Targeting MYCN in Neuroblastoma by BET Bromodomain Inhibition

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INTRODUCTION

The interplay between master regulatory transcription factors and specific chromatin-associated coactivators is an emerging hallmark of cancer. Cancer genomic discovery efforts continue to reveal mutations in epigenetic modifiers, and laboratory efforts to validate functional dependencies are ongoing (1). Moreover, notable examples of successful commercial development of drugs targeting epigenetic modifiers have come to the fore, including the development of inhibitors of enzymatic “writers,” such as DNA methyltransferases for myelodysplastic syndrome, and inhibitors of enzymatic “erasers,” such as histone deacetylases for cutaneous T-cell lymphoma.

Modulation of the epigenetic regulators known as “readers” has recently emerged as a therapeutic strategy in cancer treatment. These epigenetic “readers” are structurally diverse proteins, which recognize and bind to covalent modifications of chromatin (2). One important modification associated with open chromatin and transcriptional activation is the side-chain acetylation of lysine residues on histone tails (3). The dominant mode of recognition of acetylated lysine residues is by bromodomains present in 47 human proteins (4–6). We and others have described the therapeutic potential of targeting one bromodomain-containing family important in regulating transcription, epigenetic memory, and cell growth: The bromodomain and extraterminal domain (BET) family is composed of BRD2, BRD3, BRD4, and BRDT. Numerous hematologic malignancies and the highly malignant solid tumor NUT midline carcinoma are responsive to BET inhibition in vitro and in mouse models (7–12).

Although disease-specific indications for drugs modifying epigenetic regulators have been uncovered, precise genomic biomarkers predictive of treatment response remain elusive. To date, the best validated genetic predictor of response to BET inhibitors is in a rare genetically defined subset of poorly differentiated squamous cell carcinomas (NUT midline carcinoma), in which the presence of recurrent t(15;19) chromosomal translocation results in the expression of the twin N-terminal bromodomains of BRD4 as an in-frame fusion with the NUT protein (13). High-throughput pharmacogenomic profiling offers the opportunity to reveal new insights into selective responses to drugs in defined cancer genotypes. Initial efforts to connect drug response with genotype in the NCI60 cell line panel have since been expanded to screening campaigns in large panels of genetically characterized cancer cell lines (14–17). These efforts have revealed both expected
Nearly all of the MYCN-amplified neuroblastoma cell lines confirmed to have MYCN amplification as a predictor of response to JQ1 treatment (Fig. 1C). More recently, the unexpected connections between response to PARP inhibitors and expression of the EWS/FLI fusion protein in Ewing sarcoma was elucidated in a screen of 130 drugs in more than 600 cancer cell lines (16). In an independent study of 24 anticancer drugs in 479 human cancer cell lines, new connections were also observed between small-molecule sensitivities and cell lineage, gene expression, and genotype (17).

We conducted a high-throughput pharmacogenomic screen to identify biomarkers of response to BET bromodomain inhibitors. The prototype ligand JQ1, a novel thieno-triazolo-1,4-diazepine, which displaces BET bromodomains from chromatin by competitively binding to the acetyl lysine recognition pocket, has been validated in numerous models, nominating it as an excellent chemical probe for high-throughput screening (7–10). In this study, we therefore queried a large compendium of genotypically characterized tumor cell lines to identify predictors of sensitivity to JQ1. We identified MYCN amplification as a top predictive marker of response to JQ1 treatment and characterized the mechanistic and translational significance of this finding in neuroblastoma, the most common extracranial solid tumor diagnosed in children, and a cancer notable for frequent MYCN amplification in patients with high-risk disease.

RESULTS

High-Throughput Pharmacogenomic Profiling Reveals MYCN Amplification as a Predictor of Response to Bromodomain Inhibitors

We first conducted an unbiased screen of a collection of 673 genetically characterized tumor-derived cell lines (16) to understand response and resistance to BET bromodomain inhibition, as a way to discover new opportunities for therapeutic development. Cell lines with response to JQ1 yielding IC_{50} ≤ 1 μmol/L and the maximum effect corresponding to the minimum measured viability E_{max} > 70% were designated as sensitive and all others were designated as resistant in a stringent classification schema. Cell lines arising from the pediatric solid tumor of neural crest origin, neuroblastoma, were identified as among the most JQ1 sensitive and MYCN amplification as the most predictive marker of sensitivity. 4 cell lines of 99 sensitive cell lines are MYCN amplified and 0 cell lines of 237 resistant cell lines are MYCN amplified. The two-tailed Fisher exact test returns a P value of 0.007 (Fig. 1A and B and Supplementary Table S1). We next determined expression level of MYCN in the neuroblastoma cell lines from the primary screen (Supplementary Fig. S1A) and evaluated the correlation of MYCN protein levels with JQ1 response. MYCN protein level is also substantially correlated with response to JQ1 treatment (Fig. 1C).

We retested one of the most highly sensitive MYCN-amplified cell lines from the screen, BE(2)-C, and multiple additional MYCN-amplified neuroblastoma cell lines confirmed to have elevated levels of MYCN protein (Supplementary Fig. S1B). Nearly all of the MYCN-amplified cell lines tested responded to JQ1 and a panel of structurally distinct BET bromodomain inhibitors, each of which conferred a dose-responsive, inhibitory effect on cell viability as well as growth over time (Fig. 1D–F and Supplementary Table S2). The only exception in this cell line panel was NGP, which was comparatively insensitive to all of the BET bromodomain inhibitors tested. Importantly, cell growth was not affected in any cell lines by the (−)-JQ1 enantiomer, which lacks activity against BET bromodomains in biochemical and biologic assays, further supporting an on-target mechanism of action. Similarly, we retested one of the least sensitive cell lines from the primary screen, the MYCN–wild-type neuroblastoma cell line SK-N-AS and a second MYCN–wild-type cell line SH-SY5Y not in the primary screen. As predicted, SK-N-AS was insensitive to the effects of JQ1, and SH-SY5Y was less sensitive than the MYCN-amplified cell lines based on E_{max} (Supplementary Fig. S2A and S2B and Supplementary Table S2).

