins were first identified in NUT midline carcinoma, which is commonly driven by fusion of the bromodomains of BRD3 or BRD4 and the NUT (nuclear protein in testis) protein (10). More recently, an RNA interference screening found that knockdown of BRD4 in acute myeloid leukemia led to downregulation of c-Myc, depletion of leukemia stem cells, and disease regression (11). JQ1 competitively binds to BET bromodomain and displaces BET proteins from acetylated lysines on chromatin (7). Inhibition of the BET bromodomain with JQ1 showed potent anti-cancer effects both *in vitro* and *in vivo* in several different hematopoietic cancers as well as in NUT midline carcinoma (7, 11–14). However, cell lines derived from

solid tumors, such as lung cancer, breast cancer and cervical cancer, appear to be less sensitive (14, 15). Several studies found that inhibition of BET bromodomain by JQ1 resulted in significant downregulation of c-Myc (11–14). The ability of BET bromodomain inhibition to reduce expression of c-Myc, until now a theoretically lucrative but pharmaceutically refractory target for cancer therapies, highlights the promise of this novel therapeutic strategy to improve treatment for some cancers that require c-Myc activity.

The pleiotropic roles of c-Myc in cancer biology have been extensively documented (16). We have previously reported that c-Myc is preferentially expressed in glioblastoma stem cells and critically implicated in self-renewal, survival, and tumorigenic potential of these cells (17). Genetically engineered mouse models of glioblastoma also highlight the roles of c-Myc in promoting transformation and maintenance of glioblastoma stem cells (18, 19). Although c-Myc is rarely mutated in glioblastoma, increases in copy number and nuclear staining have been reported (20). In this study, we assessed the antineoplastic potential of JQ1 in a panel of genetically diverse glioblastoma primary xenograft lines and primary cultures. Our results suggest that inhibition of BET bromodomain may suppress glioblastoma tumors of different genetic background. However, these effects are mediated by complex mechanisms including but not limited to c-Myc–mediated transcriptional regulation.

## Materials and Methods

### Tumor samples and cell culture

T4105, T4302, and T4597 primary glioblastoma xenograft lines were generous gifts from Jeremy Rich at Cleveland Clinic (Cleveland, OH). Primary glioblastoma xenograft lines with a “GBM” prefix were kindly provided by Jann Sarkaria at Mayo Clinic (Rochester, MN). These tumor samples were originally derived from patient surgical specimens and serially passaged as subcutaneous xenograft tumors. Primary glioblastoma neurosphere cultures were directly derived from de-identified surgical specimens obtained from patients undergoing resection at Vanderbilt University Medical Center (Nashville, TN) in accordance with protocols approved by the Institutional Review Board. Cells were enzymatically dissociated from either surgical specimens or subcutaneous xenograft tumors as previously described (17, 21). For T4105, T4302, and T4597, matched cellular fractions enriched or depleted for cancer stem cells were separated by magnetic sorting using the CD133 Microbead Kit (Miltenyi Biotec). CD133+ glioblastoma stem cells or unsorted neurospheres were maintained in neurobasal media supplemented with the B27 serum substitute, in addition to 20 ng/mL EGF and 20 ng/mL basic fibroblast growth factor (bFGF; Invitrogen). CD133– cells were maintained in Dulbecco’s Modified Eagle’s Medium supplemented with 10% FBS (Invitrogen) but were cultured in stem cell media at least 24 hours before experiments to control differences in cell media.

### DNA constructs and lentivirus production

The coding sequence of Myr-Akt1 was amplified by PCR from pcDNA3-HA-Myr-Akt1 (Addgene; #9008) and subcloned into the *Xba*I and *Eco*RI sites of the pCDH-CMV-MCS-EF1-Puro lentiviral vector (System Biosciences). Bcl-xL was amplified from pSFFV-neo-

Bcl-xL (Addgene #8749) and subcloned into the *EcoRI* sites of pCDH-CMV-MCS-EF1-Puro. The pLKO.1 lentiviral vectors directing expression of p53 short hairpin RNA (shRNA; #19119) and Rb shRNA (#25641) were purchased from Addgene; shRNA sequences specific to p21 (TRCN0000040127), BRD2 (clone 1-TRCN000006309, clone 2-TRCN000006310), BRD3 (clone 1-TRCN0000021374, clone 2-TRCN0000021376), and BRD4 (clone 1-TRCN0000196576, clone 2-TRCN0000199427) were purchased from Sigma-Aldrich. The lentiviral vectors were co-transfected with the packaging vectors psPAX2 and pCI-VSVG (Addgene) into 293FT cells by Lipofectamine 2000 (Invitrogen) to produce virus. Two days following transfection, viral supernatants were collected and used to infect cells at an approximate MOI of 5. Two days after infections, cells were selected with 1 µg/mL puromycin for at least 48 hours before experiments.

### Antibodies and other reagents

The antibodies used in this study include BRD4 antibody from Bethyl Laboratories; c-Myc (9E10) and p53 (DO-1) from Santa Cruz Biotechnology; and p21 (#2947), phospho-S473 Akt (#9271), total Akt (#2920), Bcl-2 (#2870), and Bcl-xL (#2764) from Cell signaling Technology. Actin antibody was purchased from Millipore. PFI-1 was purchased from Sigma-Aldrich.

### Cell viability, cell-cycle distribution, and neurosphere formation

To determine dose response, cells were aliquoted into 96-well plate at 5,000 cells per well in triplicates. JQ1 or PFI-1 was added by 2- or 4-fold serial dilutions. Cell number was measured 5 days after plating using the CellTiter-Glo Kit (Promega) and normalized to corresponding vehicle-treated groups. IC<sub>50</sub> values were calculated by the GraphPad Prism 5 software using the 4-parameter logistic nonlinear regression model. To calculate the relative growth of cells, averaged cell titers of each group on day 1 were assigned a value of 1. All subsequent cell titer values were normalized accordingly.

Activation of caspase-3/7 was measured by the Caspase-Glo 3/7 Assay Kit (Promega) according to the manufacturer's instructions. Values of caspase activities were normalized to the corresponding cell titers to determine the relative caspase-3/7 activities. Alternatively, cells were labeled by the fluorescein isothiocyanate (FITC)-conjugated Annexin V Staining Kit (BD Biosciences) according to manufacturer's instructions, and the apoptotic subpopulation was analyzed by flow cytometry.

Cell-cycle distribution was analyzed by flow cytometry using cells stained by 10 µg/mL propidium iodide. The percentage of cells in each cell-cycle phase was determined by the ModFit LT Software.

To determine formation of neurospheres, 100 CD133+ glioblastoma cells were plated to each well in 24-well plates. Cells were treated by JQ1 with 4 replicates for each concentration. Ten days after plating, spheres containing more than 50 cells were scored.

