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

AUTHOR(S)

L Togel, R Nightingale, A C Chueh, A Jayachandran, H Tran, T Phesse, R Wu, O M Sieber, D Arango, Amardeep Dhillon, M A Dawson, B Diez-Dacal, T C Gahman, P Filippakopoulos, A K Shiau, J M Mariadason

PUBLICATION DATE

01-06-2016

HANDLE

10536/DRO/DU:30155874

Published OnlineFirst March 16, 2016; DOI: 10.1158/1535-7163.MCT-15-0724

Small Molecule Therapeutics

Molecular Cancer Therapeutics

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

Lars Tögel[1,2], Rebecca Nightingale[1,2], Anderly C. Chueh[2], Aparna Jayachandran[2], Hoanh Tran[1,2], Toby Phesse[1], Rui Wu[2], Oliver M. Sieber[3], Diego Arango[4], Amardeep S. Dhillon[5], Mark A. Dawson[5], Beatriz Diez-Dacal[6], Timothy C. Gahman[7], Panagis Filippakopoulos[6], Andrew K. Shiau[7], and John M. Mariadason[1,2]

Abstract

Inhibitors of the bromodomain and extraterminal domain (BET) protein family 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). Super-enhancers are chromatin

[1]Olivia Newton-John Cancer Research Institute and La Trobe University School of Cancer Medicine, Melbourne, Victoria, Australia. [2]Ludwig Institute for Cancer Research, Melbourne, Victoria, Australia. [3]Walter and Eliza Hall Institute of Medical Research, Melbourne, Victoria, Australia. [4]CIBBIM-Nanomedicine, Vall d'Hebron University Hospital Research Institute, Universitat Autònoma de Barcelona, Barcelona, Spain. [5]Peter MacCallum Cancer Institute, Melbourne, Victoria, Australia. [6]Ludwig Institute for Cancer Research and UK and Structural Genomics Consortium, Oxford, United Kingdom. [7]Small Molecule Discovery Program, Ludwig Institute for Cancer Research, La Jolla, California.

Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

Corresponding Author: John M. Mariadason, Olivia Newton-John Cancer Research Institute, Austin Health, 145 Studley Road, Heidelberg, Vic 3084, Australia. Phone: +61 3 94963068; E: john.mariadason@onjcri.org.au

doi: 10.1158/1535-7163.MCT-15-0724

Published OnlineFirst March 16, 2016; DOI: 10.1158/1535-7163.MCT-15-0724

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 promoting the initiation and progression of colon cancer, 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 Hauyuan Chemexpress Ltd. and ChemieTek, respectively. Trametinib (GSK1120212) was obtained from Selleck Chemicals.

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 Transcriptor 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-BRD2 (5848, Cell Signalling Technology, 1:100), rabbit anti-BRD3 (A302-368A, Bethyl Laboratories, 1:100), and rabbit anti-BRD4 (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), 1mmol/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-BRD2 (HPA042816, Sigma, 1:250), anti BRD3 (ab50818, Abcam, 1:50), anti-BRD4 (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 (IRdye680CW, Li-Cor, 1:15,000) and goat anti-rabbit (IRdye800CW; Li-Cor, 1:15,000).

Assessment of cell proliferation and cell-cycle kinetics

Cell proliferation was determined by MTS [3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt] assay using the CellTiter 96 AQ_{ueous} One Solution Assay Kit (Promega). Cell-cycle distribution was assessed by propidium iodide (PI) staining and FACS analysis as previously described (36).

Clonogenic cell growth assay

Cells (400–500) were seeded in 96-well plates as single-cell suspensions in 1× DMEM (Life Technologies) supplemented with 10% FCS and 0.45% low melting agarose (SeaPrep Agarose, Lonza) on top of a layer consisting of 1× DMEM (Life Technologies) supplemented with 10% FCS and 0.7% low melting agarose (SeaPrep Agarose, Lonza). Cells were treated with JQ1 for 14 days and colony area determined using ImageJ (37).

Animal studies

Animal studies were performed with the approval of the Austin Health Animal Ethics Committee. Eight-week-old female Balb/*c nu/nu* mice were obtained from the Australian Resources Centre, (ARC, Perth, Australia). HCT116 cells (2×10^6 cells) were injected subcutaneously into the right and left flank of each animal in a 150 μL suspension consisting of a 1:1 mixture of DMEM (Invitrogen) and BD Matrigel Basement Matrix (BD Biosciences). Once palpable tumors developed, mice were randomized to receive an

Published OnlineFirst March 16, 2016; DOI: 10.1158/1535-7163.MCT-15-0724

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 cm^3 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 with GI_{50} values ranging from 19.8 nmol/L (GP5D) through to 1367.7 nmol/L (HuTu80; Fig. 1A). In the three most sensitive cell lines (GP5D, HT29, and LIM1215), JQ1 (500 nmol/L) inhibited cell growth by 79.4% ± 7.6% (mean ± SD) relative to control compared with 12.2% ± 8.9% in the three most resistant lines (HuTu80, SW480, KM12; Fig. 1B). The acute myeloid leukemia (AML) cell lines MOLM13 and MV4;11 have previously been reported to be highly sensitive to JQ1 (5, 10, 33) and therefore were used to compared the sensitivity of colon lines to that of AML lines. The GI_{50} values of the three most sensitive colorectal cancer lines GP5D, HT29, and LIM1215 (19.8, 22.1, and 92.7 nmol/L respectively) were comparable to that of MV4;11 (41.3 nmol/L) and MOLM13 (158.5 nmol/L; Fig. 1B).

