Dual Targeting of Bromodomain and Extraterminal Domain Proteins, and WNT or MAPK Signaling, Inhibits c-MYC Expression and Proliferation of Colorectal Cancer Cells

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Abstract

Inhibitors of the bromodomain and extraterna domain (BET) family protein attenuate the proliferation of several tumor cell lines. These effects are mediated, at least in part, through repression of c-MYC. In colorectal cancer, overexpression of c-MYC due to hyperactive WNT/β-catenin/TCF signaling is a key driver of tumor progression; however, effective strategies to target this oncogene remain elusive. Here, we investigated the effect of BET inhibitors (BETi) on colorectal cancer cell proliferation and c-MYC expression. Treatment of 20 colorectal cancer cell lines with the BETi JQ1 identified a subset of highly sensitive lines. JQ1 sensitivity was higher in cell lines with microsatellite instability but was not associated with the CpG island methylator phenotype, c-MYC expression or amplification status, BET protein expression, or mutation status of TP53, KRAS/BRAF, or PIK3CA/PTEN. Conversely, JQ1 sensitivity correlated significantly with the magnitude of c-MYC mRNA and protein repression. JQ1-mediated c-MYC repression was not due to generalized attenuation of β-catenin/TCF-mediated transcription, as JQ1 had minimal effects on other β-catenin/TCF target genes or β-catenin/TCF reporter activity. BETi preferentially target super-enhancer-regulated genes, and a super-enhancer in c-MYC was recently identified in HCT116 cells to which BRD4 and effector transcription factors of the WNT/β-catenin/TCF and MEK/ERK pathways are recruited. Combined targeting of c-MYC with JQ1 and inhibitors of these pathways additively repressed c-MYC and proliferation of HCT116 cells. These findings demonstrate that BETi downregulate c-MYC expression and inhibit colorectal cancer cell proliferation and identify strategies for enhancing the effects of BETi on c-MYC repression by combinatorial targeting the c-MYC super-enhancer. Mol Cancer Ther; 15(6): 1217–26. ©2016 AACR.

Introduction

The bromodomain and extraterminal domain (BET) family of epigenetic readers, consists of four members (BRD2, BRD3, BRD4, and BRDT), that regulate RNA polymerase II (RNA Pol II)-dependent transcription. The BET family is characterized by two tandem bromodomains that recognize acetylated lysine residues and an extraterminal (ET) domain that associates with histone modifiers and chromatin remodeling factors (1). The bromodomain comprises a left-handed bundle of four α-helices linked by variable loop regions that recognize and bind to acetylated lysine residues on histones and other non-histone proteins (2). BET inhibitors (BETi) such as JQ1 and I-BET151 bind competitively to the bromodomain, inhibiting its interaction with acetylated histones and displacing the BET protein from chromatin (3, 4). BETi have demonstrated robust growth inhibition in hematologic and some solid tumor cell lines (5–10).

Among the BET family, the function of BRD4 has been the most extensively investigated. BRD4 regulates transcription at multiple levels including the initiation and elongation of transcription through its interaction with the Mediator complex, and the positive elongation factor B (P-TEFb), respectively (11–13). To induce transcriptional elongation, BRD4 recruits P-TEFb which phosphorylates the negative elongation factor (NELF) complex, as well as serine 2 in the C-terminal domain of RNA Pol II. This results in NELF dissociation from RNA Pol II paused at proximal promoter regions and in transcriptional elongation (14). A number of genes are regulated at the level of transcriptional elongation, in particular primary response genes such as FOS, JUNB, and c-MYC (15).

Consistent with the important role BET proteins play in transcriptional regulation, BETi can alter transcription of a number of genes (5, 6). In particular, genes regulated by super-enhancers are highly sensitive to BETi (16).
regions characterized by elevated levels of mediator and BRD4 occupancy, are typically organized in clusters bound by tissue-specific transcription factors, and can span up to several kb in size [16, 17].

One gene that has been a strong focus of regulation by BETi is c-MYC [6, 9, 10]. c-MYC regulates the transcription of up to 15% of all genes [18, 19] and is among the most frequently overexpressed oncogenes in human cancers [20]. Overexpression of c-MYC occurs through multiple mechanisms including gene locus amplification [8q24.21], translocation [21, 22], mutations that enhance protein stability [23], SNPs in regulatory sequences [24], and transcriptional activation through constitutively activated signaling pathways such as WNT/β-catenin/TCF [25].

