BET Bromodomain Inhibition of MYC-Amplified Medulloblastoma

Pratiti Bandopadhayay1,3,5, Guillaume Bergthold1,5, Brian Nguyen6, Simone Schubert6, Sharareh Gholamin7, Yujie Tang9, Sara Botin11, Steven E. Schumacher1,5, Rhamy Zeid2, Sabran Masoud6, Furong Yu6, Nujsabnusi Vue6, William J. Gibson1,5, Brenton R. Paolella1,5, Siddhartha S. Mitra7, Samuel H. Cheshier7, Jun Qi2, Kun-Wei Liu9, Robert Wechsler-Reya9, William A. Weiss10, Fredrik J. Swartling11, Mark W. Kieran3, James E. Bradner2,5, Rameen Beroukhim1,2,4,5, and Yoon-Jae Cho6,7,8

Medical Research Institute, La Jolla;10Department of Neurology, Pediatrics, Maintenance Program, NCI-Designated Cancer Center, Sanford-Burnham Massachusetts; Departments of6Neurology and Neurological Sciences and Institute, Stanford University Medical Center, Stanford;9Tumor Initiation and Genomics, Dana-Farber Cancer Institute and Harvard Medical School;3Pediatric Hematology/Oncology, Boston Children's Hospital;4Center for Cancer Genome Characterization, Dana-Farber Cancer Institute and Division of Pediatric Hematology/Oncology, Boston Children's Hospital;61Center for Cancer Genome Characterization, Dana-Farber Cancer Institute, Boston; 2The Broad Institute of MIT and Harvard, Cambridge, Massachusetts; Departments of 2Neurology and Neurological Sciences and 2Neurosurgery, Stanford University School of Medicine; 9Stanford Cancer Institute, Stanford University Medical Center, Stanford; 9Tumor Initiation and Maintenance Program, NCI-Designated Cancer Center, Sanford-Burnham Medical Research Institute, La Jolla; 10Department of Neurology, Pediatrics, and Neurosurgery, University of California, San Francisco, California; and 11Department of Immunology, Genetics and Pathology, Science for Life Laboratory, Rudbeck Laboratory, Uppsala University, Uppsala, Sweden

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Abstract

Purpose: MYC-amplified medulloblastomas are highly lethal tumors. Bromodomain and extraterminal (BET) bromodomain inhibition has recently been shown to suppress MYC-associated transcriptional activity in other cancers. The compound JQ1 inhibits BET bromodomain-containing proteins, including BRD4. Here, we investigate BET bromodomain targeting for the treatment of MYC-amplified medulloblastoma.

Experimental Design: We evaluated the effects of genetic and pharmacologic inhibition of BET bromodomains on proliferation, cell cycle, and apoptosis in established and newly generated patient- and genetically engineered mouse model (GEMM)-derived medulloblastoma cell lines and xenografts that harbored amplifications of MYC or MYCN. We also assessed the effect of JQ1 on MYC expression and global MYC-associated transcriptional activity. We assessed the in vivo efficacy of JQ1 in orthotopic xenografts established in immunocompromised mice.

Results: Treatment of MYC-amplified medulloblastoma cells with JQ1 decreased cell viability associated with arrest at G1 and apoptosis. We observed downregulation of MYC expression and confirmed the inhibition of MYC-associated transcriptional targets. The exogenous expression of MYC from a retroviral promoter reduced the effect of JQ1 on cell viability, suggesting that attenuated levels of MYC contribute to the functional effects of JQ1. JQ1 significantly prolonged the survival of orthotopic xenograft models of MYC-amplified medulloblastoma (P < 0.001). Xenografts harvested from mice after five doses of JQ1 had reduced the expression of MYC mRNA and a reduced proliferative index.

Conclusion: JQ1 suppresses MYC expression and MYC-associated transcriptional activity in medulloblastomas, resulting in an overall decrease in medulloblastoma cell viability. These preclinical findings highlight the promise of BET bromodomain inhibitors as novel agents for MYC-amplified medulloblastoma.

Clin Cancer Res; 20(4); 912–25. © 2013 AACR.

Introduction

Medulloblastoma is the most common malignant brain tumor of childhood (1). Patients with local disease receive surgical resection, radiotherapy, and chemotherapy, with 5-year overall survival exceeding 80% (2). These treatments cause significant therapy-related morbidity, including disabling cognitive deficits (3), growth failure, and increased risk of secondary malignancies (4). However,
Translational Relevance

Collectively, MYC, MYCN, and MYCL1 are the most commonly amplified oncogenes in medulloblastoma, and are associated with a dismal prognosis. The recent development of strategies to block MYC activity through the inhibition of bromodomain and extraterminal (BET) bromodomain proteins represents a possible novel therapeutic strategy for these tumors. Here, we report that JQ1, a potent inhibitor of BET bromodomain proteins, results in both reduced cell proliferation and prominent apoptosis using in vitro models of MYC-amplified medulloblastoma, and prolongs survival in xenograft models. We confirm effective downregulation of MYC-related pathways with JQ1 and suppression of the expression of MYC. We also show reduced cell proliferation with JQ1 treatment of cells derived from MYCN-driven tumors harvested from a genetically engineered mouse model. BET bromodomain inhibition, therefore, represents a novel therapeutic strategy for children with MYC-amplified medulloblastoma. These data support further evaluation in early-phase clinical trials.

despite intensive chemotherapy and radiotherapy, the overall survival of “high-risk” patients remains dismal, with 10-year overall survival rates as low as 20% (5). Thus, tremendous impetus exists for the development of more effective treatments, based on known molecular targets, in medulloblastoma.