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).

JQ1 is largely cytostatic in colon cancer cells

To determine the effect of JQ1 on cell cycle, we treated the three most sensitive and resistant cell lines with JQ1 for 24 hours and analyzed cell-cycle changes by PI staining and FACS analysis. In sensitive cells, JQ1 induced a 49% ± 9% decrease in the

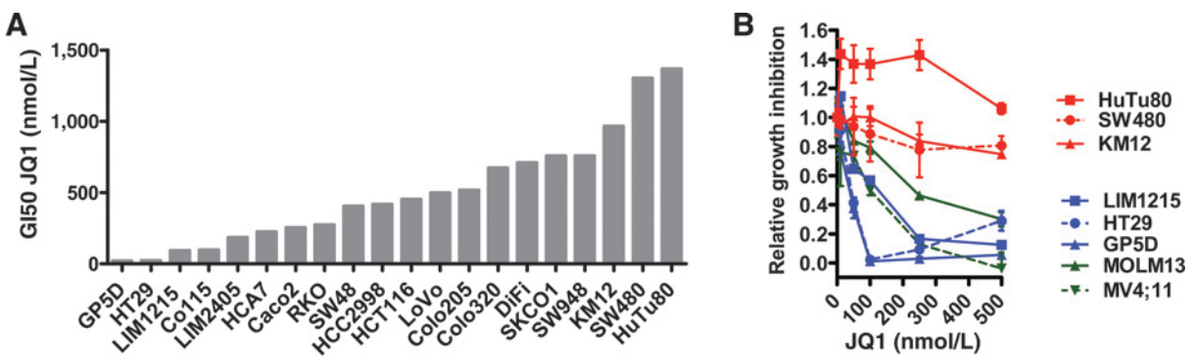

Figure 1.
Effect of JQ1 on colorectal cancer cell proliferation. A, panel of 20 colorectal cancer cell lines ranked, ordered by increasing resistance to JQ1. Response to JQ1 was determined in MTS assays by computation of GI_{50}. B, dose–response analysis of the effect of JQ1 on cell growth inhibition 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.