JQ1 Induces Cell-Cycle Arrest and Apoptosis

To further characterize the phenotypic consequences of JQ1 treatment on responsive MYCN-amplified neuroblastoma cells, we determined its effects on cell-cycle arrest and apoptosis. JQ1 treatment induced a G_{0}/G_{1} arrest and a decrease in S-phase at 24 hours by flow-cytometric evaluation of propidium iodide (PI) staining (Fig. 2A and Supplementary Fig. S3). A progressive increase in cell death was noted, based on the increase in the sub-G_{1} fraction, with marked induction of apoptosis confirmed by annexin V staining at 72 hours (Fig. 2B). In contrast, the effect of JQ1 on cell cycle and apoptosis in MYCN–wild-type neuroblastoma was less pronounced (Supplementary Fig. S4A and S4B).

Bromodomain Inhibition Downregulates MYCN and c-MYC Transcriptional Programs in Neuroblastoma

Prior research from our laboratory has shown a selective effect of BET bromodomain inhibition on the expression and function of c-MYC in hematologic malignancies (8–10). We thus hypothesized that the selective sensitivity of neuroblastoma lines to BET inhibition may relate to implications of the function of the MYCN and c-MYC transcriptional activators. We profiled 2 of the highly sensitive neuroblastoma cell lines, BE(2)-C and Kelly, treated with 1 μmol/L JQ1 for 24 hours using genome-wide transcriptional profiling coupled with gene set enrichment analysis (GSEA; ref. 18). The top 50 differentially upregulated and downregulated genes are depicted in the heatmap in Fig. 3A, and the full list is reported in Supplementary Table S3. Consistent with the established biologic role of BET coactivators in chromatin-dependent transcriptional activation, there was a preponderance of downregulated genes in comparison with upregulated genes. Acute JQ1 treatment prompted a dynamic change in transcription, with 122 genes significantly upregulated and 193 genes significantly downregulated on the basis of permutation P < 0.05 and false discovery rate (FDR) < 0.05 for signal-to-noise in the comparison of all vehicle-treated versus all JQ1-treated samples. To assess the effects of JQ1 more specifically on transcriptional programs regulated by either MYCN or c-MYC, we interrogated the data with published, validated gene signatures for statistically significant enrichment by GSEA. GSEA seeks to estimate the significance of overrepresentation of...
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**Figure 1.** MYCN-amplified neuroblastoma is sensitive to the effects of BET bromodomain inhibition. **A,** antiproliferative activity of JQ1 was profiled in more than 650 cancer cell lines, revealing a broad range of sensitivity and resistance. Red dots are neuroblastoma cell lines with MYCN amplification based on SNP 6.0 arrays and/or high levels of protein expression. Black dots indicate neuroblastoma cell lines wild-type (WT) for MYCN and poor MYCN expression. Drug response is presented as ln(IC$_{50}$), plotted against the maximum effect corresponding to the minimum measured viability ($E_{\text{max}}$). **B,** distribution of $E_{\text{max}}$ and ln(IC$_{50}$) for MYCN–wild type versus MYCN–amplified cancer cell lines based on SNP 6.0 copy number analysis. P value was calculated using nonparametric Mann–Whitney test. Red squares indicate MYCN–amplified neuroblastoma cell lines. **C,** Spearman correlation between minimum measured viability ($E_{\text{max}}$), and the expression level of MYCN was normalized by actin level (ratio of MYCN/actin). **D,** structures of BET bromodomain inhibitors. (+)-JQ1, I-BET, and I-BET151 are all active and structurally distinct, BET bromodomain inhibitors. JQ1R is the inactive (−)-JQ1 enantiomer. **E,** Dose response of neuroblastoma cell line viability with BET bromodomain inhibitor treatment was measured by a luminescent ATP detection assay. Data represent mean ± SEM of 4 biologic replicates. **F,** 4 MYCN–amplified neuroblastoma cell lines were used to determine the effects of JQ1 on growth. Values over time are shown relative to the day 0 values, with error bars representing the mean ± SD of 8 replicates per condition.

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neuroblastoma (Fig. 3B and C). Because the genes downregulated by BET bromodomain inhibition in the gene expression dataset. The majority of the MYCN- and c-MYC-related gene sets were statistically enriched among genes downregulated by BET bromodomain inhibition in neuroblastoma (Fig. 3B and C). Because the MYCN signature in the Molecular Signature Database (MSigDB) was derived in small cell lung cancer cells (19), we sought to develop a custom MYCN-upregulated signature in neuroblastoma cells, using a publicly available dataset of primary neuroblastoma tumors characterized for MYCN amplification status (20). This MYCN signature was also highly downregulated by JQ1 treatment of neuroblastoma cell lines based on GSEA (Fig. 3D and Supplementary Table S4). An open-ended enrichment analysis was next conducted, as a measure of specificity, on the entire set of transcription factor target gene signatures available from MSigDB (21). In almost all cases, gene sets defined by adjacency to MYC-binding motifs (both MYCN and c-MYC) were highly enriched in genes whose expression is suppressed by JQ1 in neuroblastoma (Fig. 3E).

Recently, we reported the unexpected downregulation of MYC expression in multiple myeloma and acute leukemia models by BET bromodomain inhibitors (8–10). Localization of BRD4 to strong enhancer elements proximal to MYC prompted the consideration that expression of amplified MYCN may, indeed, be dependent on BET bromodomains. We therefore evaluated the effects of JQ1 treatment on MYCN expression. Although not in the top 100 differentially expressed genes, MYCN expression was significantly downregulated in the genome-wide expression profiling of both neuroblastoma cell lines for 3 of 4 PrimeView representative probes, with an average reduction of 50% (Supplementary Table S5). These results were confirmed in the 3 neuroblastoma cell lines responsive to JQ1 [BE(2)-C, Kelly, and LAN-1] by reverse transcriptase PCR (RT-PCR) and immunoblot (Fig. 3F and G). In contrast, MYCN expression was not downregulated in the cell line most insensitive to JQ1, NGP. Finally, c-MYC expression level was reduced in response to JQ1 treatment across all the MYCN-amplified or nonamplified neuroblastoma cell lines (Supplementary Fig. S5). Taken together, these data provide strong evidence that the MYCN program is downregulated in neuroblastoma cell lines by JQ1 treatment, and as observed for MYC in several hematologic disease models, BET bromodomain inhibition targets the expression of MYCN itself.