Medulloblastoma is a genetically heterogeneous disease, composed of molecular subtypes characterized by differing transcriptional signatures, genomic alterations, and clinical courses (6–9). The current consensus is of at least four distinct subtypes, including Wingless (WNT), Sonic Hedgehog (SHH), and groups 3 and 4 (10). Group 3 medulloblastomas have the worst prognosis, and are commonly metastatic and refractory to standard therapy, with 10-year overall survival rates of 39% (5, 6, 10). Amplifications of one of three members of the MYC family of genes (MYC, MYCN, and MYCL1) are found in several subtypes. Group 3 tumors are often associated with amplification of MYC (11), which is the most frequently observed amplification across multiple cancer types (12). Group 3 tumors without MYC amplification are often characterized by over-expression of MYC (6) or amplification of MYCN (11). MYCL1 amplifications have been reported in a few SHH tumor cases whereas SHH and group 4 tumors are enriched with amplifications of MYCN (11).

MYC and other transcription factors complicit in cancer are poor targets for small molecule development (13). The evidence that transcription of MYC and MYCN and subsequent activation of their downstream transcriptional programs can be targeted by BET bromodomain inhibition (13, 16, 17) presents a novel therapeutic strategy for patients with MYC-amplified medulloblastoma.

Materials and Methods

Ethics statement

Ethics approval was granted by the relevant human Institutional Review Board and/or animal ethics Institutional Animal Care and Use Committee (IACUC) research committees of Dana-Farber Cancer Institute (Boston, MA) and Stanford University (Stanford, CA).

Cell lines and culture

D283, D425, D458, and D556 were generously provided by Dr. Darrell Bigner (Duke University, Durham, NC). Daoy cells were obtained from the American Type Culture Collection. Cell lines were maintained in Dulbecco’s Modified Eagle Medium (Gibco) supplemented with 10% FBS (100106; Benchmark) and 1% penicillin–streptomycin with 1% glutamine (Gibco). U228, R256, R262, and R308 were a kind gift from Michael Bobola (University of Washington, Seattle, WA). MB002 cells were derived from an autopsy specimen of the leptomeningeal compartment from a child with metastatic, treatment-refractory (chemotherapy only) medulloblastoma. The MB002 primary tumor displayed histologic features of large-cell medulloblastoma and gene expression markers consistent with group 3 medulloblastoma (Supplementary Fig. S1A; ref. 11). MB004 cells were derived from the primary surgical resection of a tumor in a child whose tumor recurred after therapy. The MB004 primary tumor displayed focal anaplasia and gene expression markers consistent with group 3 and 4 medulloblastomas (see Supplementary Fig. S1; ref. 6). MYC amplification in the MB002 and MB004 cells was confirmed with NanoString nCounter v2 Cancer CN CodeSet, which estimates a copy number of 86 genes commonly amplified or deleted in cancer (Supplementary Table S1). Human neural stem cells were derived from subventricular zone tissue surgically excised during a functional hemispherectomy in a child with resection. MB004 cells were maintained in culture media with 1:1 Dulbecco’s Modified Eagle Medium (Gibco) and neural stem cell media (Gibco) supplemented with B27 (Gibco) EGF (02653, StemCell), fibroblast growth factor (GF003; Millipore), Heparin (07980, Stem Cell), and leukemia inhibitory factor (LIF; LIF1010; Millipore). The subventricular zone (SVZ)–derived neural stem cells were maintained similarly, with the exception of the LIF supplement. The MYC- and MYCN-driven medulloblastoma GEMM cell lines were derived and cultured as previously described (18, 19). Briefly, for Myc-amplified GEMM lines, cerebellar stem cells infected with Myc and Dnp53 retroviruses were transplanted into cerebella of NOD-SCID-IL2Rgammanull (NSG) mice. When mice became symptomatic, tumors were harvested and dissociated into single cell suspensions.
Patient-derived cell lines M8002 and M8004 were authenticated using sequence-tagged site (STS) fingerprinting. Cell lines obtained from the Bigner and Babola laboratories were authenticated using SNP250k or SNP6.0 arrays, which revealed copy-number alterations consistent with previously published karyotypes (12, 20).

Short hairpin RNA suppression
Lentiviral vectors encoding short hairpin RNAs (shRNA) specific for BRD4, MYC, and the control LACZ were obtained from The RNAi Consortium (Clones and sequence: shBRD4 TRCN0000021426, 5’CGTCCGATTGATGTTCTCCAA; shMYC TRCN0000039640, 5’CAGTTGAAACACAACATTGTAAGA; and shLacZ TRCN0000231726, 5’TGTTCGCCATTACCGAACCAT). Lentivirus was produced by the transfection of 293T cells with vectors encoding each shRNA (2 μg) with packaging plasmids encoding PSPAX2 and VSVG using Lipofectamine (Invitrogen, 56532). Lentivirus-containing supernatant was collected 48 and 72 hours after transfection, pooled, and stored at –80°C. Cells were infected (a ratio of 1:4 virus media) in polybrene-containing media (2.5 μg/mL). Mean titres were infected with lentiviral plasmids encoding shRNA with 1 μg/mL of JQ1R or JQ1S for 72 hours. Apoptosis was measured with Annexin V/PI staining. The Annexin V was labeled with Alexa Fluor (A13201; Invitrogen) and flow cytometry was performed per the manufacturer’s guidelines.

Protein extraction and immunoblotting
MYC-amplified cells were lysed in boiling RIPA (radioimmunoprecipitation assay) lysis buffer containing protease and phosphatase inhibitors, and centrifuged at 13,000 × g for 10 minutes. For MYCN-amplified lines, Western blot analysis was performed as previously described (19) with the following modification: lysis buffer with 1% SDS. Supernatant was mixed with 4× SDS sample buffer, boiled for 10 minutes, and subjected to SDS-PAGE on 4% to 12% gradient gels. Blots were probed with antibodies against BRD4 (12183; Cell Signaling Technology), MYC (sc-764; Santa Cruz Biotechnology), MYCN (ab-16898; Abcam), β-tubulin (MAB 3408; Millipore), and actin (sc-1615; Santa Cruz).