### Quantitative real-time PCR

Total RNA was extracted using the Illustra RNAspin Kit (GE Healthcare) and reverse-transcribed using the iScript cDNA Synthesis Kit (Bio-Rad). Real-time PCR was carried out using SYBR-Green Mastermix (Bio-Rad) for 40 cycles of 95°C for 20 seconds and 60°C for 45 seconds. The threshold cycle ( $C_T$ ) values for each gene were normalized to the  $C_T$  values of  $\beta$ -actin. The primers used are described in Supplementary Table S1.

### Orthotopic glioblastoma model

All animal experiments were carried out in female athymic nude mice under a protocol approved by the Vanderbilt University Institutional Animal Care and Use Committee. To induce intracranial tumors, 5,000 T4302 or T4597 CD133+ cells suspended in 10  $\mu$ L PBS were implanted into the right cerebrum of nude mice. Before implantation, T4597 cells were infected with lentivirus directing expression of firefly luciferase and selected with 1  $\mu$ g/mL puromycin for 3 days. Following tumor implantation, mice were maintained for 10 days to allow tumor establishment. Starting from the day 11, mice were intraperitoneally administered with JQ1 at 50 mg/kg twice per day for indicated time. Animals were sacrificed upon development of apparent symptoms, such as lethargy or hunched posture. The median survival was determined by the Kaplan–Meier estimator using the GraphPad Prism 5.0 Software.

### *In vivo* bioluminescence imaging

Tumor progression was monitored by bioluminescence imaging weekly beginning 1 day before JQ1 treatment. Animals were intraperitoneally administered with 150 mg/kg D-luciferin (Gold Biotechnology). Ten minutes after luciferin administration, bioluminescence was read for 50 seconds on a Xenogen IVIS 200 imaging system (Caliper Life Sciences). Bioluminescence was quantified using the Living Images software (Caliper Life Sciences).

### Statistics

GraphPad Prism 5.0 was used to determine statistical significance by the Student *t* test. Significance of survival assays was determined by the log-rank test.  $P < 0.05$  was considered significant.

## Results

### Inhibition of BET proteins by small-molecule inhibitors or shRNA reduces proliferation and survival in glioblastoma

We have previously reported that knockdown of c-Myc significantly reduced proliferation, survival, and the tumorigenic potential of glioblastoma stem cells (17). The BET bromodomain inhibitor JQ1 recently emerged as a novel modulator that potently downregulates c-Myc expression in certain hematopoietic cancers (13, 14), suggesting that JQ1 has the potential of targeting other c-Myc–dependent tumors. We first assessed whether JQ1 affected proliferation and survival of functionally validated glioblastoma stem cells, enriched from freshly dissociated glioblastoma xenograft tumors by selection for expression of CD133 cell surface marker (17, 21–23). In the present study, we focused on the short-

term glioblastoma stem cell cultures and primary neurosphere cultures for samples of which the stem cell fraction has not been validated, as these *ex vivo* models more faithfully phenocopy and genocopy the original human tumors than the traditional serum-supplemented monolayer cultures (21, 22, 24). Treatment with JQ1 [abbreviation for the active enantiomer (+)-JQ1] reduced the viability of T4302 CD133+ cells and CD133+ glioblastoma stem cells derived from other primary xenograft lines in a concentration-dependent manner (Fig. 1A and Supplementary Fig. S1A). In addition, formation of neurospheres and growth by T4302 CD133+ cells were markedly decreased in the presence of JQ1 at various concentrations (Fig. 1B and Supplementary Fig. S1B). Consistent with previous studies, the enantiomer (–)-JQ1 incapable of recognizing BET bromodomain displayed limited effects (Supplementary Fig. S1C; ref. 7). Finally, another selective but less potent BET bromodomain inhibitor, PFI-1, also induced dose-dependent reduction of cell viability in T4302 CD133+ cells (Supplementary Fig. S1D). Taken together, these results suggest that the antineoplastic effects of JQ1 and PFI-1 are specifically dependent on targeting the BET bromodomain. Matched CD133– non-stem glioblastoma cells showed comparable sensitivity to JQ1 (Fig. 1A and Supplementary Fig. S1A), suggesting similar dependency on the functions of BET proteins across different cell lineages of glioblastoma.

Inhibition of BET bromodomain in hematopoietic cancers and NUT midline carcinoma consistently induces G<sub>1</sub> cell-cycle arrest and apoptosis (6, 7, 11–14). In line with these observations, we showed that JQ1 induced a significant G<sub>1</sub> cell-cycle arrest in T4302 CD133+ cells (Fig. 1C). In addition, JQ1 activated caspase-3/7 (Supplementary Fig. S1E) and resulted in accumulation of cellular subpopulations positively labeled for the early apoptosis marker, Annexin V (Fig. 1D). Similar observations were made with glioblastoma stem cells derived from multiple xenograft lines (data not shown). JQ1 also compromised proliferative potential in normal human neural progenitors but only induced modest caspase activation in comparison to that in glioblastoma stem cells (Supplementary Fig. S2).

We next asked whether genetic targeting of BET proteins led to similar repression of proliferation and survival by lentivirus-mediated expression of shRNA sequences specific to BRD2, BRD3, or BRD4. Interestingly, knockdown of any one of these BET genes significantly decreased cell growth of T4302 CD133+ cells, associated with potent caspase activation (Fig. 2 and Supplementary Fig. S3). Although a BRD3 knockout mouse model has not been reported, loss of either BRD2 or BRD4 in mouse results in embryonic lethality (25, 26). In particular, BRD2-null embryos display significant defects in neural tubes (26). Taken together, our results suggest that BET proteins possess nonredundant functions in glioblastoma, which are consistent with their proliferative and prosurvival roles in developing neural tissues.

### **JQ1 alters c-Myc–dependent and -independent transcription regulations**

The BET proteins are important transcription regulators. For example, BRD4 promotes transcription by recruiting the positive transcription elongation factor b (P-TEFb) to the RNA polymerase II complex (27, 28) or by acting as an atypical kinase that phosphorylates the carboxyl-terminal domain of RNA polymerase II (29). Studies in several types of c-Myc–driven hematopoietic cancers suggest that, upon JQ1 treatment, downregulation of c-



Myc plays a major role in mediating transcriptional changes and repression of cell-cycle progression. However, JQ1 also affects c-Myc-independent mechanisms, as ectopic c-Myc expression does not effectively attenuate JQ1-induced cell death (11, 13, 14). We therefore asked whether c-Myc was the primary downstream target of BET proteins in glioblastoma stem cells. We first showed that c-Myc was downregulated at both mRNA and protein levels upon treatment with JQ1 in T4302 CD133+ cells as well as in matched CD133- cells (Fig. 3A and Supplementary Fig. S4), albeit to a lesser degree in comparison to that observed in hematopoietic cancer cell lines (12–14). In addition, we also found that JQ1 treatment reduced expression of hTERT, Bcl-2 and Bcl-xL and modestly induced expression of the CDK inhibitor p21<sup>CIP1/WAF1</sup> (p21), which were repetitively described as BET-regulated genes in different types of hematopoietic cancer cell lines (Fig. 3A; refs. 6, 11, 13, 14). These target genes connect BET proteins to important regulatory mechanisms of proliferation and survival in glioblastoma. For example, preferential expression of telomerase and sensitivity to telomerase inhibition are recently reported in glioblastoma stem cells (30).