**Figure 2.** JQ1 treatment induces a G0-G1 arrest and apoptosis in neuroblastoma cell lines. Indicated neuroblastoma cell lines were treated with 1 μmol/L JQ1 for (A) 24, 48, and 72 hours before cell-cycle analysis or (B) 72 hours before measuring apoptosis by annexin V staining detected by flow cytometry.

 Genome-Wide Transcriptional Effects of BET Bromodomain Inhibition in Cancer Cells

Although the transcriptional programs altered by BET bromodomain inhibition in individual diseases have been established, an integrated analysis has not yet been conducted. To establish neuroblastoma-specific and canonical BET bromodomain-dependent transcriptional pathways, we analyzed 3 datasets profiling the transcriptional changes associated with JQ1 treatment in cancer cell lines [multiple myeloma (8), acute myelogenous leukemia (AML; ref. 9), and neuroblastoma] to determine whether a robust, cell context-independent JQ1 signature exists. First, we generated signatures of genes whose expression is regulated (up and down) by JQ1 treatment in neuroblastoma and used these as a gene set to query both the multiple myeloma and the AML transcriptional profiling datasets. Significant enrichment of these neuroblastoma JQ1 signatures was noted in the hematopoietic malignancies with an FDR < 0.25 (Supplementary Fig. S6A).

Next, we developed hematopoietic malignancy signatures for both upregulated and downregulated genes with JQ1 treatment, using the multiple myeloma and the AML datasets. We applied these signatures as gene sets to query our
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Figure 3. Inhibition of MYCN- and c-MYC-dependent transcription by JQ1 treatment of neuroblastoma cells. A, heatmap of the top 50 down- and upregulated genes following 24 hours of 1 μmol/L JQ1 treatment of neuroblastoma cell lines based on a signal-to-noise ratio (SNR) score and P < 0.05. Data are presented as row normalized. B, GSEA showing downregulation of MYCN- and c-MYC-dependent gene sets and representative sets of genes with proximal promoter regions containing MYCN- or MYC-MAX-binding sites in the transcriptional profiles of neuroblastoma cell lines treated with JQ1. Depicted is the plot of the running sum for the MsigDB gene set within the JQ1 neuroblastoma dataset, including the maximum enrichment score and the leading edge subset of enriched genes. C, table of selective gene sets enriched among genes downregulated by JQ1 in neuroblastoma cell lines based on GSEA (size, number of genes in each set, NES, normalized enrichment score). D, GSEA showing downregulation of a custom MYCN gene set derived from the comparison of MYCN-amplified versus MYCN-nonamplified primary neuroblastoma tumors. E, quantitative comparison of all transcription factor target gene sets available from the MsigDB by GSEA for downregulation in JQ1-treated neuroblastoma cells. Data are presented as a scatterplot of FDR versus NES for each evaluated gene set. Red indicates sets for either MYCN or c-MYC and gray for other transcription factors. F, neuroblastoma cell lines were treated with 1 μmol/L JQ1 or DMSO for 8 hours. After RNA extraction, level of MYCN transcript was quantified. Expression values are shown relative to the DMSO condition for each cell line. Error bars represent mean ± SD of 4 technical replicates. *, P < 0.001 calculated using a one-way ANOVA with Bonferroni correction comparing JQ1 treatment with DMSO within a cell line. G, Western blot analyses for MYCN on whole-cell extracts from 4 neuroblastoma cell lines treated with 1 μmol/L JQ1 or DMSO for 24 hours.
neuroblastoma transcriptional profiling data by GSEA. Both of these signatures were consistently enriched in the expected directions in neuroblastoma cell lines treated with JQ1 (Supplementary Fig. S6B). Moreover, all of the JQ1 signatures were enriched in the treatment of AML cells with a structurally distinct BET bromodomain inhibitor, I-BET151 (11), consistent with an on-target effect of JQ1 (Supplementary Fig. S7). Finally, we developed a consensus signature across all 3 datasets (Fig. 4A) and determined the relevance to primary human neuroblastoma. The consensus JQ1 signature was significantly associated with MYCN status and stage, suggesting that the signature has relevance beyond cancer cell lines grown in culture to bona fide human disease (Fig. 4B). We next determined the biologic and functional connections among those genes. Notably, although many individual gene sets were significant, marked enrichment was noted for multiple gene sets associated with MYCN and c-MYC, E2F, cell cycle, transcription/translation, metabolism, and DNA damage/repair (Fig. 4C and Supplementary Table S6). In consideration of these findings this analysis suggests that there indeed exists a consensus transcriptional signature altered by JQ1 treatment of cancer cells, with c-MYC and MYCN gene sets, and their associated biologic functions, serving as hubs. Although BET bromodomain inhibition alters a common transcriptional program across structurally distinct compounds and cancers of different lineages, we were also interested in the transcriptional changes unique to MYCN-amplified neuroblastoma. In this case, we identified a set of genes whose expression is uniquely altered in neuroblastoma, but not in the hematopoietic cancer cell lines (Supplementary Fig. S8). Here, evidence was found of the loss of lineage-related genes with JQ1 treatment, reflected in the downregulation of gene sets associated with neural crest stem cells and neurotransmitter release, as well as the downregulation of sets of genes with proximal promoter regions containing binding sites for LHX3 (LIM homeobox 3, a gene involved in neural development) and of ZIC3 (zinc fingers of the cerebellum 3; Fig. 4D and E; ref. 22). In addition, consistent with the observed phenotypic consequences of the compound in neuroblastoma cells, enrichment was noted for gene sets associated with apoptosis (Fig. 4F). A full list of enriched gene sets is presented in Supplementary Table S7.