DNA extraction and real-time reverse transcriptase PCR
DNA was extracted with the RNeasy Kit (Qiagen). cDNA was synthesized from 1 μg RNA using High Capacity RNA-to-cDNA kits (Applied Biosystems). Real-time reverse transcriptase (RT-PCR) was performed using SYBR Green master mix (Applied Biosystems). Cycling was performed as follows: 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds. This was followed with a dissociation stage of 95°C for 15 seconds, 60°C for 30 seconds, and 95°C for 15 seconds. Primers for BRD4, MYC, and β-actin are listed in Supplementary Table S2. Samples were amplified in triplicate and data were analyzed using the ΔΔCt method.

Copy-number analysis
Relative copy-number estimates were generated from published Affymetrix SNP 6.0 data for 1,073 tumors (11) using comparison data from 131 normal samples and an analytic pipeline described in detail elsewhere (Tabak and colleagues, in preparation). Briefly, signal intensities for each probe were normalized to uniform intensity values and merged to form probe set–level values using SNPFileCreator, a Java implementation of dChip (21, 22). Marker-level intensities were calibrated to DNA copy-number levels using Birdseed for single-nucleotide polymorphism (SNP) markers (23) and using the results of experiments with cell lines with varied copy numbers of the X chromosome for copy-number probes (Tabak and colleagues, in preparation). Regions of frequent germline copy-number variation were identified using a large bank of normal tissue samples and excluded from the data (Tabak and colleagues, in preparation). Noise was reduced by applying tangent normalization (12), followed by circular binary segmentation (24, 25). Data were mean centered for each sample. Amplifications were defined as greater than

Flow cytometry
Cell-cycle analysis was performed by measuring DNA content by propidium iodide (PI)–stained cells treated with 1 μmol/L of JQ1R or JQ1S for 72 hours. Apoptosis was measured with Annexin V/PI staining. The Annexin V was labeled with Alexa Fluor (A13201; Invitrogen) and flow cytometry was performed per the manufacturer’s guidelines.

Cell viability assays following treatment with JQ1 or shRNA suppression
To assess responsiveness to JQ1, 1,000 cells were plated in 96-well plates in serial dilutions of either JQ1R or JQ1S, in triplicate. Cell viability was measured by assessing ATP content at 0, 24, 48, 72, 96, and 120 hours using Cell Titre-Glo (Promega) according to the manufacturer’s instructions. Mean ± SD was calculated. Nonlinear dose–response curves were applied to the data using GraphPad Prism.

To assess the dependence of cells on BRD4 or MYC, cells were infected with lentiviral plasmids encoding shRNA. Forty-eight hours after infection, 1,000 cells were plated in each well of 96-well plates, in triplicate, in media containing puromycin (1 μg/mL). Cell viability was measured by assessing ATP content using Cell Titre-Glo (Promega), and results were normalized to baseline. Mean ± SD was calculated.

Flow cytometry
Cell-cycle analysis was performed by measuring DNA content by propidium iodide (PI)–stained cells treated with 1 μmol/L of JQ1R or JQ1S for 72 hours. Apoptosis was measured with Annexin V/PI staining. The Annexin V was labeled with Alexa Fluor (A13201; Invitrogen) and flow cytometry was performed per the manufacturer’s guidelines.

Protein extraction and immunoblotting
MYC-amplified cells were lysed in boiling RIPA (radioimmunoprecipitation assay) lysis buffer containing protease and phosphatase inhibitors, and centrifuged at 13,000 × g for 10 minutes. For MYCN-amplified lines, Western blot analysis was performed as previously described (19) with the following modification: lysis buffer with 1% SDS. Supernatant was mixed with 4× SDS sample buffer, boiled for 10 minutes, and subjected to SDS-PAGE on 4% to 12% gradient gels. Blots were probed with antibodies against BRD4 (12183; Cell Signaling Technology), MYC (sc-764; Santa Cruz Biotechnology), MYCN (ab-16898; Abcam), β-tubulin (MAB 3408; Millipore), and actin (sc-1615; Santa Cruz).

RNA extraction and real-time reverse transcriptase PCR
RNA was extracted with the RNeasy Kit (Qiagen). cDNA was synthesized from 1 μg RNA using High Capacity RNA-to-cDNA kits (Applied Biosystems). Real-time reverse transcriptase (RT-PCR) was performed using SYBR Green master mix (Applied Biosystems). Cycling was performed as follows: 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds. This was followed with a dissociation stage of 95°C for 15 seconds, 60°C for 30 seconds, and 95°C for 15 seconds. Primers for BRD4, MYC, and β-actin are listed in Supplementary Table S2. Samples were amplified in triplicate and data were analyzed using the ΔΔCt method.

Copy-number analysis
Relative copy-number estimates were generated from published Affymetrix SNP 6.0 data for 1,073 tumors (11) using comparison data from 131 normal samples and an analytic pipeline described in detail elsewhere (Tabak and colleagues, in preparation). Briefly, signal intensities for each probe were normalized to uniform intensity values and merged to form probe set–level values using SNPFileCreator, a Java implementation of dChip (21, 22). Marker-level intensities were calibrated to DNA copy-number levels using Birdseed for single-nucleotide polymorphism (SNP) markers (23) and using the results of experiments with cell lines with varied copy numbers of the X chromosome for copy-number probes (Tabak and colleagues, in preparation). Regions of frequent germline copy-number variation were identified using a large bank of normal tissue samples and excluded from the data (Tabak and colleagues, in preparation). Noise was reduced by applying tangent normalization (12), followed by circular binary segmentation (24, 25). Data were mean centered for each sample. Amplifications were defined as greater than
a relative copy number of 2.4. For samples with a relative copy number of 2.4 to 3, we applied the ABSOLUTE algorithm (26), and confirmed that each of these samples had an absolute copy number of greater than three copies.

