**BET Protein Inhibitor JQ1 Attenuates Myc-Amplified MCC Tumor Growth In Vivo**

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**Abstract**

Merkel cell carcinoma (MCC) is an aggressive neuroendocrine tumor of the skin currently with no cure. In this study, we have first demonstrated that c-Myc overexpression is common in MCC. By targeting c-Myc, bromodomain inhibitors have demonstrated antitumor efficacy in several preclinical human cancer models. Thus, we interrogated the role of c-Myc inhibition in MCC with c-Myc amplification by using the BET inhibitor JQ1. We have uncovered that c-Myc can be regulated by JQ1 in MCC cells with pathologic c-Myc activation. Moreover, JQ1 potently abrogates c-Myc expression in MCC cells and causes marked G1 cell-cycle arrest. Mechanistically, JQ1-induced cell-cycle arrest coincides with downregulation of cyclin D1 and upregulation of p21, p27, and p57, whereas JQ1 exerts no effect on apoptosis in MCC cells. Further knockdown of p21, p27, or p57 by shRNA partially protects cells from JQ1-induced cell-cycle arrest. In addition, c-Myc knockdown by shRNA generates significant cell-cycle arrest, suggesting that c-Myc overexpression plays a role in MCC pathogenesis. Most importantly, JQ1 significantly attenuates tumor growth in xenograft MCC mouse models. Our results provide initial evidence, indicating the potential clinical utility of BET protein inhibitors in the treatment of MCC with pathologic activation of c-Myc. *Cancer Res;* 74(23): 1-13. © 2014 AACR.

**Introduction**

Merkel cell carcinoma (MCC) is an aggressive skin tumor of neuroendocrine origin with a rising incidence. Its 5-year mortality rate is 46%, and there is no cure for metastatic disease (1, 2). Although a causative link between Merkel cell polyomavirus (MCC) and MCC has been proposed, the cellular mechanisms involved in MCC pathogenesis remains largely unknown (3, 4).

Interrogation of MCC tumors for mutations of both tumor-suppressor genes and oncogenes, such as p53, PTEN, Ras, B-RAF, c-kit, and β-catenin, which are frequently mutated and dysregulated in many cancers, have failed to reveal a consistent significant role for any of these genes in MCC (5, 6). Intriguingly, one study has shown that the MAPK pathway is silent, as demonstrated by lack of pathway activation and no ERK phosphorylation (7). Recently, PI3K/AKT and the mTOR pathway, the most commonly dysregulated pathway in human cancer, are found to be upregulated in MCCs, though low mutation rates of PI3K/Akt have been detected (8, 9). Interestingly, Paulson and colleagues (10) reported amplification of L-Myc in MCC by array-comparative genome hybridization (CGH) in 2008. Moreover, a recent study by Kwun and colleagues (11) suggests that MCV small T antigen stabilizes c-Myc expression by inhibiting the cellular ubiquitin ligase protein complex, suggesting that c-Myc plays a role in MCC pathogenesis.

c-Myc is a master regulator of cell proliferation and metabolism and is central to the pathogenesis of many human cancers, by the coordinated upregulation of a transcriptional program influencing metabolic adaptation, cell division, and survival (12–14). c-Myc also promotes transformation and maintenance of stem cells in genetically engineered mouse models of glioblastoma (15–17). Furthermore, conditional transgenic models featuring tunable transcriptional suppression have shown that even transient inactivation of Myc results in sustained regression of tumors (17). However, therapies directly targeting Myc hyperactivation are not currently available in the clinic.

Members of the bromodomains and extraterminal (BET) domain family of proteins (BRD2, BRD3, BRD4, and Brdt) are associated with acetylated chromatin and facilitate transcriptional activation through increasing the effective molarity of recruited transcriptional activators (18). BET proteins primarily bind to the transcriptional start sites of genes expressed during mitosis and affect the transcription of growth- and survival-promoting genes (19, 20). Recently, an RNAi screen has...
discovered that knockdown of BRD4 leads to downregulation of c-Myc in acute myelogenous leukemia (21). Subsequently, small-molecule compounds with high potency against BET proteins, such as JQ1, I-BET151, iBET176, and MS417, have been developed (22, 23). Through epigenetic mechanism, they repress downstream gene expression by competitively binding to BET proteins and displacing BET proteins from acetylated lysines on chromatin. Notably, c-Myc transcription is associated locally and globally with increases in histone lysine side-chain acetylation (18, 19, 22). Consistent with this model, inhibition of BET protein with JQ1 results in significant downregulation of c-Myc and antitumor activity in several hematopoietic malignancies as well as in NUT midline carcinoma (24–35). Thus, BET protein inhibitors are currently in phase I and II clinical trials for advanced malignancies.