We next determined whether exogenous c-Myc could protect glioblastoma stem cells from JQ1. Expression of c-Myc from a CMV promoter on a lentiviral vector was not altered by JQ1, in contrast to endogenous c-Myc (Fig. 3B and C). However, T4302 CD133+ cells expressing exogenous c-Myc remained sensitive to JQ1 (Fig. 3D). In addition, JQ1-induced p21 upregulation was not affected by ectopically expressed c-Myc (Fig. 3C), in contrast to previous observations in a multiple myeloma cell line (14). Analysis of other JQ1-targeted genes showed that exogenous c-Myc partially rescued Bcl-2 expression but not Bcl-xL (Fig. 3C), whereas expression of hTERT was fully restored by exogenous c-Myc in the presence of JQ1 (Fig. 3C). These results suggest that inhibition of BET proteins induces profound changes in gene expression via both c-Myc-dependent and -independent mechanisms in glioblastoma. In consistent with the findings in lung adenocarcinoma cell lines (15), our results also suggest that c-Myc plays a minor role in mediating the antineoplastic activities of JQ1 in glioblastoma.

### Altering core glioblastoma signaling pathways does not confer resistance to JQ1

Advances in high-throughput profiling technology have led to identification of several core signaling pathways that are recurrently altered in glioblastoma, exemplified by multiple receptor tyrosine kinases (RTK) and their downstream RAS/PI3K/AKT signaling axis (31, 32). Dysregulated activation of these pathways can be associated with intrinsic and acquired resistance to targeted therapies (33). We therefore asked whether aberrations of these pathways might affect glioblastoma response to BET bromodomain inhibition. The hyperactivation of the RTKs/RAS/PI3K/AKT signaling axis was modeled by lentivirus-directed expression of a myristoylation signal-linked Akt1 (Myr-Akt1). Expression of this constitutively active Akt1 fusion protein is sufficient to rescue HER2-amplified breast cancer cells from trastuzumab (34). In T4302 CD133+ cells, Myr-Akt1 not only drastically increased the levels of phosphorylated Akt but also surprisingly ablated JQ1-induced c-Myc downregulation (Fig. 4A). However, changes in p21 and Bcl-xL expression were not significantly affected and Myr-Akt1-expressing cells appeared to be equally or even more sensitive to JQ1 than the parental cells (Fig. 4B).

The activities of p53 and Rb tumor suppressors are compromised in essentially all glioblastoma tumors via different mechanisms (31, 32). Glioblastoma tumors with Rb mutations are less susceptible to CDK inhibitors (35). The 2 xenograft lines we used in our study, T4302 and T4597, retain wild-type p53 and Rb. Therefore, we determined whether further reducing the activities of these 2 tumor suppressors by RNA interference affected tumor sensitivity to JQ1. Knockdown of p53 with a previously published shRNA sequence significantly decreased p21 expression (Supplementary Fig. S5A; ref. 36), consistent with the role of p53 as a main transcriptional activator for p21. Yet, JQ1 treatment still resulted in an apparent induction of p21 (Supplementary Fig. S5A). The ability of JQ1 to compromise the viability of T4302 CD133+ cells was not significantly changed in the absence of p53 (Fig. 4C). Similarly, knockdown of Rb with a previously reported shRNA did not alter response to JQ1 (Fig. 4D; ref. 37). The basal levels of Rb protein in T4302 CD133+ cells were too low to be detected by immunoblotting, thus the efficacy of Rb-specific shRNA was shown by quantitative real-time PCR (qRT-PCR; Supplementary Fig. S5B) and validated by immunoblotting in HeLa cells (data not shown). Collectively, our observations suggest that these core glioblastoma signaling pathways may not be critically implicated in response to BET inhibition.

### **JQ1 shows broad activity in glioblastoma tumors of diverse genetic background**

The core signaling pathways frequently mutated in glioblastoma exhibit limited impact on tumor sensitivity to JQ1, suggesting that BET inhibition may generate broad response in heterogeneous glioblastoma samples. We therefore assessed the effects of JQ1 on the Mayo Clinic test panel of primary glioblastoma xenograft tumors that are known to carry different genetic lesions altering p53, Rb, PTEN, and EGFR (35, 38). These tumors belong to different molecular subtypes as characterized by the Cancer Genome Atlas (TCGA) research network (32). The response to JQ1 of these tumors was also compared with early-passage primary cultures established in our laboratory. All samples were derived from enzymatically dissociated tumor masses and maintained as neurospheres in media that support enrichment of stem cell-like glioblastoma cells (24). In 10 of 15 of these samples, JQ1 reduced cell viability by more than 50% at 5  $\mu\text{mol/L}$  concentration, with  $\text{IC}_{50}$  values largely ranging between 60 and 500  $\text{nmol/L}$  (Table 1). These results were comparable to previous observations in hematopoietic cancers (12–14). Consistent with the results using genetic targeting methods, tumor responsiveness to JQ1 was not specifically associated with any mutational status characterized for these samples (Table 1). Several samples were examined for changes in protein levels of c-Myc and several other JQ1-targeted genes (Fig. 5A). Induction of p21 and downregulation of Bcl-xL upon JQ1 treatment was found in nearly all samples, although to different extents (Fig. 5A). Interestingly, significant downregulation of c-Myc was only observed in those JQ1-sensitive samples (Fig. 5A). Among the 3 modestly responsive samples, c-Myc was barely detectable in GBM8, highly expressed but not affected by JQ1 in GBM22, and even increased upon JQ1 treatment in GBM10 (Fig. 5A). Cells derived from these 3 primary xenograft lines also expressed considerably more Bcl-2 and Bcl-xL than other lines (Fig. 5A). Taken together, our results show widespread and significant responsiveness to BET bromodomain inhibition in a set of genetically heterogeneous glioblastoma samples. Although these samples are not sufficient to identify any markers predictive of JQ1 sensitivity, these data do suggest that low levels of the Bcl-2



family anti-apoptotic proteins and a functional link between c-Myc and BET proteins may serve as favorable indicators for tumor responsiveness to BET bromodomain inhibition.