A BET Bromodomain Inhibitor Displaces BRD4 from the MYCN Promoter Region and MYCN Targets Are Downregulated

It was previously shown that BET bromodomain inhibitors displace BRD4 from the c-MYC promoter region, suggesting the hypothesis that BET inhibitors would similarly displace BRD4 from the MYCN promoter region (8). Using chromatin immunoprecipitation (ChIP) PCR, we observed BRD4 localization to the MYCN promoter as well as a putative enhancer region. JQ1 treatment resulted in displacement of the BRD4 coactivator protein from both elements, providing a mechanistic explanation for the observed JQ1-dependent decrease in MYCN transcribed in sensitive neuroblastoma cells (Fig. 5A). Moreover, with JQ1 treatment 2 well-reported targets of MYCN, MCM7 and MDM2, are confirmed to be downregulated by Western blotting, and expression of the neuroblastoma-specific target identified in our expression profiling analysis, PHOX2B, is also downregulated (Fig. 5B and C). As expected, there is no alteration of these targets in NGP; in which JQ1 treatment does not lead to downregulation of MYCN expression.

BRD4 Suppression by Short Hairpin RNA Recapitulates the Effects of JQ1 Treatment in Neuroblastoma Cell Lines

Previous studies have focused on the role of a specific BET family member, BRD4, which is potently inhibited by JQ1 and implicated in several malignancies, including NUT midline carcinoma, AML, B-cell acute lymphoblastic leukemia (B-ALL), and multiple myeloma (7–9). Moreover, a recent siRNA screen to identify synthetic lethal interactions with MYC overexpression revealed BRD4 as a candidate gene (23). Therefore, we studied selective suppression of BRD4 by short hairpin RNA (shRNA) in the 3 JQ1-sensitive neuroblastoma cell lines. Four shRNAs directed against BRD4 were evaluated and shown to alter relative cell viability and growth over a 6-day time course, with a dose response in all lines sensitive to JQ1 (Fig. 6A and B). As seen with JQ1 treatment, there was a striking induction of apoptosis (Fig. 6C) and downregulation of MYCN expression by RT-PCR and immunoblot with the top 3 shRNAs in the 3 JQ1-sensitive cell lines (Fig. 6D and E). Moreover, as expected, direct suppression of MYCN by shRNA resulted in growth arrest and apoptotic cell death (Supplementary Fig. S9A–S9C). However, in the JQ1-resistant neuroblastoma cell line NGP, BRD4-directed shRNAs did not alter growth or viability, induce apoptosis, or lead to suppression of MYCN expression (Fig. 6A–E). According to these results, BRD4 knockdown did not significantly diminish growth of 2 MYCN nonamplified cell lines, SH-SY5Y and SK-N-AS (Supplementary Fig. S10A and S10B).

Efficacy of JQ1 Treatment in Independent Murine Models of Neuroblastoma

Because no consensus has been reached about the most relevant in vivo mouse model for preclinical testing of a new therapy, we conducted in vivo testing of JQ1 in 3 unique models of MYCN-amplified neuroblastoma: a subcutaneous xenograft, an orthotopic primary human xenograft, and an experimentally derived mouse model of MYCN-amplified neuroblastoma (24). First, JQ1 was evaluated in a BE(2)-C cell line xenograft model established by subcutaneous injection into nonobese diabetic (NOD)–severe combined immunodeficient (SCID) IL2Rγκ−/− (NSG) mice. Tumor-bearing mice were treated with 50 mg/kg of JQ1 or vehicle delivered by daily intraperitoneal injection (n = 10 per group) until sacrifice. JQ1 treatment significantly diminished tumor volume and prolonged overall survival in these mice compared with control-treated mice, without an effect on body weight (Fig. 7A and B and Supplementary Fig. S11A), and no overt toxicities were observed. In contrast, treatment of the MYCN–wild-type SH-SY5Y subcutaneous xenograft with JQ1 did not significantly prolong survival or reduce tumor volume (Supplementary Fig. S11B and S11C). In the primary human orthotopic xenograft model, a human MYCN-amplified primary neuroblastoma tumor was obtained from a heavily pretreated child with relapsed metastatic and drug-resistant disease. Tumor pieces were
Figure 4. Transcriptional changes associated with BET bromodomain inhibition by JQ1. A, heatmap of the 36 down- and 17 upregulated genes after JQ1 treatment, with a consistent direction of regulation in neuroblastoma, multiple myeloma, and AML and fold-change greater than 2 and P ≤ 0.05. Data are presented as row normalized. B, heatmap of the JQ1 consensus signature developed in A, evaluated in a dataset profiling genome-wide expression of primary neuroblastoma tumors. JQ1 consensus signature genes denoted in blue are downregulated and those in red are upregulated with JQ1 treatment. Data are presented as row normalized. The neuroblastoma samples cluster into 2 groups, which are associated with the MYCN amplification status of the tumors (P < 0.002 by a two-tailed Fisher exact test) and with high stage (stage III and IV) versus low stage (all others; P < 0.002 by a two-tailed Fisher exact test). C, a relaxed consensus JQ1 downregulation signature was identified on the basis of the absolute fold change ≥ 1.5 and P value and FDR ≤ 0.05 and was interrogated in a functional enrichment analysis across the MSigDB. The results were visualized with the Enrichment Map software, which organizes the significant gene sets into a network called an "enrichment map." In the enrichment map, the nodes correspond to gene sets and the edges reflect significant overlap between the nodes according to a two-tailed Fisher exact test. The hubs correspond to collections of gene sets with a unifying class label according to gene ontology (GO) biologic processes. The size of the nodes is correlated with the number of genes in the gene set. D, table describing the results of a two-tailed Fisher exact tests for the MSigDB signatures enriched with genes selectively downregulated by JQ1 in neuroblastoma cells. Heatmap of the genes uniquely regulated by JQ1 in neuroblastoma in the neural development (E) and apoptosis-related (F) gene sets. Data are presented as row normalized.
implanted into the kidney capsule of nude mice, and the mice were treated once daily with 50 mg/kg of JQ1 intraperitoneally for 28 days, starting 7 days after orthotopic transplantation. As shown in Fig. 7C, JQ1 treatment significantly prolonged survival as compared with vehicle treatment.