Despite two previously published studies, the role of c-Myc in MCC pathogenesis remains poorly defined. In this study, we have revealed that c-Myc overexpression is common in MCC fresh tumors examined and primary human MCC cell lines. c-Myc inhibition by the BET protein inhibitor JQ1 induces cell-cycle arrest and decreased MCC cell proliferation. Most importantly, JQ1 significantly attenuated xenograft tumor growth in vivo. Thus, our results establish the therapeutic rationale for BET protein inhibitors in the management of MCC with pathologic activation of c-Myc.

Materials and Methods

Cell lines

In accordance with institutional approvals for the human study protocol, we have established three primary human MCC cell lines (MCC-2, MCC-3, and MCC-5) derived from lymph node metastases of 3 patients with MCC as previously described (36, 37). Primary MCC cells were cultured with RPMI-1640 medium containing 10% FBS and 10% penicillin–streptomycin–t-glutamine and incubated at 37°C in a humidified incubator with 5% CO2. Fresh medium was added every other day.

Reagents

Human embryonic kidney (293T/17; ATCC) cells were cultured in DMEM supplemented with 10% FBS and 5 mg/mL of sodium pyruvate. The following antibodies were used for immunoblotting analyses or IHC: c-Myc, β-actin, and cleaved caspase-3 (Cell Signaling Technology); BRD4, p21, p27, p57, and cyclinD1 (Santa Cruz Biotechnology). Additional reagents in the study include: TransIT-LT1 transfection reagent (Millipore) and staurosporine (Selleck Chemicals). Immunohistochemistry

Briefly, 5-μm paraffin sections were deparaffinized with xylene and graded ethanol, and antigen retrieval was performed by microwaving in 0.01 mol/L sodium citrate for 20 minutes. Endogenous tissue peroxidase activity was blocked with 1% hydrogen peroxide at room temperature (RT) for 1 hour. The sections were further blocked with normal goat or horse serum at RT for 1 hour following incubation with primary antibody dilution at 4°C overnight. Secondary antibody was applied to the slides for 1 hour at RT before developing in the horseradish peroxidase detection system and freshly prepared diaminobenzidine as the chromagen (brown). Sections were counterstained with hematoxylin. Immunostained slides were viewed on an Olympus BX51 Research System Microscope by 20× and 40× UPlanApo air objective lenses (Olympus America). Images were photographed using a high-resolution interline CCD camera (CoolSNAP cf Photometrics) and acquired with automated microscopy acquisition software (MetaMorph version 7.7; Molecular Devices).

Gene-expression analysis

Total RNA was isolated from primary MCC cell lines and MCC fresh tissues with the RNAesy Kit (Qiagen). cDNA was generated from mRNA using the Reverse Transcription Kit (Applied Biosystems). Quantitative real-time-PCR (qRT-PCR) was performed with a StepOne Plus Real-Time PCR System (Applied Biosystems). The following TaqMan Gene Expression Assays primers were used: Hs00905030_m1 (c-Myc), Hs00355782_m1 (p21), Hs01597588_m1 (p27), Hs00175938_m1 (p57), Hs00211334_m1 (MRPS2) and Hs00765553_m1 (cyclin D1), Hs01062014_m1 (Notch1), Hs00765730_m1 (NFkB). Triplicate runs of each sample were normalized to MRPS2 mRNA to determine relative expression.