Because JQ1 treatment widely affects expression of p21 and Bcl-xL, we assessed their functions downstream of BET proteins. Although rarely mutated in cancer, p21 is a potent inhibitor of G<sub>1</sub> cyclin-dependent kinases and an important cell-cycle regulator. Knockdown of p21 using a previously published shRNA sequence significantly attenuated G<sub>1</sub> cell-cycle arrest induced by JQ1 in T4302 CD133+ cells (Fig. 5B; ref. 39). In addition, ectopically expressed Bcl-xL partially protected T4302 CD133+ cells via counteracting apoptosis induced by JQ1 treatment (Fig. 5C and D). Although p21 and Bcl-xL alone are certainly not adequate to fully explain the consequences of BET bromodomain inhibition, our results suggest that these 2 proteins play broad and important roles in mediating the ability of BET proteins to regulate glioblastoma proliferation and survival.

### JQ1 represses glioblastoma progression in an orthotopic tumor model

Matzuk and colleagues recently report that JQ1 shows excellent permeability across the blood–brain barrier ( $AUC_{\text{brain}}/AUC_{\text{plasma}} = 98\%$ ; ref. 40). We therefore assessed its therapeutic effects on an orthotopic glioblastoma model that recapitulated the pathologic hallmarks of glioblastoma (21). In a preliminary test, mice bearing T4302 intracranial tumors were sacrificed at 2, 4, or 8 hours after a single intraperitoneal injection of 100 mg/kg JQ1. RNA was extracted from grossly dissected tumor masses and subjected to qRT-PCR with human-specific primers. A decrease of c-Myc and Bcl-xL expression was detected as early as 2 hours after JQ1 administration, followed by apparent hTERT and Bcl-2 reduction at 4 hours (Fig. 6A). Significant p21 upregulation, which may require precedent degradation of existing c-Myc protein, was not seen until 8 hours after drug administration (Fig. 6A). These results recapitulated observations in *ex vivo* cultures and thus confirmed JQ1 activities in intracranial tumors. In the first *in vivo* assay, intracranial tumors were established using 5,000 T4302 CD133+ cells per mouse. Ten days after tumor implantation, mice received 50 mg/kg JQ1 twice a day via intraperitoneal injection for 20 days. The median survival of the JQ1-treated arm was 37 days in comparison to 31 days of the vehicle-treated arm ( $P = 0.0026$  by the log-rank test; Fig. 6B). The second experiment was carried out with tumors established using 5,000 T4597 CD133+ cells infected with lentivirus directing expression of firefly luciferase. JQ1 was administered from day 11 to 45 after tumor implantation, resulting in an extension of the median survival from 37 to 48 days ( $P = 0.0089$  by the log-rank test; Fig. 6C). Tumor progression was monitored weekly by bioluminescence imaging in the second test (Fig. 6D). Interestingly, 1 week after JQ1 administration, the growth rate and the sizes of JQ1-treated tumors were slightly higher than those of the control arm, as determined by bioluminescence intensity (Supplementary Fig. S6). Nevertheless, JQ1-treated tumors appeared to progress at a slower rate while treatment continued and thus were soon overgrown by tumors in the control arm (Supplementary Fig. S6). In both *in vivo* experiments, no significant weight loss or other toxicity was observed in association with drug administration. These results establish *in vivo* the feasibility of BET bromodomain inhibitors as a novel therapeutic approach for treating glioblastoma.

## Discussion

Epigenetic regulation is a rapidly evolving central theme to oncology. Aberrations in epigenetic regulation are increasingly identified in human cancers (41). Targeting epigenetic modulators holds great promise as cancer therapies, exemplified by vorinostat and romidepsin for treating cutaneous T-cell lymphoma (41). However, successful efforts for developing epigenetic therapy have been largely restricted to hematopoietic cancers via targeting chromatin-modifying enzymes and also obscured by concerns over the lack of specificity of these enzymes and their drugs (41, 42). Recently developed small-molecule inhibitors against BET bromodomain appear to be highly selective for a few closely related targets and affect only a few hundred of genes in different context, thus promoting epigenetic therapy to a new level (4). In the present study, we showed that inhibition of the BET epigenetic readers with JQ1 compromised cellular proliferation and survival of glioblastoma cells. Our complementary genetic and pharmaceutical approaches validated that the antineoplastic effects of JQ1 were mediated by selectively targeting the BET proteins. Remarkably, JQ1 displayed broad efficacy across a panel of genetically diverse glioblastoma samples, highlighting the therapeutic potential of BET bromodomain inhibitors for treating complex solid tumors, such as glioblastoma, in addition to NUT midline carcinoma and hematopoietic cancers.

Pharmaceutical inhibition of BET bromodomain has attracted extensive interest as a novel anti-cancer strategy since its invention 2 years ago (7, 8). One compelling feature of this approach is its ability to induce effective downregulation of c-Myc (11, 13), a long pursued “undruggable” cancer target. Several types of hematopoietic cancers, such as acute myeloid leukemia, Burkitt lymphoma, and multiple myeloma, are well characterized by recurrent c-Myc amplification, translocation, and activation (16). In these largely c-Myc-driven cancers, ectopically expressed c-Myc protected cells from BET inhibition-induced growth suppression in selected cell lines, although protection against cell death is limited (11, 13, 14). Our results also showed that BET inhibition decreased c-Myc expression in glioblastoma. However, in glioblastoma, downregulation of c-Myc upon BET inhibition was rather modest and appeared to only play a minor role in mediating the effects of JQ1. Although c-Myc may not be the primary BET target in glioblastoma, we noticed that c-Myc levels were not significantly reduced by JQ1 in tumors showing relatively moderate response to JQ1 such as GBM8, GBM10, and GBM22. In contrast, JQ1-induced c-Myc downregulation were significant in JQ1-sensitive samples. These results suggest that the ability of JQ1 to downregulate c-Myc expression may be associated with sensitivity to BET inhibition. Among other targets affected by BET inhibition, p21 and Bcl-xL provided partially protection against JQ1 when genetically manipulated. These 2 genes were also widely responsive to JQ1 in various glioblastoma samples. Therefore, it appears that p21 and Bcl-xL play important roles in BET-mediated regulation of cellular proliferation and survival in glioblastoma. However, the BET-targeted gene expression profiles and the precise molecular mechanisms mediating the actions of BET inhibitors in glioblastoma, a highly complex disease, remain to be defined and warrant future studies on a broader scale.