Finally, we tested the effects of JQ1 therapy in a genetically engineered mouse model (GEMM) of MYCN-amplified neuroblastoma (TH-MYCN; ref. 24). TH-MYCN mice with palpable tumors were treated with JQ1 or vehicle, as above, once daily for 28 days. A marked improvement in survival for JQ1-treated mice was observed (Fig. 7D). Tumor biopsy specimens were obtained from mice receiving therapy with JQ1 or vehicle and assessed for pharmacodynamic effects. As shown in Fig. 7E, JQ1 conferred an inhibitory effect on cellular proliferation evidenced by Ki67 staining, an increase in apoptosis by cleaved caspase-3 staining, and an inhibitory effect on the expression of MYCN itself and its downstream target MCM7 (Fig. 7F).

DISCUSSION

Neuroblastoma, a tumor arising from primitive neural crest cells in the sympathetic nervous system, is the most common extracranial solid tumor in children (25). Although progress has been substantial for treating patients with low-risk disease, the majority of children with neuroblastoma present with high-risk disease, and many of these children will succumb to neuroblastoma despite intensive chemotherapy with autologous stem cell transplantation, surgery, and radiation (26). New treatment approaches are clearly needed for these patients, as the delivery of dose-intensified, cytotoxic chemotherapy is already maximized. The pathologic activation of MYCN plays a central role in high-risk neuroblastoma, with MYCN amplification identified in 25% of primary neuroblastoma tumors and nearly half of high-risk cases (26–29). Genomic studies of neuroblastoma tumors, including massively parallel sequencing, have failed to reveal other recurrent and imminently targetable molecular lesions, with the exception of mutations in ALK in neuroblastomas, which often coharbor MYCN amplification (20, 30–34). The central role of the MYCN oncogene in neuroblastoma tumorigenesis, and its limited expression in mature, normal tissues, make it a compelling target for drug development. Indeed, our data also support the use of other...
Figure 6. Effect of BRD4 downregulation on neuroblastoma cell lines. A–E, the 3 most JQ1-sensitive neuroblastoma cell lines were transduced with shRNAs targeting BRD4 or a control shRNA. A, RNA was extracted at day 3 post transduction and BRD4 transcript level was quantified. Shown are the expression values relative to the shRNA control–transduced cells. Error bars represent mean ± SD of 4 technical replicates. *, P < 0.05; **, P < 0.01, calculated using a one-way ANOVA, with Bonferroni correction. B, viability values over days post transduction are shown relative to the day 0 (time of seeding) values, with error bars representing the mean ± SD of 8 replicates per condition. C, apoptosis analysis was conducted on day 6 post transduction. D, Western blot analyses showing BRD4 and MYCN protein levels on day 4 post transduction with 3 BRD4-directed shRNAs. E, MYCN transcript levels were quantified 4 days after transduction. The expression values are relative to cells infected with a control shRNA. Error bars represent mean ± SD of 4 technical replicates. *, P < 0.001 calculated using a one-way ANOVA, with Bonferroni correction, comparing each BRD4-directed shRNA with the control shRNA within a cell line.
Figure 7. Testing of JQ1 in multiple in vivo models of MYCN-amplified neuroblastoma. A, mice were injected with BE(2)-C cells subcutaneously and treated with JQ1 or vehicle once tumors reached 100 mm³. After 15 days, tumor volume was measured. Error bars indicate mean ± SD of 5 mice. *, \( P = 0.01 \), calculated using nonparametric Mann–Whitney test. B, effects of JQ1 treatment on survival in the BE(2)-C xenograft model. Day 0 indicates the first day of treatment, and mice were treated until time of sacrifice. C, a human MYCN-amplified primary neuroblastoma tumor was implanted into kidney capsule of nude mice, and mice were treated once daily with JQ1 for 28 days, starting 7 days after orthotopic transplantation. D, TH-MYCN mice with palpable tumors were treated with JQ1 or vehicle once daily for 28 days. Statistical significance (A–C) was determined by log-rank (Mantel–Cox) test for the survival curves, as shown. Day 0 indicates the day of treatment initiation. E, staining for Ki-67 (red), cleaved caspase-3 (green), and 4′,6-diamidino-2-phenylindole (DAPI; blue) in GEMM tumors treated with either vehicle or JQ1, as indicated. F, Western blot analysis indicating the expression of MYCN and MCM7 in GEMM tumors treated with either vehicle or JQ1, as indicated.

BET bromodomain inhibitors, expanding the possibilities for future clinical testing.

Even though numerous examples now exist of genotypic predictors of chemical phenotypes, particularly for kinase inhibitors (35–37), we are still lacking genetic/genomic predictors of response to most drugs in current clinical use and in early clinical testing. In this study, we reveal the power of large-scale screening of highly annotated collections of cancer cell lines to uncover a strong genetic predictor of response to BET bromodomain inhibitors. Many of the cancer cell lines in our high-throughput screening study, particularly among the solid tumors, were insensitive to JQ1, suggesting that JQ1 is not a general cytotoxic agent. Although nearly all of the prior literature supports the use of BET bromodomain...
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Inhibitors in hematologic malignancies, our work shows a strong indication for the use of BET bromodomain inhibitors in a genetically defined solid tumor, MYCN-amplified neuroblastoma. Similar to the observation that MYC is transcriptionally regulated by bromodomain inhibitors, we showed that MYCN expression is repressed both by JQ1 treatment and by shRNA directed against BRD4. Importantly, in the one MYCN-amplified cell line (NGP) with no response to JQ1 or other structurally distinct BET bromodomain inhibitors, no downregulation of MYCN expression at either a transcriptional or a protein level occurred with either JQ1 treatment or BRD4-directed shRNA. Although the precise mechanism for failure to suppress MYCN expression in NGP by BET bromodomain inhibition is still under investigation, these data support the assessment of MYCN downregulation as a putative approach to response stratification in clinical trials investigating this compound class. Furthermore, whereas in primary human neuroblastoma tumors, MYCN amplification is strongly correlated with the JQ1 expression signature derived from cell lines, some primary human tumors cluster with the JQ1 signature but lack MYCN amplification, suggesting that additional predictors of response to JQ1 remain to be discovered.