Published OnlineFirst March 16, 2016; DOI: 10.1158/1535-7163.MCT-15-0724

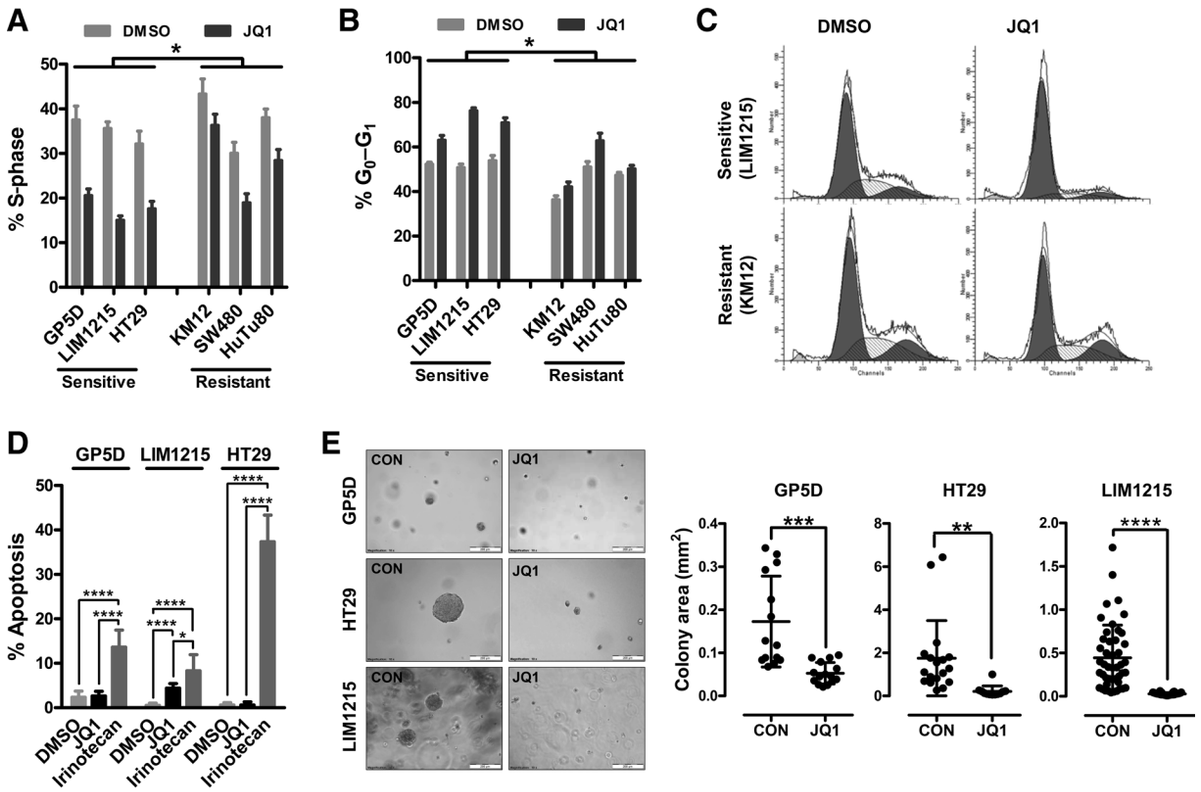

Figure 2.

Effect of JQ1 on cell-cycle distribution, apoptosis, and clonogenic growth. The 3 most sensitive (GP5D, HT29, LIM1215) and resistant (KM12, SW480, HuTu80) colorectal cancer lines were treated with JQ1 (500 nmol/L) or vehicle (0.1% DMSO) for 24 hours and changes in S-phases (A) and G_0–G_1 (B) determined by FACS analysis. Values shown are mean ± SEM of 3 independent experiments. *, $P < 0.05$, Student t test. C, representative FACS plots of JQ1-sensitive LIM1215 and JQ1-resistant KM12 cells treated for 24 hours with DMSO or JQ1. D, effect of JQ1 on apoptosis. The 3 most sensitive cell lines were treated with DMSO (0.1%) or JQ1 (500 nmol/L) for 72 hours and apoptosis determined by FACS analysis. Cells were also treated with the known apoptosis-inducing agent irinotecan (25 μmol/L). Values shown are the mean ± SEM of $n = 3$ independent experiments performed in triplicate. *, $P < 0.05$; ****, $P < 0.0001$, Student t test. E, JQ1 reduces anchorage-independent growth. Cells were grown in soft agar in the presence or absence of JQ1 (500 nmol/L) for 14 days and stained with crystal violet. Values shown are mean ± SD of a representative experiment performed in triplicate (**, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$, Student t test).

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 G_1 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 G_1 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 cancers cell lines 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).