BET bromodomain inhibition displayed broad antineoplastic effects in a range of glioblastoma tumors carrying different genetic lesions. Some of these samples significantly

differ in response to conventional chemoradiotherapy or molecular targeted agents that block signaling pathways frequently activated in glioblastoma. For examples, Sarkaria and colleagues report that the EGFRvIII-expressing GBM6 is resistant to EGFR inhibition by erlotinib, whereas GBM12 is sensitive (43). Nonetheless, *ex vivo* cultures derived from these 2 xenograft lines were both highly sensitive to JQ1. In addition to these samples, glioblastoma stem cells genetically engineered to alter the core glioblastoma signaling pathways, such as Akt, p53, or Rb pathways, maintained sensitivity to JQ1. In glioblastoma and most other solid cancers, key tumor promoting pathways are often altered by combinations of genetic events, compromising the potential of single-targeted agents in the absence of predictive biomarkers. For example, distinct cellular subclones amplified for EGFR, PDGFR, or MET can co-exist in some glioblastoma tumors, providing one explanation as to why targeting one of these kinases only affects a small subset of patients (44, 45). According to the TCGA database (accessed via the cBio Cancer Genomics Portal), genetic alterations of BET proteins are very rare in glioblastoma. Our results showed that BRD2, BRD3, and BRD4 possessed nonredundant functions in regulating proliferation and survival in glioblastoma. We also showed that BET inhibition compromised proliferation of normal human neural progenitor cells derived from fetal tissues. Data generated from genetically engineered mouse models suggest that BET proteins, particularly BRD2, are critically implicated in neurogenesis (25, 26). Taken together, we speculate that BET proteins are commonly required for both proliferating normal neural tissues and their transformed counterparts. The potential of BET inhibitors to broadly target glioblastoma tumors carrying different genetic lesions has important clinical implication, as it suggests that this novel therapeutic strategy has the potential to generate measurable response in patients with glioblastoma, even in the absence of guidance by BET mutations or other predictive biomarkers.

Another interesting feature of JQ1 is its ability to target both glioblastoma stem cells and matched non-stem cancer cells. Glioblastoma is among several human cancers following a hierarchical organization that stem cell-like cells critically drive the pathology of cancer and give rise to multilineage non-stem cancer cells that have restricted tumorigenic potential (22). Glioblastoma stem cells are particularly important therapeutic targets as they appear to be more resistant to conventional chemotherapy and radiotherapy than their progenies and propel tumor recurrence (21, 46). However, non-stem cancer cells are not simply bystanders. Recent studies provide evidence suggesting that differentiation of non-stem cancer cells can be reversible under certain circumstances so that these cells may confer tumorigenesis (47). It is also reported that non-stem cancer cells participate in microenvironmental support of cancer stem cells (48). Therefore, while eradication of glioblastoma stem cells is greatly desired, it is also important that the non-stem cancer cell subpopulation can be effectively compromised. Normal lineage commitment is associated with epigenetic changes (49). Similarly, the phenotypic difference between cancer stem cells and their differentiated progenies can be driven by epigenetic mechanisms, although direct experimental evidence is lacking (49). Therefore, our results suggest that BET protein-regulated epigenetic mechanisms are shared by the glioblastoma cell hierarchy despite potentially significant difference in epigenetic regulations among these cellular subpopulations.

Results of this study established the concept and explored the feasibility of treating glioblastoma with a new class of epigenetic agents that targets the BET bromodomain proteins. Notably, this therapy displays broad activity across a set of genetically or epigenetically distinct glioblastoma samples, suggesting that BET family proteins regulate essential functions conserved to a substantial percentage of this heterogeneous disease. While targeting signaling pathways frequently dysregulated in glioblastoma has experienced significant setbacks in clinical development over the past decade, the encouraging preclinical results of BET bromodomain inhibition suggest a new avenue to treat this deadly disease. Given the excellent brain-penetrating capacity of JQ1, further study is warranted to fully explore its potential in additional cancer types or other conditions of the central nervous system.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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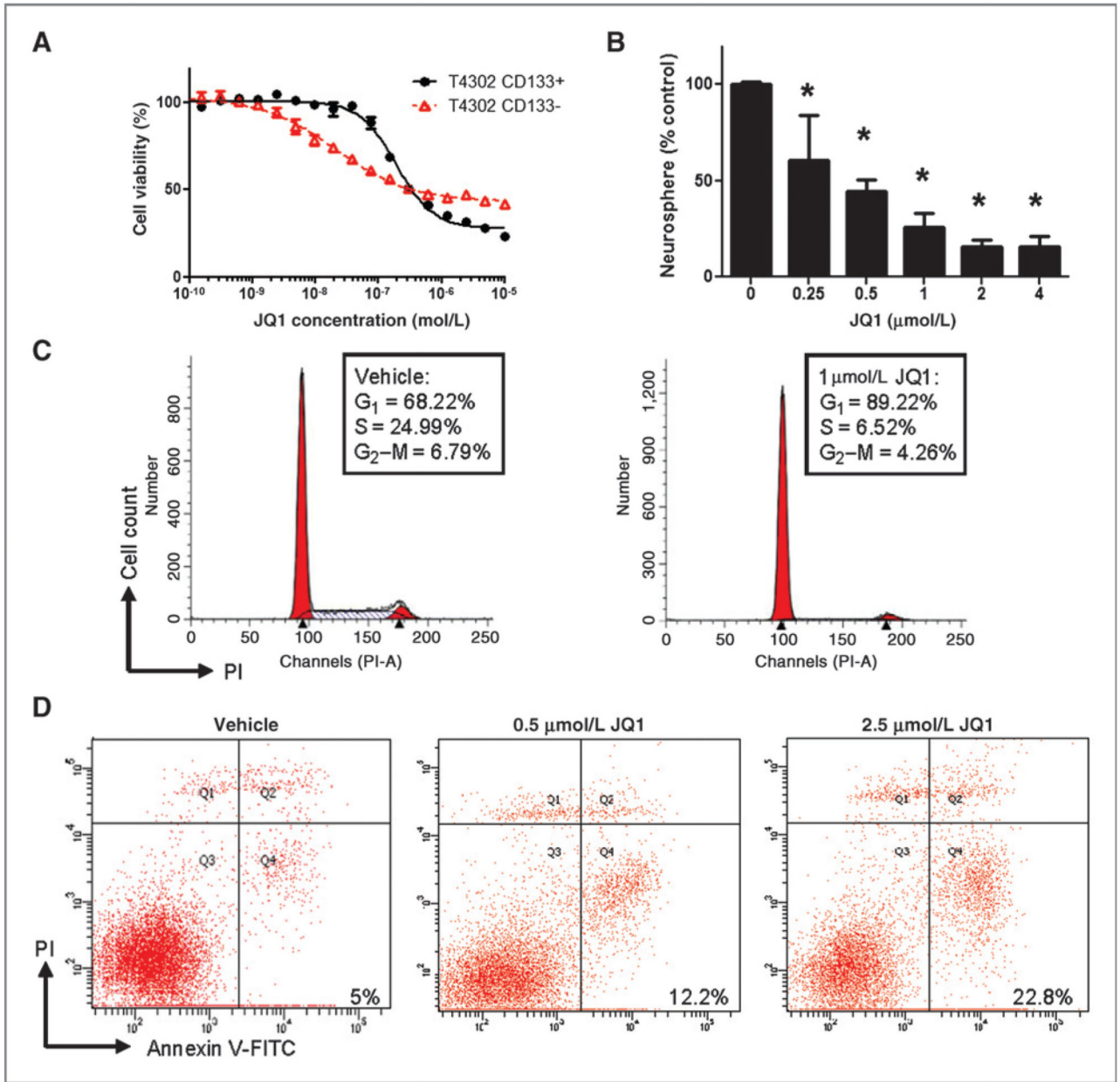
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### Translational Relevance