In addition to identifying a consensus JQ1 gene expression signature shared by JQ1-responsive cell lines, we also identified a gene signature uniquely regulated in neuroblastoma, including genes associated with neural cellular identity. For example, one of the genes whose expression is uniquely modulated by BET bromodomain inhibition in neuroblastoma is PHOX2B, a gene encoding a transcription factor involved in the differentiation of neural crest cells. PHOX2B is mutated in neuroblastoma, particularly in association with familial neuroblastoma and with congenital central hypoventilation syndrome and Hirschsprung disease (38–40). Similarly, tyrosine hydroxylase expression is uniquely modulated by BET bromodomain inhibition with JQ1 in neuroblastoma, a likely explanation for the downregulation of MYCN expression in our GEMM model of neuroblastoma in which MYCN is driven from a tyrosine hydroxylase promoter. The even more dramatic effects of JQ1 on the downstream target of MYCN, MCM7, could be explained by the contribution of a direct inhibitory effect of JQ1 on the interaction of BRD4 and MYCN at MCM7 promoter sites.

Although it will be important in future studies to extend testing of BET bromodomain inhibition to other diseases with MYCN amplification, such as medulloblastoma (41), the open-source nature of publicly available screening data infinitely expands the use of any individual laboratory’s screening efforts by enabling remixing of the data. For example, if we relax the criteria for identifying candidate biomarkers of sensitivity versus resistance to JQ1 to significant ($P \leq 0.05$) values for a two-tailed Fisher exact test at the 25th percentile (sensitivity) or the 75th percentile (resistance) of the ln($IC_{50}$) distribution, NOTCH1 activation is also correlated with response to JQ1, nominating diseases with aberrant NOTCH1 activation, such as T-cell acute lymphoblastic leukemia (T-ALL; ref. 42) and chronic lymphocytic leukemia (CLL; ref. 43), for future study (Supplementary Table S8). Similarly, expression of GNAS, MDM2, and NF2 should be explored as predictors of resistance and the combination of MDM2 inhibitors with bromodomain inhibitors tested. Moreover, the recent report that ALK(F1174L)/MYCN tumors exhibit increased MYCN dosage due to ALK(F1174L)-induced activation of the phosphoinositide 3-kinase (PI3K)/AKT/mTOR and mitogen-activated protein kinase (MAPK) pathways suggests the possibility of combining ALK inhibitors with BET bromodomain inhibitors in neuroblastoma (44).

In summary, we have leveraged high-throughput, cell-based screening of genetically characterized cancer cell lines to identify MYCN amplification as a strong predictor of sensitivity to BET bromodomain inhibition. We have confirmed this finding in MYCN-amplified neuroblastoma and executed on the preclinical validation of this hypothesis. With a survival advantage observed in 3 independent in vivo models of neuroblastoma treated with JQ1, including a highly chemotherapy-resistant genetically engineered MYCN mouse model, the clinical development of bromodomain inhibitors for testing in children with relapsed or refractory neuroblastoma is already under way.

**Methods**

**Genomic Characterization of Cancer Cell Lines**

A total of 84 of the most frequently mutated, amplified, deleted, or rearranged cancer genes were examined in the cell line collection. Sixty-five genes were sequenced to base-pair resolution across all coding exons for each gene by capillary sequencing. The presence of 7 of the most commonly rearranged cancer genes (e.g., BCR–ABL and EWS–FLIJ) was determined across the drug screen cell line panel by the design of breakpoint-specific sequence primers that enabled detection of the rearrangement following capillary sequencing. In addition, analysis of microsatellite instability (MSI) was conducted according to the guidelines set down by the International Workshop on Microsatellite Instability and RER Phenotypes in Cancer Detection and Familial Predisposition (45). Samples were screened using the markers BAT25, BAT26, DSS346, D2S123, and D17S250 and were characterized as MSI if 2 or more markers showed instability. Total integral copy number values across the footprints of the cancer genes were determined from Affymetrix SNP6.0 microarray data, using the PICNIC algorithm to predict copy number segments in each of the cell lines (46). For a gene to be classified as amplified, the entire coding sequence must be contained in one contiguous segment defined by PICNIC and have a total copy number of 8 or more. Deletions must occur within a single contiguous segment with copy number 0. A complete description of the characterization of the cancer cell line collection used in this study is available from the Cancer Genome Project webpages (47).

**Cell Line Screening**

Cell lines in an exponential phase of growth were seeded at optimal density previously determined to provide maximum signal in control (untreated) wells, while maintaining growth through the experimental exposure. Cells were seeded on day 1, with drug delivered the following day. Nine concentrations of (+)JQ1 were applied to cells at a stepwise 2-fold dilution series. After 72 hours of drug exposure, cells were fixed and stained with Syto60 (Invitrogen) for fluorescence measurement, as described previously (48). Duplicate experiments were variably carried out for quality assessment during the screening phase. Stricter quality control criteria were applied, and estimates of IC$_{50}$ were derived from the 9 dose–response curves, as described previously (16). Drug response is presented as the ln(IC$_{50}$) plotted against the $E_{max}$ (the maximum effect corresponding to the minimum measured viability). Enrichment for a given organ of origin (673 cell lines) or genotype (473 cell lines with genotype information)
in the sensitive cell lines was tested using a two-tailed Fisher exact test-based approach. Cell lines with response to JQ1 yielding IC$_{50} \leq 1 \mu$mol/L and IC$_{max} \geq 70\%$ were designated as resistant, and all others were designated as resistant in the most stringent classification schema. With less stringent criteria, cell lines were assigned to the sensitive or resistant group thresholds corresponding to the 25th to 75th percentile of the distribution of ln(IC$_{50}$) values. A two-tailed Fisher exact test was used to determine the statistical enrichment of a given genotype (or organ) in the sensitive or resistant group at each threshold. In this analysis, mutation is defined as the presence of a specific gene fusion, a sequence change, or a copy number change across 84 cancer genes.