Published OnlineFirst March 16, 2016; DOI: 10.1158/1535-7163.MCT-15-0724

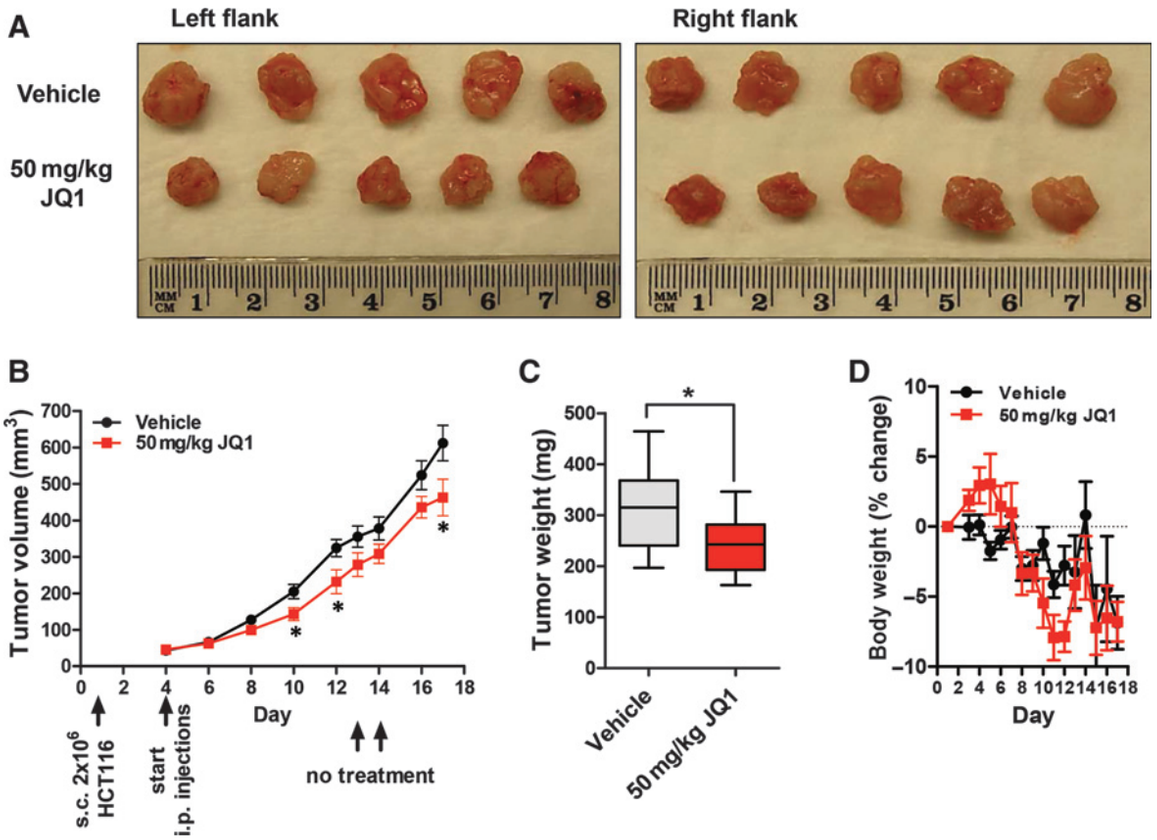

Figure 3.
Effect of JQ1 on tumor growth *in vivo*. A and C, HCT116 cells were injected into the right and left flank of Balb/*c nu/nu* mice (day 0). On day 4, mice were randomized to receive vehicle or JQ1 (50 mg/kg). Mice were treated daily for 18 days except for days 12 to 14, when mice in both groups 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.

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 cells 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).

Published OnlineFirst March 16, 2016; DOI: 10.1158/1535-7163.MCT-15-0724

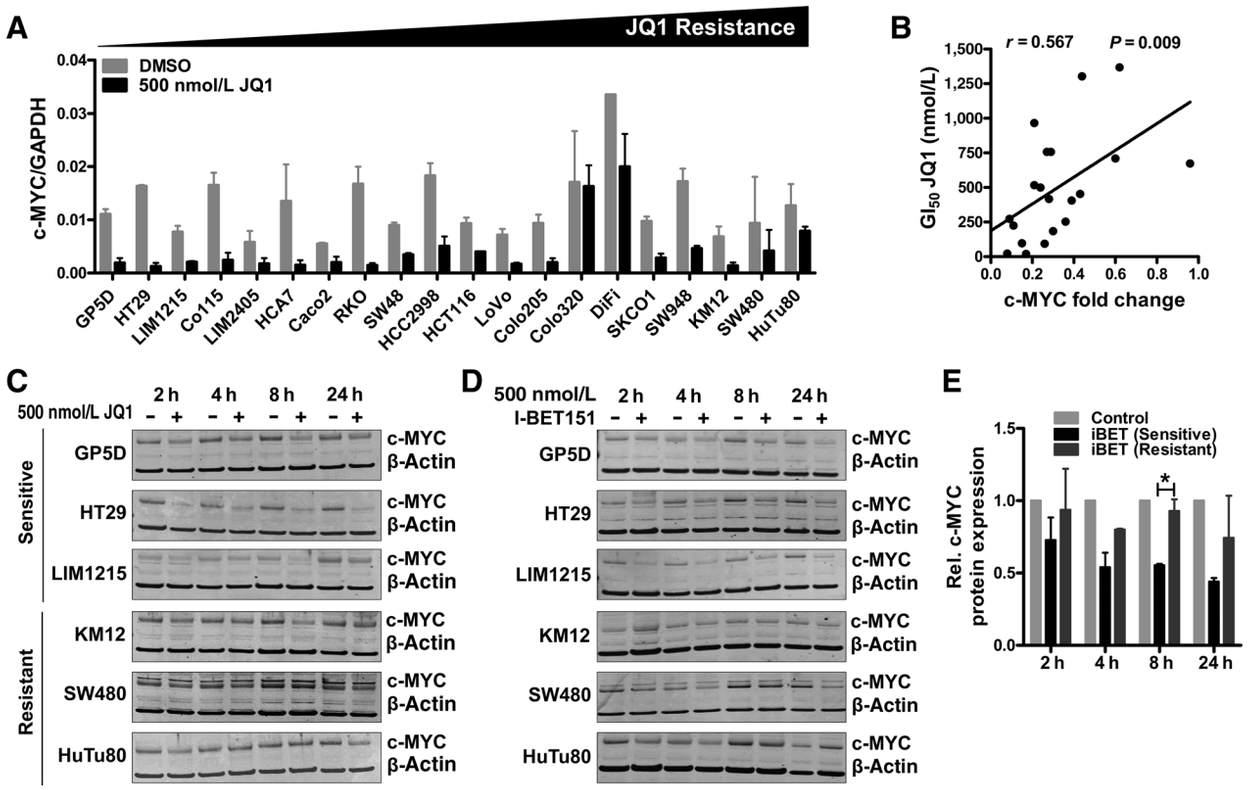

Figure 4.