In colon cancers, signaling through the WNT/β-catenin/TCF pathway is constitutively activated in more than 90% of cases, mostly due to inactivating mutations in the APC gene or in some cases activating mutations in CTNNB1 (β-catenin). β-Catenin/TCF complexes bind to consensus sites in the c-MYC promoter to drive its expression [25]. c-MYC gene amplification has also been reported in approximately 10% of colon cancers, collectively resulting in c-MYC overexpression in more than 70% of cases, a feature associated with poorer outcome [26].

The importance of c-MYC in the initiation and progression of colorectal cancer was demonstrated by in vitro studies where knockdown of c-MYC inhibits the growth of colon cancer cell lines [27, 28] and confirmed by studies of the mouse intestine, in which the pro-proliferative phenotype induced by APC inactivation was rescued by parallel inactivation of c-MYC [29–31].

Given the ability of BET inhibitors to repress c-MYC expression in a range of tumor types [7, 9, 32, 33] and the importance of c-MYC in mediating the effects of BETi, we sought to determine the effect of BET inhibitors on the growth of colorectal cancer cells and the role of c-MYC in mediating these effects. By analyzing a panel of 20 colon cancer cell lines, we found that sensitivity to JQ1 was significantly associated with the magnitude of repression of c-MYC. We also identified novel combinatorial strategies to enhance the efficacy of BETi through targeting of the c-MYC super-enhancer, which additively inhibited c-MYC expression and proliferation of colon cancer cells.

**Materials and Methods**

**Chemicals and reagents**

All chemicals were obtained from Sigma-Aldrich unless stated otherwise. The BET inhibitors (+)JQ1 (3) and I-BET151 (GSK1210151A, ref. 5) were obtained from Selleck Chemicals. Trametinib (GSK1120212) was obtained from ChemieTek Ltd. and ChemiTek, respectively. 

**Cell lines and cell culture**

Colon cell lines used in this study were obtained from the ATCC or other investigators as previously described [34] and were maintained in DMEM (Invitrogen), supplemented with 10% FCS and 1% GlutaMAX (Invitrogen). The unique identity of each cell line was authenticated by short tandem repeat (STR) profiling. MV4;11 and MOLM13 were kindly provided by Mark Dawson. The microsatellite instability (MSI), CpG island methylator phenotype (CIMP), and mutation status of the cell lines have been previously described by us and others [34, 35].

**Gene expression analysis by qPCR**

Total RNA was purified employing the High Pure RNA isolation kit (Roche) and reverse transcribed using the Transcripter High Fidelity cDNA Synthesis Kit (Roche). Gene expression levels were determined by qPCR in technical triplicates using PowerSYBR green (Applied Biosystems) on a Viia 7 Real-Time system (Life Technologies). Primers used are listed in Supplementary Table S1.

**Immunohistochemistry**

IHC was performed on formalin-fixed, paraffin-embedded sections of primary colon cancers collected under an IRB-approved protocol. Sections were incubated with rabbit anti-β-catenin (5848, Cell Signalling Technology, 1:100), rabbit anti-β-catenin (A302-368A, Bethyl Laboratories, 1:100), and rabbit anti-β-catenin (ab128874, Abcam 1:100), overnight at 4°C, and a horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (DAKO Envision + Labeled Polymer HRP, K4011, Agilent Technologies) for 30 minutes.

**Western blot analysis**

For assessment of BRD protein expression, cells were lysed in 50 mmol/L HEPES, pH 8.0, 100 mmol/L KCl, 2 mmol/L EDTA, 0.1% NP-40, 10% glycerol, 1 mmol/L dithiothreitol (DTT, Roche), 1 mmol/L PMSF (Sigma-Aldrich), protease inhibitor cocktail (Sigma-Aldrich). For assessment of all other proteins, cells were lysed in RIPA buffer (Sigma-Aldrich). Antibodies used for immunoblotting were: anti-β-catenin (HPA042816, Sigma, 1:250), anti-β-catenin (ab50818, Abcam, 1:50), anti-β-catenin (ab128874, Abcam, 1:1,000), anti-β-actin (sc-47778, Santa Cruz, 1:2,000 or A5316, Sigma, 1:5,000), anti-CTNNB1 (610154, BD Transduction Laboratories, 1:1,000), anti-FOSL1 (R20, Santa Cruz, 1:2,000), and anti-c-MYC (N262, Santa Cruz, 1:200). Secondary antibodies used were fluorescent-labeled goat anti-mouse (IRDye800CW, Li-Cor, 1:15,000) and goat anti-rabbit (IRDye680CW, Li-Cor, 1:15,000).
intraperitoneal daily dose of 50 mg/kg JQ1, or vehicle (20% hydroxypropyl-β-cyclodextrin, 5% DMSO, 0.2% Tween-80 in saline), for 18 days. Animals in both groups were not treated on days 12 to 14 due to weight loss in the JQ1 group. Tumor growth was monitored every second day by caliper measurement until the end of the experimental period or when tumors reached 1 cm³ in size. At this point, tumors were extracted and weighed.