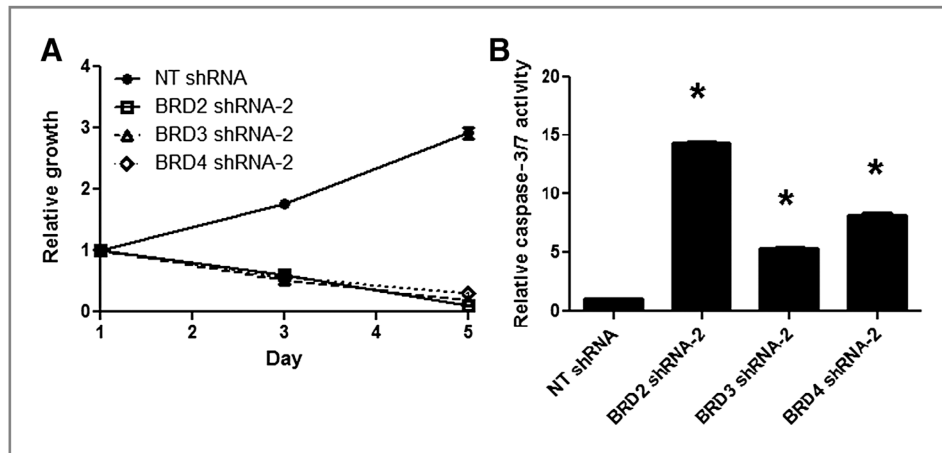
Glioblastoma is a highly heterogeneous cancer, characterized at diagnosis by complex genetic and signaling aberrations. Experimental targeted agents, mostly inhibitors of kinases aberrantly activated in glioblastoma, have shown limited efficacy in clinical trials. A novel class of small-molecule inhibitors selectively targeting the bromodomains of BET epigenetic readers recently displayed therapeutic potential in several types of hematopoietic cancers and NUT midline carcinoma. One of these compounds, JQ1, exhibits excellent brain penetrating capacity. In this study, we showed that JQ1 induced significant antineoplastic effects in glioblastoma samples of different genetic background and further confirmed its *in vivo* efficacy. Our observations suggest that certain epigenetic mechanisms, such as the one mediated by BET proteins, may be commonly required by glioblastoma. Therefore, targeting BET epigenetic readers holds promise to provide benefits for a considerable percentage of patients with glioblastoma.



**Figure 1.**

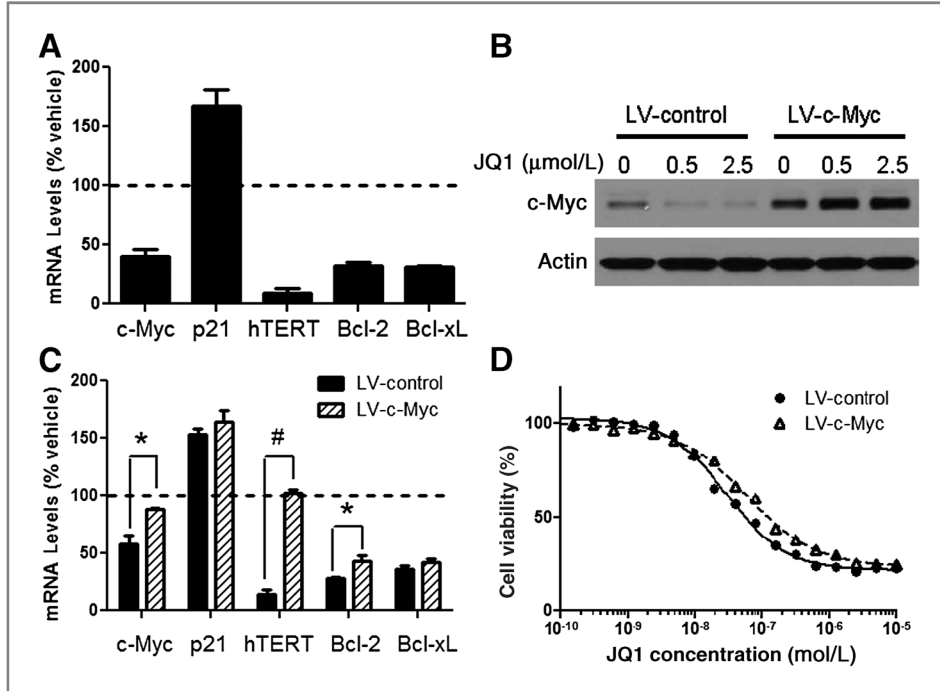
JQ1 reduces proliferation and survival of glioblastoma stem cells and matched non-stem cancer cells. A, CD133+ and CD133- cells derived from T4302 primary glioblastoma xenograft tumor were exposed to JQ1 with a 2-fold serial dilution starting at 10 μmol/L for 17 dilutions in total. Cell viability was determined 5 days posttreatment and normalized to the vehicle-treated group (dimethyl sulfoxide). Data points are presented as mean ± SD (*n* = 3) unless otherwise indicated. B, neurosphere formation of T4302 CD133+ cells were measured in the presence of JQ1 at indicated concentrations. \*, *P* < 0.05 by Student *t* test. C, T4302 CD133+ cells were exposed to vehicle or 1 μmol/L JQ1 for 48 hours. Cell-cycle distribution was determined by the ModFit LT software. Data are representative of 3

independent experiments. D, presence of the Annexin V apoptotic marker was determined 72 hours after treatment. The percentage of cells stained positively for Annexin V but negatively for propidium iodine (PI) is indicated. Data are representative of 3 independent experiments.