JQ1-mediated c-MYC repression correlates with growth inhibition. A, *c-MYC* mRNA expression is inhibited by JQ1. Each of the 20 colon colorectal cancer lines were treated with vehicle (0.1% DMSO) or JQ1 (500 nmol/L) for 6 hours and *c-MYC* mRNA expression determined by qPCR. Values shown are mean ± SD of $n = 2$ to 3 experiments. Cell lines are rank ordered from most to least sensitive to JQ1-induced growth inhibition as per Fig. 1A. B, linear regression analysis of JQ1 GI_{50} values (*y*-axis) versus the magnitude of *c-MYC* mRNA repression following JQ1 treatment JQ1 (*x*-axis; $r = 0.567$, $P = 0.017$, Spearman correlation). C and D, BETi preferentially inhibit c-MYC protein expression in sensitive cell lines. JQ1-sensitive GP5D, HT29, and LIM1215 cells and JQ1-resistant KM12, SW480, and HuTu80 cells were treated with JQ1 (500 nmol/L; C) or I-BET151 (500 nmol/L; D) for 2 to 24 hours and c-MYC protein expression determined by Western blotting. E, pooled densitometric analysis of c-MYC protein expression pre- and post-iBET treatment in the 3 sensitive and resistant cell lines. Values shown are mean ± SEM, $n = 3$; *, $P < 0.05$.

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% ± 1.0% and protein expression by 78% ± 2.8% ($n = 2$ 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.

Published OnlineFirst March 16, 2016; DOI: 10.1158/1535-7163.MCT-15-0724

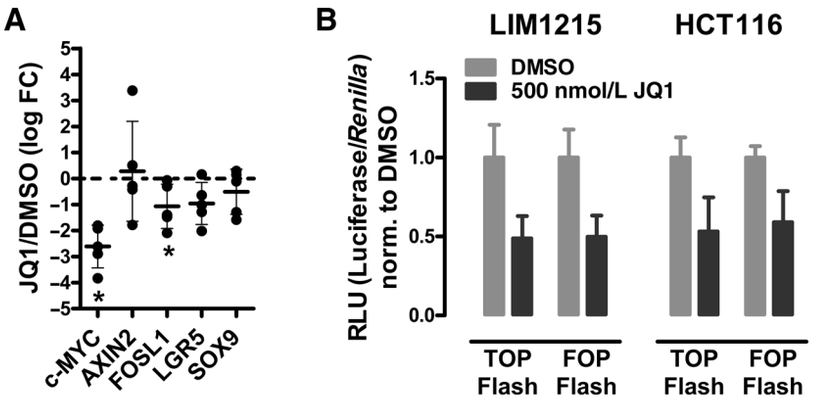

Figure 5.
Effect of JQ1 treatment on WNT/β-catenin/TCF signaling. A, JQ1-induced transcriptional changes on β-catenin/TCF target genes in the 5 most sensitive cell lines (LIM2405, Co115, LIM1215, HT29, GP5D) assessed by qPCR after 6 hours treatment with 500 nmol/L JQ1 ($n = 2$-3, mean ± SD; *, $P < 0.05$, Student t test). B, effect of JQ1 treatment on WNT/β-catenin/TCF reporter assay. Cells were transfected with a β-catenin/TCF reporter constructs treated with JQ1 (500 nmol/L) for 6 hours, and luciferase reporter activity determined. Values shown are mean ± SD of two biologic replicates performed in triplicate.