**Results**

**BRD2, BRD3, and BRD4 are expressed in colon cancer cells**

To confirm that members of the BET family are expressed in colon cancer cells, we examined BRD2, BRD3, and BRD4 protein expression in a panel of 20 colon cancer cell lines by Western blotting. Variable expression of BRD2, BRD3, and BRD4 expression was observed across the cell lines, with each cell line displaying expression of at least one of the three BRD proteins (Supplementary Fig. S1). We also examined the expression of BRD2, BRD3, and BRD4 in primary colon cancers by IHC. Robust nuclear staining of BRD4 was evident in the majority of tumors examined. Staining of BRD2 and BRD3 was weaker overall but evident in a subset of cases (Supplementary Fig. S2). Collectively these findings establish that BRD2, BRD3, and BRD4 are expressed in colon cancer cells.

**JQ1 inhibits proliferation of a subset of colon cancer cell lines**

To determine whether the BET family represents a potential therapeutic target in colon cancer, we determined the effect of the BETi JQ1 on the proliferation of a panel of 20 colon cancer cell lines over 72 hours. A continuum of response to JQ1 was observed across the cell lines, with each cell line displaying expression of at least one of the three BRD proteins (Supplementary Fig. S1). We also examined the expression of BRD2, BRD3, and BRD4 in primary colon cancers by IHC. Robust nuclear staining of BRD4 was evident in the majority of tumors examined. Staining of BRD2 and BRD3 was weaker overall but evident in a subset of cases (Supplementary Fig. S2). Collectively these findings establish that BRD2, BRD3, and BRD4 are expressed in colon cancer cells.

To determine which of the BET family members are expressed in colon cancer cells, we examined BRD2, BRD3, and BRD4 protein expression in a panel of 20 colon cancer cell lines by Western blotting. Variable expression of BRD2, BRD3, and BRD4 expression was observed across the cell lines, with each cell line displaying expression of at least one of the three BRD proteins (Supplementary Fig. S1). We also examined the expression of BRD2, BRD3, and BRD4 in primary colon cancers by IHC. Robust nuclear staining of BRD4 was evident in the majority of tumors examined. Staining of BRD2 and BRD3 was weaker overall but evident in a subset of cases (Supplementary Fig. S2). Collectively these findings establish that BRD2, BRD3, and BRD4 are expressed in colon cancer cells.

**JQ1 is largely cytostatic in colon cancer cells**

To determine whether the BET family represents a potential therapeutic target in colon cancer, we determined the effect of the BETi JQ1 on the proliferation of a panel of 20 colon cancer cell lines over 72 hours. A continuum of response to JQ1 was observed across the cell lines, with each cell line displaying expression of at least one of the three BRD proteins (Supplementary Fig. S1). We also examined the expression of BRD2, BRD3, and BRD4 in primary colon cancers by IHC. Robust nuclear staining of BRD4 was evident in the majority of tumors examined. Staining of BRD2 and BRD3 was weaker overall but evident in a subset of cases (Supplementary Fig. S2). Collectively these findings establish that BRD2, BRD3, and BRD4 are expressed in colon cancer cells.