**Cell Culture**

Neuroblastoma cell lines were maintained in Dulbecco modified Eagle medium (Cellgro) supplemented with 10% FBS (Sigma-Aldrich) and 1% penicillin–streptomycin with glutamine (Cellgro). BE(2)-C and SH-SY5Y were obtained from the American Type Culture Collection, and Kelly, LAN-1, SK-N-AS, and NGP were kindly provided by Dr. Rani George (Dana-Farber Cancer Institute). The identity of all lines was verified by small tandem repeat profiling conducted by the Shannon McCormack Advanced Molecular Diagnostics Laboratory at the Dana-Farber Cancer Institute.

**Compounds, Cellular Viability, and Growth**

JQ1R, JQ1S, I-BET, and I-BET151 were synthesized by Dr. Jun Qi (Dana-Farber Cancer Institute). For the testing of multiple BET bromodomain inhibitors in 4 neuroblastoma cell lines, cells were seeded onto 384-well tissue culture–treated plates at a density of 2,000 per well in a volume of 50 μL/well. Addition of inhibitor was conducted with a JANUS Workstation (PerkinElmer) using a 384-well pinhead tool that is calibrated to deliver 100 nL drug per well. After 72 hours of incubation with inhibitor, cells were analyzed for cell viability using the ATP kit (PerkinElmer) Luminescence Assay Kit per the manufacturer’s instructions, using ATP content as a surrogate for viable cell number. Luminescence was read on an EnVision 2104 Multilabel Plate Reader (PerkinElmer). Nonlinear dose–response curves were fitted to the data using Graphpad Prism software. To assess growth, neuroblastoma cell lines were incubated with JQ1 in 384-well plates. ATP content was measured on days 0, 2, 3, and 5, using CellTiter Glo (Promega) according to the manufacturer’s instructions.

**Flow Cytometry**

Cell cycle was determined by measuring DNA content using PI staining. Apoptosis was measured using the annexin V fluorescein isothiocyanate (FTTC) Apoptosis Detection Kit per the manufacturer’s protocol (BD Pharmingen).

**Protein Extraction and Immunoblotting**

Protein was extracted from cells by lysis with Cell Signaling Lysis Buffer (Cell Signaling Technology) containing Complete EDTA-Free Protease Inhibitor Cocktail (Roche Diagnostics) and PhosSTOP Phosphatase Inhibitor (Roche Diagnostics). Immunoblot analyses were run as previously described (49). Primary antibodies included anti-MYCN (Abcam 16898), anti-BRD4 (Abcam 75898), anti-Vinculin (Abcam 18058), anti-MCM7 (Cell Signaling Technology 4018S), anti-MDM2 (Santa Cruz Biotechnology sc-813), and anti-Actin (Novemakers MS1295P).

**RNA Extraction and Real-Time RT-PCR**

RNA was extracted from cells with the RNasy Kit and on-column DNA digestion (Qiagen). Primers and probes for MYCN (Hs00232074_m1), BRD4 (Hs04188087_m1), PHOX2B (Hs00243679_m1), and the control gene RPL13A (Hs01926559_g1) were obtained from Applied Biosystems. Data were collected in technical quadruplicate, analyzed using the ΔΔC$_T$ method, and plotted as percentage of transcript compared with the negative control condition.

**shRNA Studies**

Virus was created by transfecting 293T cells with packaging plasmid (pCMV-d8.9), envelope plasmid (VSV-G), and a pLK0.1 hairpin plasmid (see Supplementary Table S9 for hairpin target sequences), using FuGENE 6 per the manufacturer’s instructions (Promega). It was filtered with a 0.4 μL/mL filter before use. Neuroblastoma cell lines were transduced in 10-cm plates with 2 mL virus and 8 μg/mL Polybrene (Sigma-Aldrich) for 2 hours at 37°C, and then 8 mL medium was added to each plate. On day 2 post transduction, 2 μg/mL (BE(2)-C) or 1 μg/mL (all other cell lines) polybrene (Sigma-Aldrich) was added to select for infected cells, and 4 days post transduction, experiments to evaluate cell growth, apoptosis, and protein and transcript levels were begun.

**Genome-Wide Expression Analysis**

BE(2)-C and Kelly cells were treated in triplicate with 1 μmol/L JQ1 or dimethyl sulfoxide (DMSO) for 24 hours. RNA was extracted, and a decrease in MYCN transcript was confirmed by real-time RT-PCR, as described earlier. The samples were profiled using the Affymetrix PrimeView Human Gene Expression Array (Affymetrix) at Beth Israel Deaconess Medical Center (Boston, MA). The computational analysis pipeline was conducted on the Genespace bioinformatics platform (50).

**Data Processing**

The CEL data files were subjected to quality control and passed tests based on distance between arrays, array intensity distribution, and variance mean dependence, which are implemented in the ArrayQuality R package (Bioconductor). The raw expression data were processed and log$_2$ transformed by applying the RNA algorithm in the Affymetrix package (Bioconductor). All probe sets with an average log$_2$ expression less than 4 were considered underexpressed and were designated as “filtered.” Of the 49,293 probe sets on the Affymetrix PrimeView Human Gene Expression Array, 45,925 probe sets remained after filtering. The data from the 45,925 probe sets were further collapsed to 19,108 nonredundant genes with distinct Human Genome Organisation (HUGO) symbols, by assigning to each gene the probe set with the maximum average expression intensity. Gene expression data are available from Gene Expression Omnibus (GEO; accession no. GSE43392).