**Genome-wide expression analysis**

Previously published microarray expression and copy-number data (11) were obtained from the Gene Expression Omnibus (GEO; GSE37385 and GSE37382). The expression data were obtained using the GEOImporter module in GenePattern. $Z$ scores of gene expression values of genes within samples were calculated. For analyses of gene sets enriched among samples exhibiting high expression of MYC family members ($z$ score $> 1$ for MYC, $z$ score $> 0.85$ for MYCL1, and $z$ score $> 1.5$ for MYCN), gene set enrichment analysis (GSEA; refs. 27, 28) was performed using the C2 canonical pathway (CP) gene sets and seven additional gene sets from The Molecular Signatures Database (MSigDB) that represent MYC activation signatures (Supplementary Table S2). Gene sets with a nominal $P$ value of less then 0.05 were considered significant.

To examine the effect of JQ1 on global gene transcription, cell lines were treated with JQ1R or JQ1S (1 $\mu$mol/L for 24 hours) and RNA was extracted. Gene expression profiles were assayed using Affymetrix Human Gene 1.0 ST microarrays (Affymetrix). Affymetrix CEL files were normalized using Robust Multi-Array average (RMA) (29). Expression-array data have been deposited in the GEO portal under the accession number GSE51020. Comparative marker selection analysis (30) between JQ1S- and JQ1R-treated cells was performed in GenePattern using the default settings.

The recently described JQ1 consensus signature (16) was applied to the gene expression profiles using the 52 genes identified as being significantly differentially expressed following treatment with JQ1. Agglomerative hierarchical clustering was performed using pairwise complete linkage and a Pearson correlation metric across both samples and genes. To identify gene sets differentially expressed following treatment with JQ1, GSEA was performed using the same customized C2 (CP) gene sets (MSigDB) with the seven additional MYC activation gene sets. Gene sets with a nominal $P$ value of less then 0.05 were considered significant.

**In vivo experiments**

In vivo efficacy studies were performed in accordance with protocols approved by the IACUC at Stanford University. Briefly, MB002 cells were transduced with a GFP-luciferase lentiviral expression construct and FACS sorted to obtain 30,000 GFP-luciferase–positive cells that were then injected with stereotaxic guidance into the cerebellum of 4- to 6-week-old NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ mice (The Jackson Laboratory). To confirm engraftment, mice were administered d-luciferin (75 mg/kg; Promega) and imaged on a Xenogen IVIS2000 (PerkinElmer) 14 days after injection. Mice were randomized into treatment and control groups ($n = 5$ mice/group) and administered JQ1-S (50 mg/kg in 1:10 solution of DMSO:10% cyclodextrin) or vehicle alone (1:10 solution of DMSO:10% cyclodextrin), daily via intraperitoneal injection, until euthanasia was required. Tumor growth was monitored by in vivo imaging systems (IVIS) imaging at 14 and 21 days of treatment. Statistical significance for Kaplan–Meier analysis was determined by the log-rank (Mantel–Cox) test.

For immunohistochemistry analysis of xenografted medulloblastomas, 4- to 6-week-old NSG mice received intracerebellar injections of MB002 cells (30,000 cells) and were administered JQ1-S (50 mg/kg twice daily; $n = 3$) or vehicle ($n = 3$), for five doses and then euthanized. Brains were carefully dissected and either frozen in RNAlater (Qiagen) or preserved in 4% paraformaldehyde and subsequently embedded in paraffin. RNA was extracted from frozen cerebellum using the RNeasy Kit (Qiagen) as per the manufacturer’s instructions.

**Immunohistochemistry**

JQ1- and vehicle-treated MB002 xenografts were harvested, rinsed in PBS, and fixed in 4% paraformaldehyde overnight at 4°C. Then, 5-μm-thick sections were mounted on poly-d-lysine–coated slides and treated with xylene, followed by several changes of graded alcohol. Antigen retrieval was performed by application of citrate buffer pH 6.00 for 20 minutes. Slides were then incubated with anti-Ki67 (Lab Vision; SP6 RM-9106-S, lot 9106S1210D) overnight at 4°C. Cells were washed with several changes of PBS, and secondary antibody conjugated to horseradish peroxidase was applied and detected using the Dako Envision Kit for 3,3′-diaminobenzidine.

**Immunofluorescence**

Primary medulloblastoma cells (MB002) were cultured in 12-well plates at a density of $1 \times 10^5$ cells per well and treated with vehicle, (S)-JQ1 (500 nmol/L and 1 $\mu$mol/L for 6 and 12 hours). Cells were centrifuged at 1,000 rpm for 5 minutes, washed in PBS, and mechanically dissociated for 5 minutes at 37°C. Single cell suspensions were transferred to coverslips precoated with poly-L-lysine (10 μg/mL in double-distilled water, catalog number P6516; Sigma-Aldrich) and then fixed with 4% paraformaldehyde for 15 minutes. Immunolabeling was carried out with the antibody anti-BAD (C-7; 1:400, SC8044; Santa Cruz), detected by Cy3-conjugated secondary Donkey anti-mouse antibody (1:200; JacksonImmuno Research) and visualized by confocal fluorescence microscopy (Leica DM5500 B; Leica Microsystems).