**Association between JQ1 response and molecular subgroups of colon cancer**

To identify potential biomarkers of JQ1 response, cell lines were separated according to established colon cancer subgroups or mutation status of commonly altered oncogenes and tumor suppressor genes and sensitivity compared. The mutation status of the cell lines is listed in Supplementary Table S2. Cell lines with microsatellite instability (MSI) were significantly more sensitive to JQ1 than microsatellite-stable (MSS) lines (P = 0.038, Mann-Whitney unpaired t-test). Conversely, no association between JQ1 sensitivity and CIMP status was observed (Supplementary Fig. S3). As KRAS and BRAF mutations, and similarly PIK3CA and PTEN mutations occur in a mutually exclusive manner in colon cancer, cell lines were classified as WT or mutant based on the collective mutation status of these genes. No association between JQ1 response and cell lines wild-type or mutant for RAS/BRAF, PIK3CA/PTEN, or TP53 was observed, nor was there a correlation between JQ1 response and basal expression of BRD2, BRD3, and BRD4 expression (Supplementary Fig. S3). As MYCN-amplified neuroblastoma and medulloblastoma cell lines have been shown to be particularly sensitive to JQ1 (9, 38), we examined JQ1 response in 17 of the 20 colon cancer cell lines for which we had c-MYC amplification status information (34). c-MYC copy number varied from 2 (n = 13) to >6 (n = 4, Colo320, HT29, SKCO1, SW480) across the cell line panel. In contrast to neuroblastoma, no significant difference in JQ1 sensitivity was observed between c-MYC–amplified and nonamplified lines, with if at all, c-MYC amplified lines tending to be more resistant to JQ1 (Supplementary Fig. S3). Similarly, no correlation between basal c-MYC mRNA and protein expression and JQ1 response was observed (Supplementary Fig. S4).

**Figure 1.**

Effect of JQ1 on colorectal cancer cell proliferation. A, panel of 20 colorectal cancer cell lines ranked, ordered by increasing resistance to JQ1. Response to JQ1 was determined in MTS assays by computation of GI50. B, dose-response analysis of the effect of JQ1 on cell growth inhibition in the 3 most sensitive (GP5D, HT29, LIM1215) and resistant (KM12, SW480, HuTu80) colorectal cancer lines and 2 BETi-sensitive AML cell lines (MOLM13, MV4;11). Growth inhibition was determined by MTS after 72 hours.
The proportion of cells in S-phase, significantly greater than the 26% ± 11% decrease in resistant cells (mean ± SD, n = 3, P < 0.05, Fig. 2A and C). In parallel, JQ1 increased the percentage of cells in G1 by 40% ± 11%, significantly greater than the magnitude of induction in resistant cells (15% ± 8%, n = 3, P < 0.05, Fig. 2B and C).

These results indicate that JQ1 sensitivity in colon cancer cells is associated with a block in the transition from G1 to S-phase. Previous reports in other tumor types have suggested that JQ1 induces apoptosis (7, 10). To address this in colon cancer cells, we investigated the effect of JQ1 on apoptosis induction in the three most sensitive lines. In LIM1215 cells, JQ1 modestly increased the percentage of apoptotic cells from 0.5% ± 0.5% to 4.4% ± 1.0% (P < 0.005) but had no effect on apoptosis in GP5D (2.4% ± 1.4% vs. 2.6% ± 1.0%) or HT29 (0.6% ± 0.6% vs. 0.6% ± 0.7%) cells. In comparison, the topoisomerase I inhibitor and known cytotoxic agent irinotecan increased the percentage of apoptotic cells in GP5D cells to 13.7% ± 3.8%, in HT29 cells to 37.4% ± 6.0%, and in LIM1215 to 8.3% ± 3.6% (Fig. 2D). These findings indicate that the effect of JQ1 on colon cancer cells line is primarily cytostatic.

To extend these findings, we also examined the effect of JQ1 on anchorage-independent growth. We tested the three JQ1-sensitive cell lines, GP5D, HT29, and LIM1215, in this assay. JQ1 significantly reduced colony size in all three sensitive cell lines but had minimal effect on colony number (data not shown), consistent with its largely cytostatic effect in colon cancer cell lines (Fig. 2E).

JQ1 inhibits the growth of colorectal cancer xenografts in vivo

We next determined the effect of JQ1 on the growth of colorectal cancer xenografts in vivo. To test this, we utilized the HCT116 cell line which exhibits intermediate sensitivity to JQ1 in vitro, and which rapidly and reproducibly develops tumors when grown as xenografts in vivo. HCT116 cells were engrafted into the right and left flank of nude mice and treatment commenced after 4 days when palpable tumors had formed. Animals were treated daily, except for days 12 to 14, for 18 days. JQ1 treatment induced a modest but statistically significant inhibition of tumor growth when assessed by caliper measurements at days 10, 12, and 18 (Fig. 3B) and when assessed by tumor weight at the completion of the experiment on day 18 (Fig. 3A and C). Notably, a decrease in body weight began to develop in the JQ1 treatment group after 12 days of treatment. We therefore introduced a treatment holiday between days 12 and 14 to enable the mice to recover from body weight loss (Fig. 3D).
JQ1-induced growth inhibition correlates with the magnitude of c-MYC repression