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

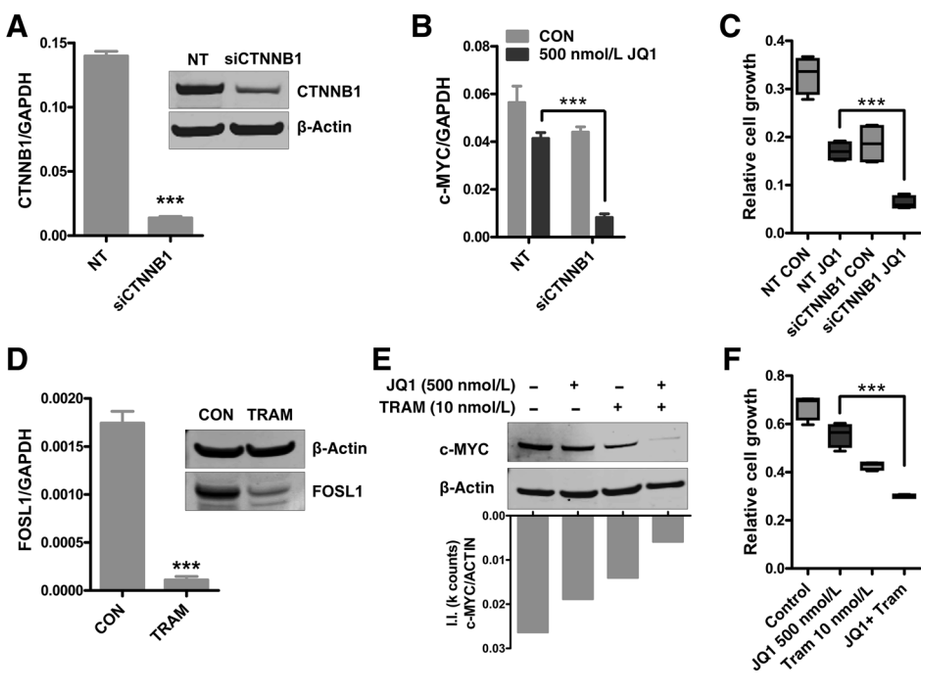

Figure 6.
A–C, combinatorial targeting of the *c-MYC* super-enhancer in HCT116 cells with BET family and WNT/β-catenin/TCF pathway inhibition. A, knockdown efficiency of β-catenin mRNA and protein repression in HCT116 cells 96 hours posttransfection with β-catenin targeting siRNAs. B and C, effect of combined β-catenin (*CTNNB1*) knockdown and JQ1 treatment (500 nmol/L JQ1, 72 hours) on *c-MYC* mRNA expression (B) and cell proliferation (C). Data shown in B are mean ± SD of a representative experiment performed in triplicate. D–F, combinatorial targeting of *the c-MYC* super-enhancer in HCT116 cells with BET family and MEK/ERK pathway inhibition. D, MEK inhibitor trametinib (10 nmol/L) decreases mRNA (bar graph) and protein expression (insert) of the MEK/ERK target gene *FOSL1* after 24 hours. Values shown are mean ± SD of three independent experiments. E and F, effect of JQ1 (500 nmol/L) and trametinib (10 nmol/L) treatment, alone and in combination on c-MYC protein expression (E) and proliferation (F) of HCT116 cells. Data shown in C and F are mean ± min/max value of a representative experiment performed in quadruplicate. ***, $P < 0.001$, Student t test.

Published OnlineFirst March 16, 2016; DOI: 10.1158/1535-7163.MCT-15-0724

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 GI_{50} values in the low nanomolar range. A previous study reported JQ1 IC_{50} 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 *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 cells 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-tria-zolo-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 antimigratory 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.

Authors' Contributions

Conception and design: L. Tögel; A.C. Chueh, A.K. Shiau, J.M. Mariadason
Development of methodology: L. Tögel; A.C. Chueh, B. Diez-Dacal, T.C. Gahman, J.M. Mariadason
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L. Tögel; R. Nightingale, A.C. Chueh, A. Jayachandran, T. Phesse, R. Wu, O.M. Sieber, J.M. Mariadason
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L. Tögel; R. Nightingale, A.C. Chueh, A. Jayachandran, H. Tran, T. Phesse, D. Arango, A.S. Dhillon, T.C. Gahman, P. Filippakopoulos, A.K. Shiau, J.M. Mariadason
Writing, review, and/or revision of the manuscript: L. Tögel; A.C. Chueh, A. Jayachandran, H. Tran, T. Phesse, O.M. Sieber, D. Arango, A.S. Dhillon, M.A. Dawson, T.C. Gahman, P. Filippakopoulos, A.K. Shiau, J.M. Mariadason
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L. Tögel; R. Nightingale, A.K. Shiau, J.M. Mariadason
Study supervision: P. Filippakopoulos, J.M. Mariadason

Grant Support

This work was supported, in part, by grants from the CASS Foundation (L. Tögel), Ludwig Cancer Research, NHMRC SRF 1046092 (J.M. Mariadason), NHMRC project grant 1026555 and the Operational Infrastructure Support Program, Victorian Government, Australia. P. Filippakopoulos is supported by a Wellcome Trust Career-Development Fellowship (095751/Z/11/Z).

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 31, 2015; revised February 19, 2016; accepted March 6, 2016; published OnlineFirst March 16, 2016.