**Comparative Marker Analysis**

The 12 samples available in the data were separated into 2 groups: 1 μmol/L JQ1-treated samples versus DMSO-treated samples. The Comparative Marker Selection module from GenePattern 3.5.0 (50) was used to identify individual genes differentially expressed between the JQ1- and the DMSO-treated groups (51). A 2-sided signal-to-noise ratio (SNR) test followed by 1,000 permutations of phenotype labels was conducted for this analysis. The settings for the SNR parameters were log-transformed data, yes; complete, no; balanced, no; and smooth $P$ values, yes. A permutation $P \leq 0.05$, a Benjamini–Hochberg FDR $\leq 0.05$, accounting for multiple hypothesis testing, and an absolute fold change $\geq 2$ served as a cutoff for significant genes. Heatmaps were created with GENE-E software (50).

**GSEA**

The GSEA software (50) was used to identify pathways, or groups of functionally related genes, deregulated by the JQ1 treatment (18). The goal of GSEA is to identify groups of genes sharing common biologic function (gene sets), which are distributed at the top or at the bottom of the ranked list of differentially expressed genes. GSEA assigns to each gene set an enrichment score calculated as a running sum statistic by walking down the ranked list of differentially expressed genes, increasing the sum when encountering...
genes in the gene set, and decreasing it when encountering genes not in the gene set. The significance of the enrichment score is estimated on the basis of a permutation P value and adjusted for multiple hypotheses testing through FDR. The set of differentially expressed genes accounting for the enrichment signal is called the leading edge.

GSEA was run on the collections of 3,272 curated gene sets (2c) and 615 transcription factor targets (c3) from version 3.1 of the MSigDB (50). Gene sets with less than 15 genes or more than 500 genes were excluded from the analysis. Gene sets with an FDR ≤ 0.25 and a nominal P ≤ 0.05 were considered significant. The gene-ranking metric in the weighted enrichment score was the 2-sided t-test for survival curves. All mice were monitored until euthanasia was required, in accordance with the University of California, San Francisco, Institutional Animal Care and Use Committee (IACUC) guidelines.

**Disclosure of Potential Conflicts of Interest**

J. Qi is a consultant/advisory board member of Tensha Therapeutics. J.E. Bradner is employed as a Scientific Founder in Tensha Therapeutics, is a consultant/advisory board member, and has ownership interest (including patents) in the same. No potential conflicts of interest were disclosed by the other authors.

**Authors’ Contributions**

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**In Vivo Studies**

Cell line xenograft tumors were established by implanting 2 million BE(2)-C-LucNeo cells or 2 million SH-SY5Y cells in 30% Matrigel (BD Biosciences) subcutaneously in the flanks of 6-week-old female NSG mice (The Jackson Laboratory). Serial bioluminescence imaging was conducted after injection with 75 mg/kg of o-luciferin (Promega) using a Xenogen IVIS Spectrum instrument (PerkinElmer). Eight days after tumor inoculation, mice with established tumors, defined as increasing bioluminescence and measurable tumor volume, were divided into cohorts to be treated with 50 mg/kg JQ1 or vehicle (10% DMSO in 5% dextrose) delivered by daily intraperitoneal injection (n = 10 per group). Because of rapid tumor growth and saturation, bioluminescence imaging was abandoned as an endpoint. Tumor volume was determined using the equation volume = 0.5 × length × width². Mice were sacrificed when tumors reached 2 cm in the longest linear dimension. Statistical significance was determined by log-rank (Mantel-Cox) test for survival curves. This study was conducted using protocols approved by the Dana-Farber Cancer Institute Animal Care and Use Committee.

**TH-MYCN mice with palpable tumors (mean age at detection was 60 ± 15 d) were treated with JQ1 via intraperitoneal injection (50 mg/kg in 1:10 solution of DMSO:10% cyclodextrin) or vehicle (1:10 solution of DMSO:10% cyclodextrin), once daily for 28 days (n = 5 per group). In the primary human orthotopic model, a human MYCN-amplified primary neuroblastoma tumor was obtained from a child with relapsed, drug-resistant metastatic disease (SFNB-06). Tumor pieces (4 mm³) were implanted into kidney capsule of nude mice and maintained as a continuous xenograft. Human primary SFNB-06 mice were treated once daily for 28 days, starting 7 days after orthotopic transplantation (dosage as above; n = 5 per group).**

Statistical significance was determined by log-rank (Mantel-Cox) test for survival curves. All mice were monitored until euthanasia was required, in accordance with the University of California, San Francisco, Institutional Animal Care and Use Committee (IACUC) guidelines.

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**Neuroblastoma JQ1-Specific Signature**

A collection of 116 genes significantly downregulated (absolute fold change ≥ 2 and P value and FDR ≤ 0.05) in the JQ1-treated neuroblastoma cell lines versus control and unchanged in any of the other 2 JQ1-treated studies was identified. The small size of the neuroblastoma JQ1-specific signature, even in a relaxed format, necessitated the use of a two-tailed Fisher exact test rather than GSEA to identify the functional relatedness of these genes.

**ChIP Studies**

ChIP studies were carried out as described previously, using specific primers for the MYCN locus (8). Primers were designed to amplify 2 sites within the promoter region (S⁻³) and a negative control region outside the promoter region: MYCN promoter site 1 (forward) TTTGACATCTGGACTACC and (reverse) TTT-GGTGTGCCTGGTGCAG; MYCN promoter, site 2 (forward) TCTCGGGAGCTGGTTGAGG and (reverse) TCTCGGATGGCT-ACAGTCT; MYCN-negative region (forward) GTATACCGCTCATC- TCCCCG and (reverse) TTTGACACGCTCAAAGGCC. Enrichment data were analyzed by calculating the immunoprecipitated DNA percentage of input DNA for each sample (triplicate PCR reactions).
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