JQ1 alters the expression of multiple genes in hematologic and solid tumor cell lines, including repression of c-MYC (5, 6). To determine whether JQ1 represses c-MYC expression in colon cancer cell lines and if this is linked to JQ1-induced growth inhibition, we treated the panel of 20 colon cell lines with 500 nmol/L JQ1 for 6 hours and examined changes in c-MYC mRNA by qPCR. JQ1 repressed c-MYC expression in the majority of cell lines (Fig. 4A). Furthermore, a regression analysis demonstrated a significant correlation between the magnitude of JQ1-mediated c-MYC repression and the extent of cell growth inhibition ($r = 0.53$; $P = 0.017$; Fig. 4B). Consistent with the effects on c-MYC mRNA, c-MYC protein levels were also reduced to a greater extent in the three most sensitive cell lines following JQ1 treatment (Fig. 4C). To further confirm these findings, we examined the response of the three most sensitive and resistant colorectal cancer lines to another bromodomain inhibitor, I-BET151, which has a different chemical scaffold to JQ1. Similar to effects induced by JQ1, I-BET151 preferentially reduced c-MYC expression in the sensitive cell lines (Fig. 4D). Densitometric analysis demonstrated this difference to be statistically significant at the 8h time point ($n = 3$, $P = 0.009$, unpaired t test; Fig. 4E).

JQ1-mediated repression of c-MYC is not due to generic downregulation of WNT/β-catenin/TCF signaling in colon cancer cells

A key driver of c-MYC overexpression in colon cancer cells is direct transcriptional activation by the β-catenin/TCF transcriptional complex (25). To determine whether the repression of c-MYC reflected a general JQ1-mediated inactivation of β-catenin/TCF–driven transcription, we determined the effect of JQ1 on expression of the established β-catenin/TCF target genes AXIN2, FOSL1, LGR5, and SOX9 in the five most sensitive cell lines. Similar to the effect on c-MYC expression, JQ1 significantly reduced expression of FOSL1; however, the magnitude of repression was markedly less than that observed for c-MYC. Conversely, JQ1 did not significantly affect expression of AXIN2, LGR5, or SOX9 (Fig. 5A). To further confirm these findings, we examined the effect of JQ1 on β-catenin/TCF reporter activity (TOPFlash), and the control reporter, FOPFlash (39), in two colon cancer cell lines. JQ1 inhibited activity of both TOP and FOPFLASH to a similar extent indicating it does not significantly impact on β-catenin/TCF–driven transcription. Collectively, these findings indicate that JQ1-mediated repression of c-MYC is not due to a general deregulation of β-catenin/TCF–driven transcription (Fig. 5B).

Figure 3.
Effect of JQ1 on tumor growth in vivo. A and C, HCT116 cells were injected into the right and left flank of Balb/c nu/nu mice (day 0). On day 4, mice were randomized to receive vehicle or JQ1 (50 mg/kg). Mice were treated daily for 18 days except for days 12 to 14, when mice in both groups were not treated because of weight loss in the JQ1 treatment group. Tumor size was monitored every second day by caliper measurements and by weighing following excision on day 18. Data represented are the mean ± SEM (B and D) and mean ± min/max group values (C). * $P < 0.05$, unpaired Student t test.

D, relative change of body weight in mice treated with vehicle or JQ1. Values shown are the mean difference in body weight ± SD relative to starting weight.
Combinatorial targeting of the c-MYC super-enhancer enhances JQ1-mediated repression of c-MYC in colon cancer cells

While JQ1 effectively downregulated c-MYC expression and inhibited cell proliferation in a subset of colon cancer cells, it also had relatively modest effects in other colon cancer lines. We therefore sought to establish strategies for enhancing the magnitude of JQ1-induced c-MYC repression, to enhance JQ1-induced growth inhibition of these lines. Genes regulated by super-enhancers have been shown to be particularly sensitive to JQ1 treatment (16). Furthermore, as super-enhancers contain both high levels of BRD4 occupancy and concentrated binding of effector transcription factors from key oncogenic signaling pathways, it has been suggested that combinatorial blockade of these signaling pathways, along with super-enhancer components, may be required for efficient transcriptional repression of genes regulated by these elements (40). A super-enhancer located about 500 kb upstream of the c-MYC transcription start site was previously identified in HCT116 colon cancer cells (40) and shown to be enriched for binding of TCF4 (TCF7L2) and FRA1 (FOSL1), effector transcription factors of the WNT/β-catenin/TCF and MEK/ERK pathways, respectively, which are constitutively activated in HCT116 cells (34). We therefore reasoned that treatment with JQ1 in combination with WNT or MAPK pathway inhibition may further repress c-MYC expression in these cells.