References

1. Shi J, Vakoc CR. The mechanisms behind the therapeutic activity of BET bromodomain inhibition. Mol Cell 2014;54:728–36.
2. Filippakopoulos P, Picaud S, Mangos M, Keates T, Lambert JP, Barsyte-Lovejoy D, et al. Histone recognition and large-scale structural analysis of the human bromodomain family. Cell 2012;149:214–31.
3. Filippakopoulos P, Qi J, Picaud S, Shen Y, Smith WB, Fedorov O, et al. Selective inhibition of BET bromodomains. Nature 2010;468: 1067–73.
4. Nicodeme E, Jeffrey KL, Schaefer U, Beinke S, Dewell S, Chung CW, et al. Suppression of inflammation by a synthetic histone mimic. Nature 2010;468:1119–23.
5. Dawson MA, Prinjha RK, Dittmann A, Giotopoulos G, Bantscheff M, Chan WI, et al. Inhibition of BET recruitment to chromatin as an effective treatment for MLL-fusion leukaemia. Nature 2011;478:529–33.
6. Delmore JE, Issa GC, Lemieux ME, Rahl PB, Shi J, Jacobs HM, et al. BET bromodomain inhibition as a therapeutic strategy to target c-Myc. Cell 2011;146:904–17.
7. Ott CJ, Kopp N, Bird L, Paranal RM, Qi J, Bowman T, et al. BET bromodomain inhibition targets both c-Myc and IL7R in high-risk acute lymphoblastic leukemia. Blood 2012;120:2843–52.
8. Banerjee C, Archin N, Michaels D, Belkina AC, Denis GV, Bradner J, et al. BET bromodomain inhibition as a novel strategy for reactivation of HIV-1. J Leukoc Biol 2012;92:1147–54.
9. Puissant A, Frumm SM, Alexe G, Bassil CF, Qi J, Chanthery YH, et al. Targeting MYCN in neuroblastoma by BET bromodomain inhibition. Cancer Discov 2013;3:308–23.
10. Mertz JA, Conery AR, Bryant BM, Sandy P, Balasubramanian S, Mele DA, et al. Targeting MYC dependence in cancer by inhibiting BET bromodomains. Proc Natl Acad Sci U S A 2011;108:16669–74.
11. Jiang YW, Veschambre P, Erdjument-Bromage H, Tempst P, Conaway JW, Conaway RC, et al. Mammalian mediator of transcriptional regulation and its possible role as an end-point of signal transduction pathways. Proc Natl Acad Sci U S A 1998;95:8538–43.
12. Jang MK, Mochizuki K, Zhou M, Jeong HS, Brady JN, Ozato K. The bromodomain protein Brd4 is a positive regulatory component of P-TEFb and stimulates RNA polymerase II-dependent transcription. Mol Cell 2005;19:523–34.
13. Yang Z, Yik JH, Chen R, He N, Jang MK, Ozato K, et al. Recruitment of P-TEFb for stimulation of transcriptional elongation by the bromodomain protein Brd4. Mol Cell 2005;19:535–45.
14. Peterlin BM, Price DH. Controlling the elongation phase of transcription with P-TEFb. Mol Cell 2006;23:297–305.
15. Adelman K, Lis JT. Promoter-proximal pausing of RNA polymerase II: emerging roles in metazoans. Nat Rev Genet 2012;13:720–31.
16. Loven J, Hoke HA, Lin CY, Lau A, Orlando DA, Vakoc CR, et al. Selective inhibition of tumor oncogenes by disruption of super-enhancers. Cell 2013;153:320–34.
17. Whyte WA, Orlando DA, Hnisz D, Abraham BJ, Lin CY, Kagey MH, et al. Master transcription factors and mediator establish super-enhancers at key cell identity genes. Cell 2013;153:307–19.
18. Fernandez PC, Frank SR, Wang L, Schroeder M, Liu S, Greene J, et al. Genomic targets of the human c-Myc protein. Genes Dev 2003;17: 1115–29.
19. Li Z, Van Calcar S, Qu C, Cavenee WK, Zhang MQ, Ren B. A global transcriptional regulatory role for c-Myc in Burkitt's lymphoma cells. Proc Natl Acad Sci U S A 2003;100:8164–9.
20. Beroukhim R, Mermel CH, Porter D, Wei G, Raychaudhuri S, Donovan J, et al. The landscape of somatic copy-number alteration across human cancers. Nature 2010;463:899–905.
21. Shou Y, Martelli ML, Gabrea A, Qi Y, Brents LA, Roschke A, et al. Diverse karyotypic abnormalities of the c-myc locus associated with c-myc dysregulation and tumor progression in multiple myeloma. Proc Natl Acad Sci U S A 2000;97:228–33.
22. Taub R, Kirsch I, Morton C, Lenoir G, Swan D, Tronick S, et al. Translocation of the c-myc gene into the immunoglobulin heavy chain locus in human Burkitt lymphoma and murine plasmacytoma cells. Proc Natl Acad Sci U S A 1982;79:7837–41.
23. Yeh E, Cunningham M, Arnold H, Chasse D, Monteith T, Ivaldi G, et al. A signalling pathway controlling c-Myc degradation that impacts oncogenic transformation of human cells. Nature Cell Biol 2004;6:308–18.
24. Wright JB, Brown SJ, Cole MD. Upregulation of c-MYC in cis through a large chromatin loop linked to a cancer risk-associated single-nucleotide polymorphism in colorectal cancer cells. Mol Cell Biol 2010;30: 1411–20.
25. He TC, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, et al. Identification of c-MYC as a target of the APC pathway. Science 1998; 281:1509–12.
26. Kozma L, Kiss I, Szakall S, Ember I. Investigation of c-myc oncogene amplification in colorectal cancer. Cancer Lett 1994;81:165–9.
27. Hongxing Z, Nancai Y, Wen S, Guofu H, Yanxia W, Hanju H, et al. Depletion of c-Myc inhibits human colon cancer colo 320 cells' growth. Cancer Biother Radiopharm 2008;23:229–37.
28. Zhang X, Ge YL, Tian RH. The knockdown of c-myc expression by RNAi inhibits cell proliferation in human colon cancer HT-29 cells in vitro and in vivo. Cell Mol Biol Lett 2009;14:305–18.
29. Sansom OJ, Meniel VS, Muncan V, Phesse TJ, Wilkins JA, Reed KR, et al. Myc deletion rescues Apc deficiency in the small intestine. Nature 2007;446: 676–9.
30. Yekkala K, Baudino TA. Inhibition of intestinal polyposis with reduced angiogenesis in ApcMin/+ mice due to decreases in c-Myc expression. Mol Cancer Res 2007;5:1296–303.
31. Ignatenko NA, Holubec H, Besselsen DG, Blohm-Mangone KA, Padilla-Torres JL, Nagle RB, et al. Role of c-Myc in intestinal tumorigenesis of the ApcMin/+ mouse. Cancer Biol Ther 2006;5:1658–64.
32. Cho H, Herzka T, Zheng W, Qi J, Wilkinson JE, Bradner JE, et al. RapidCaP, a novel GEM model for metastatic prostate cancer analysis and therapy, reveals myc as a driver of Pten-mutant metastasis. Cancer Discov 2014; 4:318–33.
33. Zuber J, Shi J, Wang E, Rappaport AR, Herrmann H, Sison EA, et al. RNAi screen identifies Brd4 as a therapeutic target in acute myeloid leukaemia. Nature 2011;478:524–8.
34. Mouradov D, Sloggett C, Jorissen RN, Love CG, Li S, Burgess AW, et al. Colorectal cancer cell lines are representative models of the main molecular subtypes of primary cancer. Cancer Res 2014;74:3238–47.
35. Medico E, Russo M, Picco G, Cancelliere C, Valtorta E, Corti G, et al. The molecular landscape of colorectal cancer cell lines unveils clinically actionable kinase targets. Nature Commun 2015;6:7002.
36. Mariadason JM, Rickard KL, Barkla DH, Augenlicht LH, Gibson PR. Divergent phenotypic patterns and commitment to apoptosis of Caco-2 cells during spontaneous and butyrate-induced differentiation. J Cell Physiol 2000;183:347–54.
37. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. Nature Methods 2012;9:671–5.
38. Bandopadhayay P, Bergthold G, Nguyen B, Schubert S, Gholamin S, Tang Y, et al. BET bromodomain inhibition of MYC-amplified medulloblastoma. Clin Cancer Res 2014;20:912–25.
39. Korinek V, Barker N, Morin PJ, van Wichen D, de Weger R, Kinzler KW, et al. Constitutive transcriptional activation by a b-catenin-Tcf complex in $APC^{-/-}$ colon carcinoma. Science 1997;275:1784–87.
40. Hnisz D, Schuijers J, Lin CY, Weintraub AS, Abraham BJ, Lee TI, et al. Convergence of developmental and oncogenic signaling pathways at transcriptional super-enhancers. Mol Cell 2015;58:362–70.