For statistical analysis, $P$ values were calculated using the Fisher, t tests, or Pearson, as appropriate. ANOVA with correction was used for the comparison of multiple groups.
Results

Medulloblastomas exhibit several indicators of MYC pathway activation

We evaluated indicators of MYC pathway activation using integrated data sets of genome-wide copy-number estimates from 1,071 medulloblastomas and corresponding gene expression data for 282 medulloblastomas (11). These tumors comprised 79 Wnt subgroup medulloblastomas, 265 SHH subgroup tumors, 168 group 3 medulloblastomas, and 313 group 4 tumors, as determined by exon array or nanoString analysis (11). Subtype designation was not available for 245 tumors. We evaluated copy numbers and expression of all three MYC family members (MYC, MYCN, and MYCL1) and nine signatures of MYC pathway activation obtained from the Gene Set Enrichment Database.

We found that 23% of all medulloblastomas exhibit amplifications of one or more of MYC, MYCN, or MYCL1 (9%, 12%, and 2% of all tumors, respectively; Fig. 1A). Group 3 tumors exhibit amplifications of MYC family members approximately three times as frequently (44% of cases) as the other subtypes (13%, 19%, and 16% among Wnt, SHH and group 4 tumors, respectively). This enrichment was most profound for MYC; group 3 tumors accounted for 70% of medulloblastomas exhibiting MYC amplifications. Amplifications of MYCN were observed slightly more often in SHH and group 4 tumors (38% and 35% of all MYCN amplifications, respectively) than in group 3 tumors (10%).

Amplifications of MYC and MYCN were anticorrelated ($P < 0.05$; Fig. 1B), suggesting that they have similar functional effects. Anticorrelated genetic alterations often indicate functional redundancies (31–33) because redundant alterations are not required by the same tumor. Although there was a trend toward anticorrelation between amplification of MYCL1 and either MYC or MYCN, this did not reach significance ($P = 0.8$ and 0.8, respectively), perhaps because MYCL1 amplifications were observed so infrequently.

Amplifications of each MYC family member were also associated with increased expression of its gene transcript ($P < 0.0001$ in all cases). Indeed, high expression of MYCN or MYCL1 tends to be found exclusively in medulloblastomas with amplification of those genes. High MYC expression, however, is often present in samples without MYC amplification, suggesting alternative mechanisms for its upregulation (14). We assessed MYC pathway activation by summing the expression levels of 68 previously published genes known to be upregulated by MYC (14). We obtained similar results with GSEA using nine signatures of MYC activation (Supplementary Table S2) and comparing tumors with high expression of any MYC to tumors with low expression of all MYCs. Among the nine MYC activation signatures present in our gene sets, six were significantly associated with high MYC expression, five with MYCN, and four with MYCL1, respectively (Supplementary Table S3).

Medulloblastomas that exhibited indicators of MYC activation also exhibited high expression of genes observed to be downregulated with treatment with the BET bromodomain inhibitor JQ1 (16). Genes downregulated by JQ1 were previously identified in tumors from multiple lineages, including multiple myeloma, leukemia, and neuroblastoma (16). We found a positive correlation between the expression of genes that are targeted by JQ1 and MYC activation signatures in medulloblastoma (Fig. 1D). We also observed positive correlations between expression of genes targeted by JQ1 and amplifications of MYC ($P = 0.003$) and MYCN ($P < 0.05$). The finding that medulloblastomas with indicators of MYC activation exhibit gene expression profiles that are opposite to the signature of JQ1 is not surprising, because the JQ1 signature has already been shown to reflect the downregulation of MYC activity (16). Nevertheless, these findings raise the hypothesis that treatment with JQ1 may limit MYC activity and suppress the proliferation of MYC-driven medulloblastomas.

JQ1 reduces cell proliferation in MYC- and MYCN-amplified medulloblastoma cells

We examined the efficacy of treatment with JQ15 in six patient-derived medulloblastoma cell lines documented to have MYC amplification relative to five non-MYC-amplified medulloblastoma lines, and human neural stem cells (34, 35). Western immunoblotting of the patient-derived cell lines confirmed increased expression of MYC in lines with MYC amplification (Supplementary Fig. S1B). No patient-derived MYCN-amplified medulloblastoma cell lines have been generated to date; therefore, we evaluated JQ1 activity in the setting of Mycn amplification using tumor cells derived from recently developed mouse models of group 4 MYNC-amplified medulloblastomas (18, 36). We also evaluated JQ1 activity in a murine model of group 3 MYC-driven medulloblastomas. The activity of JQ1 was initially assessed by comparing proliferation rates in the presence of the active stereoisomer of JQ1 (JQ1S) to proliferation rates in the presence of an inactive stereoisomer, JQ1R (13).

In all MYC-amplified patient-derived cell lines, treatment with JQ1S for 48 hours at doses less than 1 μmol/L resulted in 57% to 69% reduction in cell viability compared with treatment with JQ1R (Fig. 2A). Each MYC-amplified line had an IC$_{50}$ of less than 1 μmol/L. In contrast, cell viability of non-MYC-amplified cell lines (Fig. 2B) and neurons derived from the SVZ (Fig. 2C) was not substantially altered by treatment with either JQ1S or JQ1R. Cells with MYC amplification exhibited up to 5-fold increases in cell
number by day 4 of treatment with JQ1R, but showed no increase in cell numbers with JQ1S treatment over the same time period (Supplementary Fig. S2A).