To target the WNT/β-catenin/TCF signaling pathway, we used siRNAs against the TCF4 interacting partner, β-catenin (CTNNB1), which resulted in downregulation of β-catenin mRNA expression by 90% and protein expression by 78% (n = 2 independent experiments; Fig. 6A). CTNNB1 knockdown decreased c-MYC expression which was further enhanced by combination treatment with JQ1 (Fig. 6B) and associated with a further reduction in cell proliferation (Fig. 6C). To target the MEK/ERK pathway, we used the MEK inhibitor trametinib. As expected, trametinib markedly decreased mRNA and protein expression of the MEK/ERK target gene, FOSL1 (Fig. 6D). Trametinib treatment also decreased c-MYC protein expression, which was further enhanced by combination treatment with JQ1 (Fig. 6E) and reflected in an increased inhibition of cell proliferation (Fig. 6F). These findings demonstrate that combinatorial blockade of effector transcription factors of the c-MYC super-enhancer alongside super-enhancer components can further downregulate c-MYC expression and induce more robust antiproliferative effects in colorectal cancer cells.
Discussion

Nonresectable metastatic colorectal cancer remains an incurable disease for which novel therapies are urgently needed. c-MYC is a key driver of colon cancer cell proliferation; however, therapeutic targeting of this transcription factor has been elusive to date. Bromodomain inhibitors were originally developed as a potential treatment for midline carcinoma, a rare tumor driven by a fusion event involving the NUT gene and the BET bromodomain proteins, BRD4 and to a lesser extent BRD3 (41, 42). However, subsequent preclinical studies have demonstrated that these agents may have activity in a much broader range of tumor types.

Central to this extended effect has been the finding that BETi repress c-MYC expression in several tumor types (5, 6), and the demonstration that MYC overexpression can overcome BETi-induced tumor growth inhibition (38).

The importance of c-MYC for the growth of colon cancer cells prompted us to investigate the activity of BETi in this tumor type. First, expression of the targets of these inhibitors, BRD2, BRD3, BRD4, was confirmed in colon cancer cell lines and...
primary tumors. Subsequently, screening of a panel of 20 colorectal cancer cell lines with the BETi JQ1, identified a subset of cell lines particularly sensitive to this agent with GI50 values in the low nanomolar range. A previous study reported JQ1 IC50 values in more than 650 cancer cell lines, including 35 cell lines derived from the intestine, of which 11 were also analyzed in our screen (9). A comparison of both datasets revealed a strong overall concordance in the relative sensitivity of these cell lines. Furthermore, cell lines derived from hematologic cancers, including the AML cell lines MOLM13 and MV4;11, have been shown to be particularly sensitive to bromodomain inhibitors both in vitro and in vivo (5–7, 10, 33). Notably, we found that the in vitro sensitivity of these two AML cell lines was comparable to that of the most sensitive colorectal cancer lines.

Separation of the cell lines according to the major molecular subtypes of colon cancer revealed increased sensitivity of MSI cell lines to JQ1. The mechanistic basis for this difference is unknown; however, MSI tumors tend to be diploid and harbor fewer chromosomal alterations compared with MSS tumors. Whether these global genomic differences impact on the higher order organization of the c-MYC promoter and enhancer, and its subsequent regulation in MSS versus MSI tumors, is a point worthy of investigation. Conversely, we did not observe any significant associations between JQ1 response and CIMP status of the cell lines, or of mutation status of BRD2, BRD3, or BRD4 protein expression.

Recently, MYCN-amplified neuroblastoma and medulloblastoma cell lines were identified as being exquisitely sensitive to bromodomain inhibitors from a screen of multiple cancer cell subtypes of colon cancer revealed increased sensitivity of MSI cell lines to JQ1. The mechanistic basis for this difference is unknown; however, MSI tumors tend to be diploid and harbor fewer chromosomal alterations compared with MSS tumors. Whether these global genomic differences impact on the higher order organization of the c-MYC promoter and enhancer, and its subsequent regulation in MSS versus MSI tumors, is a point worthy of investigation. Conversely, we did not observe any significant associations between JQ1 response and CIMP status of the cell lines, or of mutation status of TP53, KRAS/BRAF or PIK3CA/PTEN, or of BRD2, BRD3, or BRD4 protein expression.