Published OnlineFirst March 16, 2016; DOI: 10.1158/1535-7163.MCT-15-0724

41. French CA, Ramirez CL, Kolmakova J, Hickman TT, Cameron MJ, Thyne ME, et al. BRD-NUT oncoproteins: a family of closely related nuclear proteins that block epithelial differentiation and maintain the growth of carcinoma cells. Oncogene 2008;27:2237–42.
42. Stelow EB.A review of NUT midline carcinoma. Head Neck Pathol 2011; 5:31–5.
43. Rajagopalan H, Jallepalli PV, Rago C, Velculescu VE, Kinzler KW, Vogelstein B, et al. Inactivation of hCDC4 can cause chromosomal instability. Nature 2004;428:77–81.
44. Baratta MG, Schinzel AC, Zwang Y, Bandopadhayay P, Bowman-Colin C, Kutt J, et al. An in-tumor genetic screen reveals that the BET bromodomain protein, BRD4, is a potential therapeutic target in ovarian carcinoma. Proc Natl Acad Sci U S A 2015;112:232–7.
45. Sears R, Nuckolls F, Haura E, Taya Y, Tamai K, Nevins JR. Multiple Ras-dependent phosphorylation pathways regulate Myc protein stability. Genes Dev 2000;14:2501–14.
46. Hu Y, Zhou J, Ye F, Xiong H, Peng L, Zheng Z, et al. BRD4 inhibitor inhibits colorectal cancer growth and metastasis. Int J Mol Sci 2015; 16:1928–48.
47. Huels DJ, Ridgway RA, Radulescu S, Leushacke M, Campbell AD, Biswas S, et al. E-cadherin can limit the transforming properties of activating beta-catenin mutations. EMBO J 2015;34:2321–33.

Published OnlineFirst March 16, 2016; DOI: 10.1158/1535-7163.MCT-15-0724

Molecular Cancer Therapeutics

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

Lars Tögel, Rebecca Nightingale, Anderly C. Chueh, et al.

Mol Cancer Ther 2016;15:1217-1226. Published OnlineFirst March 16, 2016.