Treatment with JQ1S also reduced the proliferation of cells derived from a murine model of group 3–4 medulloblastoma with MYCN overexpression. In these cells, treatment with JQ1 reduced viability by 75% compared with treatment with JQ1R (Fig. 2D). Medulloblastoma cells from a mouse model with MYC overexpression exhibited an even greater (91%) sensitivity to treatment with JQ1S (Fig. 2D). Taken together, these data show that JQ1 is efficacious in reducing cell viability in medulloblastoma cell lines driven by MYC and also in cells from a murine model of MYCN-driven medulloblastoma, with

Figure 1. Amplifications of the MYC isoforms MYC, MYCN, or MYCL1 are common and mutually exclusive in medulloblastoma. A, fractions of tumors in the Wnt, SHH, group 3, and group 4 subtypes of medulloblastoma with amplifications of MYC, MYCN, or MYCL1 in 1,071 medulloblastoma samples. B, left, scatter plot depicting copy numbers of different MYC isoforms in 1,071 medulloblastoma samples. P value depicts significant anticorrelation between MYC and MYCN. Right, Venn diagram showing medulloblastomas with high expression of MYC (z score > 1), MYCN (z score > 1.5), or MYCL1 (z score > 0.85). P value depicts significant anticorrelation between MYC and MYCN. C, left, correlation of MYC activation scores with copy numbers of MYC isoforms and correlation of MYC activation scores with gene expression of MYC isoforms. P values depict significant differences in MYC activation scores. ANOVA P value < 0.0001. D, left, association between MYC activation and JQ1 consensus score. P value depicts significant correlation as determined by the Pearson correlation test. Right, the association between JQ1 consensus score and MYC amplifications. P values depict significant differences in JQ1 scores. ANOVA P value < 0.05.
minimal effect observed in non-MYC-amplified medulloblastoma cells or neural stem cells.

**JQ1 reduces cell viability by inducing G1 arrest and apoptosis in MYC-amplified medulloblastoma cell lines**

Treatment with JQ1S significantly affected cell cycling of the MYC-driven cell lines. We profiled cell cycling by flow-cytometry measurement of PI-stained cells treated for 72 hours with either 1 μmol/L of JQ1S or JQ1R. In six patient-derived MYC-amplified cell lines, and one Mycn-driven GEMM-derived cell line, we observed that treatment with JQ1S resulted in G1 arrest, with an increased percentage of cells in G1 and reduction of cells in S phase (Fig. 3A). Across all MYC-amplified lines, treatment with JQ1S reduced the number of cells in S phase by approximately 50% compared with JQ1R (21% ± 3% vs. 43% ± 5%; P < 0.001) and increased the number of cells in G1 (73 ± 4% vs. 51 ± 6%; P < 0.001; Fig. 3B).

Treatment with JQ1S also induced apoptosis in patient-derived MYC-amplified cells. We assessed the induction of apoptosis in cells treated with 1 μmol/L JQ1S or JQ1R for 72 hours by flow cytometry analysis of Annexin V/PI staining (Fig. 3C). MB004 was observed to have an increase in the number of necrotic cells, whereas in all other cell lines, there was a clear increase in apoptosis noted. When the results of all cell lines were pooled (including MB004), JQ1S almost tripled the number of apoptotic cells (8% ± 2% vs. 22.5% ± 4%, P = 02; Fig. 3D). As an independent correlation of induction of apoptosis, we treated MB002 cells with JQ1 and observed induction of the apoptotic protein, BAD, within 6 hours of treatment (Fig. 3D. right panel). These data suggest that JQ1S reduces the cell viability of MYC-driven medulloblastoma cell lines by inducing G1 arrest and apoptosis.

**BRD4 suppression attenuates expression of MYC in medulloblastoma cells**

We hypothesized that the effects of JQ1 in the medulloblastoma cells were mediated in large part by the decreased activity of MYC through inhibition of BRD4. BRD4 has previously been shown to be a cofactor for MYC-dependent transcription in multiple cell types (13), and JQ1 has previously been shown to have higher affinity for binding domains of BRD4 than other bromodomain-containing proteins (13).
We, therefore, suppressed the expression of either MYC or BRD4 in each of the six MYC-amplified patient-derived cell lines using MYC- and BRD4-directed shRNAs. We measured the proliferation and compared the results with controls treated with LacZ-targeted shRNAs. Suppression of MYC and BRD4 protein levels was confirmed by immunoblotting (Fig. 4A and Supplementary Fig. S3).

In all cell lines, suppression of either MYC or BRD4 resulted in greater than 50% reductions in proliferation relative to cells treated with LacZ-targeted shRNAs (Fig. 4B). We observed absolute decreases in cell numbers among four lines with both MYC suppression and BRD4 suppression.

In all cell lines, suppression of BRD4 was also associated with a reduction of MYC mRNA, by an average of 45% relative to the LacZ-suppressed controls ($P < 0.0001$; Fig. 4C). We also observed a reduction in MYC protein levels in cells following suppression of BRD4 (Fig. 4A and Supplementary Fig. S3). This was most pronounced in the D425 and D556 cell lines. Suppression of BRD4 has previously been shown to decrease the MYC expression in other cell types (13). The finding that BRD4 suppression resulted in attenuation of MYC mRNA and protein suggested that JQ1 also exerted its effects in part through suppression of MYC.

**JQ1 suppresses MYC activation pathways and expression of MYC and MYCN themselves**

To determine the transcriptional effects of JQ1 treatment, we performed genome-wide expression profiling of five patient-derived MYC-amplified cell lines treated with 1 µmol/L of either JQ1S or JQ1R for 24 hours. We identified 43 genes that were significantly downregulated by JQ1S (false discovery rate (FDR) < 0.1; Supplementary Table S4). No genes were significantly upregulated at this significance threshold.

The JQ1S-treated cells exhibited significant enrichment of a signature of JQ1 treatment derived from multiple myeloma, leukemia, and neuroblastoma cells ($P < 0.0001$; Fig. 5A; 16), and significant attenuation of MYC activity ($P < 0.05$ in all cases; Fig. 5B). An unbiased screen of pathways altered by treatment with JQ1S (using the gene set enrichment algorithm and the C2 (CP) set of signatures).
indicated significant alteration of 46 pathways \( (P < 0.05; \text{Supplementary Table S5}) \). Four of these 46 pathways represented MYC activation signatures (Fig. 5B), and were downregulated following treatment with JQ1S (compared with cells treated with JQ1R).