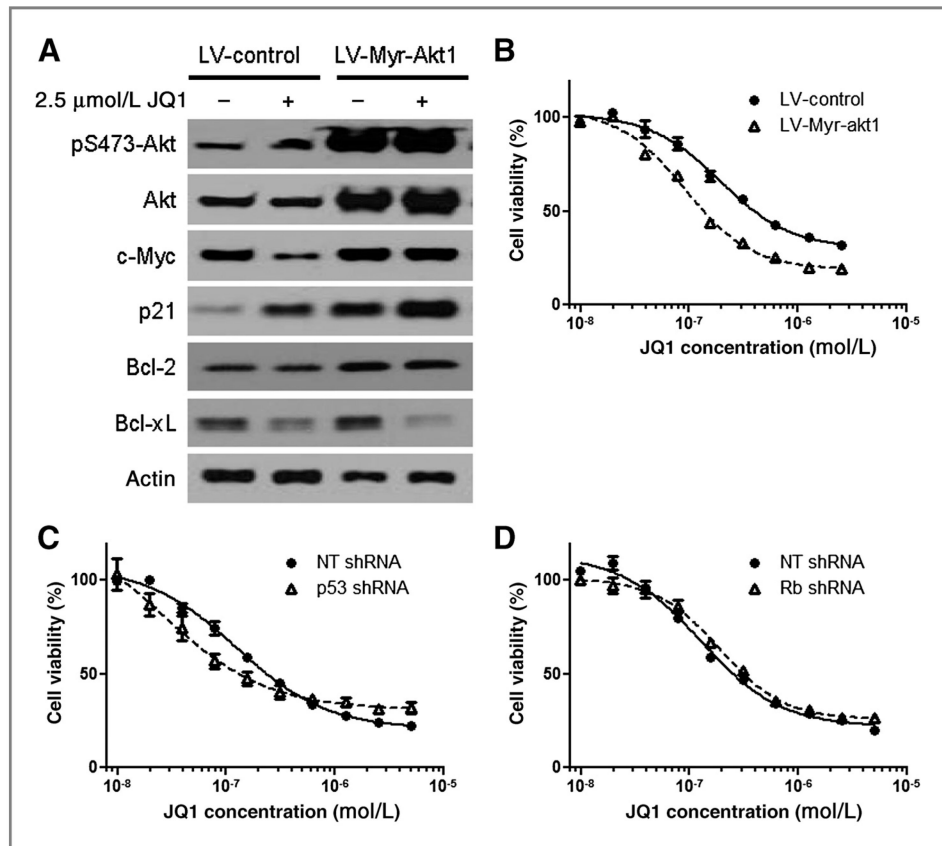
**Figure 2.**

Knockdown of BET proteins leads to antiproliferative and antisurvival effects resembling JQ1 treatment. A, T4302 CD133+ cells were infected with lentivirus directing expression of nontargeting shRNA (NT) or shRNA sequences specific to BRD2, BRD3 or BRD4 (clone 2). After selection with 1  $\mu$ g/mL puromycin for 2 days, relative cell growth was determined for the following 5 days. B, three days after puromycin selection, relative caspase-3/7 activity was determined by normalizing the activity of caspase-3/7 to the corresponding cell titers. \*,  $P < 0.001$  by Student  $t$  test.

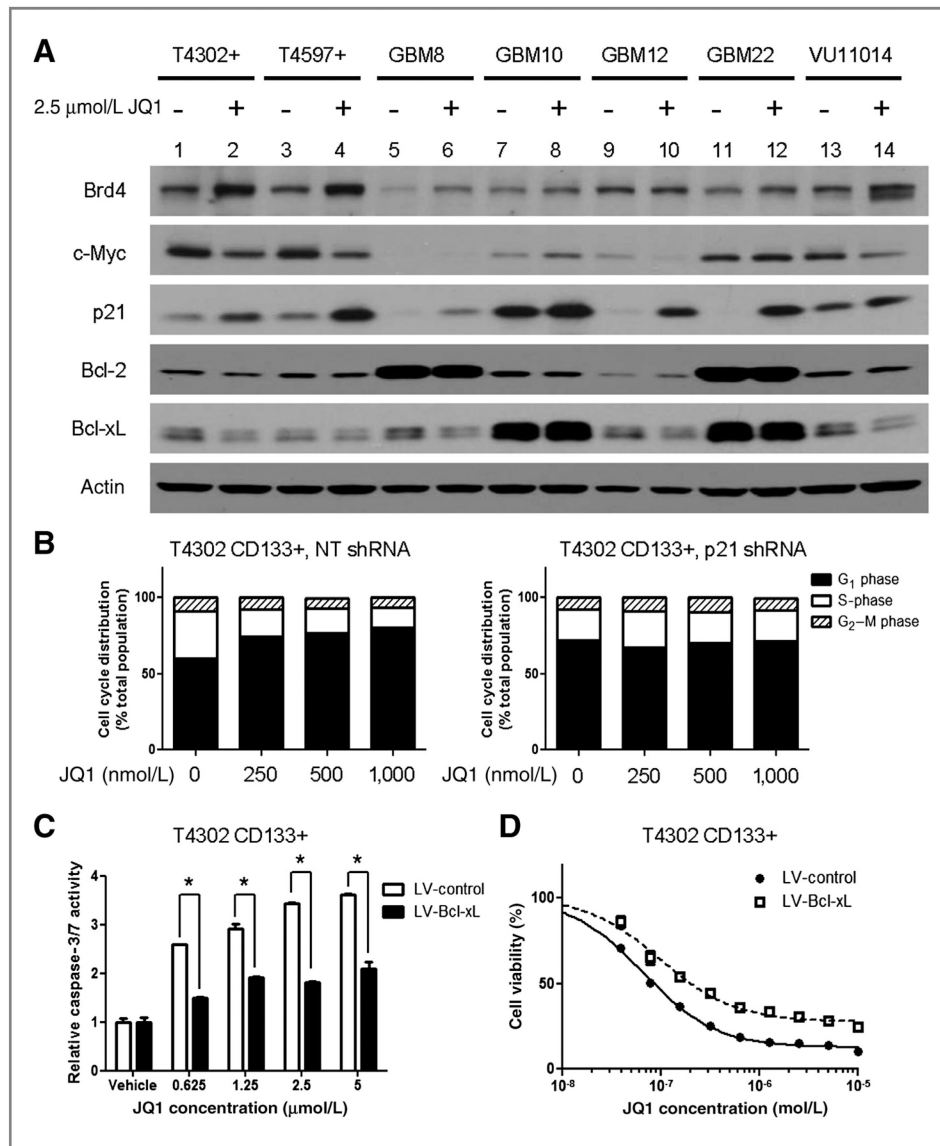


**Figure 3.** c-Myc plays a relatively minor role in mediating the antineoplastic effects of JQ1. A, qRT-PCR of c-Myc, p21, hTERT, Bcl-2, and Bcl-xL in T4302 CD133+ cells treated with 2.5  $\mu\text{mol/L}$  JQ1 for 24 hours.  $\beta$ -Actin was used as loading control for qRT-PCR or immunoblotting throughout this study. B, T4302 CD133+ cells were infected with control lentivirus or virus directing expression of c-Myc under control of a CMV promoter. The protein levels of c-Myc were determined 24 hours after JQ1 treatment. C, qRT-PCR of selected JQ1-responsive genes in control or exogenous c-Myc-expressing T4302 CD133+ cells 24 hours after treatment with JQ1 at 2.5  $\mu\text{mol/L}$ . \*,  $P < 0.05$ ; #,  $P < 0.001$  by Student  $t$  test. D, dose-response curves of JQ1 in control or exogenous c-Myc-expressing T4302 CD133+ cells determined as described in Fig. 1A.