Recently, MYCN-amplified neuroblastoma and medulloblastoma cell lines were identified as being exquisitely sensitive to bromodomain inhibitors from a screen of multiple cancer cell lines (9, 38). In contrast, we observed no significant correlation between c-MYC amplification status and JQ1 response in colorectal cancer cells. Similarly, basal c-MYC mRNA or protein expression levels were not predictive of JQ1 response. The lack of correlation between basal levels of c-MYC expression and JQ1 response is possibly a reflection of the multiple mechanisms of c-MYC regulation in colon cancer cells, including gene amplification (26), transcriptional (25), and posttranslational mechanisms (43), and the ability of JQ1 to impact primarily on only one of these processes—c-MYC transcription (5, 6).

Consistent with this mechanism, a key finding of this study is the observation that JQ1 treatment robustly represses c-MYC mRNA and protein expression in colon cancer cell lines and that the magnitude of c-MYC mRNA and protein downregulation correlates significantly with the magnitude of JQ1-induced cell growth. These findings are also consistent with previous reports in multiple myeloma (6, 44), acute lymphoblastic lymphoma (7), and medulloblastoma (38) cell lines where a correlation between MYC repression and growth inhibition was observed.

Colon cancer cell lines with intermediate sensitivity to JQ1, or which were JQ1-refractory, showed less robust downregulation of c-MYC following drug treatment. We therefore explored approaches for enhancing the efficacy of JQ1 on c-MYC repression and subsequent cell proliferation. Specifically, as genes regulated by super-enhancers have been shown to be particularly sensitive to BET inhibition (16), and as the c-MYC super-enhancer in HCT116 cells is enriched for binding of terminal transcription factors from the WNT/β-catenin/TCF and MAPK/ERK signaling pathways, we explored combinatorial targeting of these pathways with JQ1. This strategy significantly increased the magnitude of c-MYC downregulation with a parallel enhancement of HCT116 cell growth inhibition.

The findings in HCT116 cells suggest that the c-MYC super-enhancer is a key point of integration of the WNT and MAPK signaling pathways. Given that approximately 50% of colorectal cancers harbor concurrent mutations which simultaneously hyperactivate these pathways, this combination strategy may have broad applicability in this tumor type. We also note that in addition to the role of MEK/ERK signaling in driving c-MYC transcription, ERK also stabilizes c-MYC protein by phosphorylating serine 62 (45). The effects of JQ1 and trametinib combination treatment on downregulating c-MYC protein levels may therefore be mediated by both transcriptional and posttranslational mechanisms.

While our findings establish a strong link between the magnitude of c-MYC repression and JQ1-induced growth inhibition, it may not be the only mechanism through which JQ1 induces its effects. Notably, Hu and colleagues, recently reported sensitivity of colon cancer cell lines to the BET inhibitor MS417, which shares the same thieno-1,2,4-triazolo-1,4-diazepine scaffold as JQ1 (46). This study demonstrated that in addition to inhibiting tumor cell growth, MS417 inhibited the migration and metastasis of colon cancer cells both in vitro and in vivo. While the role of c-MYC was not investigated, the authors demonstrated increased expression of E-cadherin (CDH1) following BET inhibition, which may explain the antimitogenic and metastatic effects observed. In addition, increased E-cadherin has recently been linked to perturbation of the WNT/β-catenin pathway which could also result in reduced tumor cell growth (47). Whether the effects of these inhibitors on CDH1 induction are directly mediated or secondary to inhibition of c-MYC would be worthy of investigation.

In summary, these findings demonstrate that pharmacologic inhibition of the BET family abrogates the growth of colon cancer cell lines in vitro and in vivo, identifying this family of proteins as potential treatment targets in colorectal cancer. Mechanistically, we demonstrate that response to these agents correlates significantly with the magnitude of inhibition of c-MYC, an established driver of colorectal cancer. In addition, we identify two rational combination strategies to enhance BETi-mediated repression of c-MYC through combinatorial targeting of the c-MYC super-enhancer in colorectal cancer cells.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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