In all patient-derived MYC-amplified cell lines, treatment with 1 \( \mu \text{mol/L} \) of JQ1S also led to decreased expression of MYC itself. Levels of MYC mRNA declined by an average of 46\% (range, 29\%–78\%; \( P < 0.0001; \text{Fig. 5C} \)), and these changes were associated with decreased expression of the MYC protein. We also examined the expression of MYC mRNA in three cell lines treated with lower doses of JQ1S, and found attenuation of MYC mRNA expression at doses of JQ1S as low as 125 nmol/L (Supplementary Fig. S2B). Treatment of Mycn-overexpressing cells derived from tumors from the Glt1 \textit{Mycn} GEMM model with 0.5 \( \mu \text{mol/L} \) of JQ1 for 72 hours was associated with the suppression of Mycn mRNA and protein expression (Fig. 5C).

We next overexpressed MYC from an exogenous retroviral promoter in D283 cells and treated them with JQ1 to assess whether forced expression of MYC could rescue...
the cells from the effects of JQ1 (Fig. 5D). In cells infected with the empty pBabe retroviral vector, we observed reduced cell viability following treatment with JQ1S. In contrast, in cells infected with pBabe MYC, we observed minimal effect on cell viability following treatment with JQ1S.

Taken together, these results confirm that BET bromo-domain inhibition with JQ1 results in the downregulation of MYC and MYC activation pathways, resulting in reduced cell proliferation. JQ1 treatment also reduces MYC and MYCN expression in patient-derived MYC-amplified medulloblastoma cell lines and in cells derived from a MYCN-driven medulloblastoma mouse model. Forced expression of MYC from an exogenous promoter rescues D283 cells from the effects of JQ1S.

**Treatment with JQ1 prolongs survival of MYC-amplified medulloblastoma xenografts**

We examined the efficacy of JQ1 in vivo, using orthotopic xenografts generated by intracerebellar injections of MB002 cells. Compared with vehicle control, mice undergoing daily treatment with JQ1 (50 mg/kg/d) exhibited a significantly prolonged survival (Fig. 6A) with a slower rate of tumor growth at 14 and 21 days postinjection as indicated by bioluminescence (Fig. 6B). This was statistically significant on day 14 (P, 0.03). A cohort of
mice was sacrificed following five doses of JQ1 or vehicle treatment. We extracted RNA from the cerebellum of vehicle- or JQ1-treated mice and assessed the expression of MYC mRNA (Fig. 6C). We observed significant down-regulation of MYC mRNA expression following treatment with JQ1. Ki67 immunostaining confirmed a reduced proliferative index in tumors treated with JQ1 compared with vehicle controls (82% vehicle vs. 27% JQ1 treated, \( P < 0.0001 \); Fig. 6D).

Discussion

Our data provide the first preclinical evidence for a potential therapeutic role of BET bromodomain inhibition for MYC-amplified medulloblastoma. Furthermore, we show redundancy between amplification of the different MYC genes. We observed responsiveness to JQ1 therapy of all patient cell lines harboring MYC amplifications, and in a murine cell line of a Mycn-driven medulloblastoma model.

MYC-amplified medulloblastomas are characteristically resistant to conventional treatments, including radiotherapy and chemotherapy. In this study, the use of JQ1 as a single agent was able to reduce cell proliferation and tumor growth and prolong the survival of mice with intracranial xenografts. Thus, our data provide rationale for the development of clinical trials to assess the role of these agents in the treatment of patients with medulloblastoma.

Ongoing work, however, is required to determine optimal strategies to incorporate these agents into upfront therapy for children with newly diagnosed MYC-amplified medulloblastoma. Specifically, further work is required to assess strategies to combine BET bromodomain inhibition with other treatment modalities such as radiotherapy and chemotherapy. Specific predictors of sensitivity and resistance to JQ1 remain to be elucidated.

The anticorrelation between amplifications of MYC and MYCN, and the correlations between these amplifications (and amplifications of MYCL1) and expression of genes upregulated by MYC, indicate that these amplifications are associated with increased MYC activity. However, some samples without MYC amplification had high expression of MYC or of other genes upregulated by MYC. This observation...
suggests that other factors are also involved in the regulation of MYC activation pathways, and that some tumors without MYC amplification may benefit from therapeutics targeting MYC, including BET bromodomain inhibition. Although we observed minimal responsiveness of the non-MYC–amplified medulloblastoma cell lines to JQ1, it is possible that these lines may not represent the full spectrum of non-MYC–amplified medulloblastomas. For example, none of our non-MYC–amplified lines included either the Wnt or SHH subtypes. We are unable to speculate whether these subtypes may also have sensitivity to treatment with BET bromodomain inhibition.

Importantly, the inhibition of MYC family members and activation pathways may result in downregulation of other cell-signaling pathways in tumors that do not harbor amplification of MYC isoforms. This is particularly relevant in the SHH subtype. Both MYC (37) and MYCN (38–40) have been reported to interact with and regulate transcription factors involved in SHH signaling, raising the possibility that inhibition of MYC activation may also result in inhibition of the SHH pathway, and other pathways important in the pathogenesis of medulloblastoma.