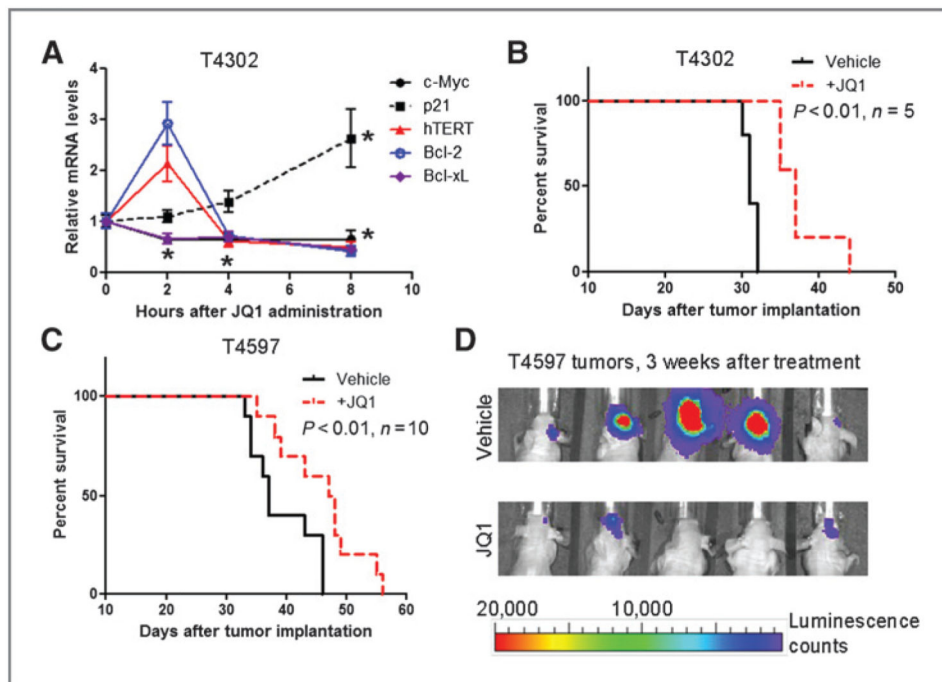


**Figure 4.**

Hyperactivation of Akt or knockdown of p53 or Rb does not compromise the efficacy of JQ1. T4302 CD133+ cells were infected with control lentivirus or virus directing expression of myristoylated Akt1 (A and B), p53-specific shRNA (C), or Rb-specific shRNA (D). A, immunoblotting was conducted 24 hours after JQ1 treatment. B–D, dose–response curves of JQ1 in control or genetically engineered T4302 CD133+ cells determined as described in Fig. 1A.



**Figure 5.** JQ1 induces broad antineoplastic effects in genetically heterogeneous glioblastoma samples partially via p21 upregulation and Bcl-xL downregulation. A, immunoblotting of indicated proteins in selected glioblastoma spheroid cultures treated with 2.5  $\mu\text{mol/L}$  JQ1 for 24 hours. B, T4302 CD133+ cells were infected with lentivirus directing expression of nontargeting shRNA (NT) or shRNA sequences specific to p21. Following puromycin selection, cells were exposed to JQ1 at indicated concentrations for 48 hours. Cell-cycle distribution was determined by the ModFit LT software. C, three days after treatment with JQ1 at indicated concentrations, relative caspase activity was determined in control or exogenous Bcl-xL-expressing T4302 CD133+ cells as described in Fig. 2B. \*,  $P < 0.01$  by Student t test. D, dose-response curves of JQ1 in control or exogenous Bcl-xL-expressing T4302 CD133+ cells.



**Figure 6.**

JQ1 represses orthotopic glioblastoma progression. A, expression of indicated target genes was determined by qRT-PCR in samples extracted from intracranial T4302 tumors following a single dose of JQ1 at 100 mg/kg at varying time points as indicated. \*,  $P < 0.05$  by Student *t* test. Mice bearing intracranial T4302 (B) or T4597 (C) tumors were treated intraperitoneally with 50 mg/kg JQ1, twice daily, from day 11 after tumor implantation to day 30 (T4302) or day 45 (T4597). The  $P$  values were calculated by the log-rank test. D, representative bioluminescence images of T4597 intracranial tumors 3 weeks after JQ1 treatment.

**Table 1**

Response to JQ1 in various glioblastoma samples

ID	IC <sub>50</sub> , nmol/L	E <sub>max</sub> , %	Subtype (32)	EGFR (38)	p53 (38)	Rb (35)	PTEN (38)
GBM6	150	90	Classic	vIII	R273C	Gain	
GBM8	400	47	Classic				HD
GBM10	200	25	Mesenchymal			Loss	
GBM12	120	95	Classic		5,3 Osplice		
GBM16	300	29	Mesenchymal		R273C	Loss	HD
GBM22	300	44	Mesenchymal			Loss	
GBM39	200	52	Mesenchymal	vIII			
GBM43	2000	86	Mesenchymal		F270C		
GBM46	500	40	Proneural	vIII		Loss	
T4105	300	83	ND	ND	ND	ND	ND
T4302	160	85	ND	ND	ND	ND	ND
T4597	60	88	ND	ND	ND	ND	ND
VU10369	500	59	ND	ND	ND	ND	ND
VU10827	130	65	ND	ND	ND	ND	ND
VU11014	700	60	ND	ND	ND	ND	ND

NOTE: Glioblastoma samples with a “GBM” prefix were short-term neurosphere cultures derived from primary glioblastoma xenograft lines serially passaged in mice. Samples with a “T” prefix were prospectively enriched CD133+ glioblastoma stem cells. Samples with a “VU” prefix were primary cultures directly isolated from patient surgical specimens (passage 3–7). IC<sub>50</sub> values were determined after 5-day JQ1 treatment. The maximal effects (E<sub>max</sub>) represented the inhibition rates of JQ1 at 5 μmol/L. Data represent the mean of 3 independent experiments.

Abbreviations: vIII, EGFR variant III; HD, homozygous deletion; ND, not determined.