We show that MYC-amplified medulloblastomas can be targeted by epigenetic modulation of oncogenes. BRD4 is an epigenetic reader that binds to e-N-lysine acetylation motifs (41). It is increasingly recognized that genomic alterations may result in global epigenetic dysregulation in pediatric brain tumors (42–44). Indeed, group 3 medulloblastomas have been found to harbor somatic copy-number alterations of genes involved in chromatin modeling (45, 46). These alterations frequently affect genes of modifiers of H3K27 methylation (46). Thus, it is possible that targeting other chromatin modifiers may also have activity in this group of tumors. Further work is required to define the interplay between methylation and acetylation of these histone marks to help determine whether the combination of different classes of epigenetic modulators may improve their efficacy.

We were unable to obtain any patient-derived medulloblastoma cell lines that harbor amplifications of either MYCN or MYCL1. Our integrative analysis of copy number and expression profiles suggest that both isoforms are associated with increased expression of MYC activation pathways, suggesting that BET bromodomain inhibition may be effective in tumors that harbor these amplifications. Indeed, BET bromodomain inhibition has been shown to suppress MYCN expression in patient-derived neuroblastoma cell lines (16). We have shown efficacy of JQ1 treatment in a cell line generated from a MYCN-driven mouse model; however, ideally this should be validated in patient-derived MYCN-amplified medulloblastoma cell lines.

In summary, we show that BET bromodomain inhibition results in the suppression of MYC activation pathways in MYC-driven medulloblastoma models, resulting in reduced cell viability, induction of G1 arrest, and apoptosis with prolongation of survival in xenograft models. These data provide a rationale for early-phase clinical trials for BET bromodomain inhibitors for children with this aggressive disease.

Disclosure of Potential Conflicts of Interest
R. Beroukhim is a consultant/advisory board member of Novartis. The Dana-Farber Cancer Institute has licensed drug-like derivatives of JQ1 prepared in the Bradner laboratory to Tensha Therapeutics for clinical translation as cancer therapeutics. Dana-Farber and Dr. Bradner have been provided minority equity shares in Tensha. Dr. Qi has a consultant agreement with Tensha Therapeutics.

Authors’ Contributions
Conception and design: P. Bandopadhyay, G. Berghold, J. Qi, R. Wechsler-Reya, M.W. Kieran, R. Beroukhim, Y.-J. Cho
Development of methodology: P. Bandopadhyay, S. Gholamin, S. Bolin, Y.-J. Cho
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): P. Bandopadhyay, G. Berghold, R. Nguyen, S. Gholamin, Y. Tang, R. Zeid, S. Masoud, N. Yue, W.I. Gibson, S. Mitra, S. Cheshier, K.-W. Liu, F.J. Swartling, Y.-J. Cho
Writing, review, and/or revision of the manuscript: P. Bandopadhyay, G. Berghold, R.R. Paolella, S. Cheshier, R. Wechsler-Reya, W.A. Weiss, M.W. Kieran, J.E. Bradner, R. Beroukhim, Y.-J. Cho
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): P. Bandopadhyay, G. Berghold, R. Nguyen, S. Schubert, S. Gholamin, F. Yu, J. Qi, R. Beroukhim, Y.-J. Cho
Study supervision: P. Bandopadhyay, S. Mitra, R. Beroukhim, Y.-J. Cho

Acknowledgments
The authors thank Vida Shokouhi and John Coller of the Stanford Functional Genomics Core, Christopher DelHeer and Karen Krantz at NanoString Technologies, Inc., Steve Avoliconc at Histo-Tec Laboratory, and John Daley and Suzan Lazo-Kallanian of the DFCI Flow Cytometry Core Facility for their technical assistance. The authors also thank Drs. Scott Pomeroy and Tenley Archer, Boston Children’s Hospital (Boston, MA), for their assistance and sharing of reagents.

Grant Support
This work was financially supported by the following sources: St. Baldrick’s Foundation Scholar Award (Y.-J. Cho), Jerome L. Hechtman Faculty Scholar Endowment (Y.-J. Cho), NIH K08-Cal176287 (Y.-J. Cho and W.A. Weiss); Stanford Center for Children’s Brain Tumors (Y.-J. Cho and S. Cheshier); Pediatric Low-Grade Astrocytoma Foundation (P. Bandopadhyay, G. Berghold, M.W. Kieran, and R. Beroukhim); Friends of DFIC (P. Bandopadhyay); the Sonagt Foundation (R. Beroukhim); Grey Matters Foundation (R. Beroukhim); Stop&Shop Pediatric Brain Tumor Program (P. Bandopadhyay and M.W. Kieran); the Mill Foundation for Kids (M.W. Kieran); Men’s Collaborative for Women’s Cancers (J.E. Bradner and R. Beroukhim); Damon-Runyon Cancer Research Foundation (J. Qi and J.E. Bradner); Nuovo-Soldati Foundation (G. Berghold); Philippe Foundation (G. Berghold); R01-Cal133091 (W.A. Weiss and R. Wechsler-Reya); NIH R01-Cal148699 (W.A. Weiss); Swedish Childrenhood Cancer Foundation (S. Bolin and F.J. Swartling); the Swedish Cancer Society (S. Bolin and F.J. Swartling); the Swedish Research Council (S. Bolin and F.J. Swartling); the Swedish Society of Medicine (S. Bolin and F.J. Swartling); the Swedish Research Council (S. Bolin and F.J. Swartling). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received August 19, 2013; revised November 6, 2013; accepted November 26, 2013; published OnlineFirst December 2, 2013.
References


Clinical Cancer Research

BET Bromodomain Inhibition of MYC-Amplified Medulloblastoma

Pratiti Bandopadhayay, Guillaume Bergthold, Brian Nguyen, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-13-2281

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2013/12/02/1078-0432.CCR-13-2281.DC1

Cited articles
This article cites 45 articles, 22 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/20/4/912.full#ref-list-1

Citing articles
This article has been cited by 22 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/20/4/912.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.