Immunoblotting

 Cultured cells were washed with ice-cold PBS and lysed in 1× RIPA buffer containing 1 mmol/L DTT and Complete Mini EDTA-free protease inhibitor cocktail. After incubation on ice for 30 minutes, the cell lysates were clarified by centrifugation at 14,000 rpm for 15 minutes at 4°C. Ten to 30 μg of total protein was subjected to 8% or 12% SDS-PAGE gels and transferred electrophoretically onto polyvinylidene difluoride membrane by a semidry blotting system (Bio-Rad). The membrane was blocked in 5% fat-free milk/Tris–buffered saline 0.1% Tween 20 for 1 hour at RT and incubated with primary antibodies at 4°C overnight, followed by secondary antibodies conjugated with horseradish peroxidase (Santa Cruz Biotechnology). Then the membrane was subjected to Western blot analysis with ECL detection reagent. The xenograft tumor tissue was homogenized in 2% SDS lysis buffer and then processed for Western blotting as described above.

MCV detection

DNA was prepared using the DNeasy Kit (Qiagen). DNA quality was confirmed by GAPDH. PCR was performed with 120 ng of genomic DNA using the Taq DNA polymerase (Invitrogen) in a final volume of 50 μL for 30 to 35 cycles. Primer sets for LT3 and MCPVS1 were used as published previously (36).

Cell proliferation and viability assays

Cell proliferation analysis was performed using the Cell Counting Kit-8 (CCK-8 kit; Sigma) and manually cell counting. MCC cell lines were plated at 1 × 103 cells per well in
96-well plates, allowed to recover for 3 hours and then exposed to serial concentrations of JQ1 (Selleck Chemicals) for 24, 48, 72, and 120 hours, respectively. CCK-8 (10 μL) was added to each well and incubated at 37°C for another 4 hours before measuring using a spectrophotometer at 450 nm. MCC cell lines were plated at 10 × 10^3 cells per well in 6-well plates, allowed to recover for 3 hours, and then exposed to serial concentration of JQ1 (Selleck Chemicals) for 24, 48, 72, and 120 hours, respectively. Cells were collected at different time points and counted manually with trypan blue exclusion staining (ViCell; Beckman Coulter).

**Methycellulose colony assay**

MCC cells clonogenic formation was assayed by culturing MCC cells in complete methycellulose (Methocult GF+H4435; STEMCELL Technologies) according to the manufacturer’s protocol. Briefly, MCC cells (3,000 cells) were resuspended in 1 mL complete methycellulose with JQ1 (800 nmol/L) or vehicle, and incubated in a humidified incubator. Colony formation was assayed after 21 days in culture by microscopy. Colonies consisting of at least 40 cells were counted.

**Apoptosis and cell-cycle analysis**

Apoptosis was detected by flow cytometry using Annexin V–FITC according to the manufacturer’s protocol (BD Biosciences). Briefly, MCC cells (1 × 10^6 cells) were plated in 6-well plates for 3 hours followed by treatment with JQ1 for 72 hours before Annexin V and propidium iodide (PI) staining (BD Biosciences FACS Aria). Cells were resuspended in the binding buffer with only Annexin V or PI served as controls. For each dye, appropriate electronic compensation of the instrument was performed to avoid overlapping of the two emission spectra. For cell-cycle analysis, MCC cells (1 × 10^6 cells) were seeded in 6-well plates for 3 hours followed by treatment with JQ1 for 72 hours and then were labeled with 10 μmol/L bromodeoxyuridine (BrdUrd) for 2 hours. BrdUrd incorporation was detected using Alexa Fluor 488–conjugated mouse anti-BrdUrd antibody (BD Biosciences-Pharminen) followed by 7-AAD staining (BD Biosciences FACS Aria) for cell-cycle analysis per the manufacturer’s protocol.

**Lentiviral transduction**

Lentivector directing expression of shRNA specific to p21 (TRCN0000040123), p27 (TRCN0000009856), p57 (TRCN0000010484), and c-Myc (TRCN0000039642) were purchased from Sigma-Aldrich and nonvtargeting PLKO.1 scrambled shRNA (plasmid 1864) was purchased from Addgene. To generate lentivirus media, 293T/17 cells were cotransfected with gene transfer vectors and virus packaging vectors ΔH8.2 and VSVG by TransIT-LT1 transfection reagent (Mirus). Two days following transfection, viral supernatants were collected and MCC cells were transduced with viral supernatant for 48 hours in fibronectin-coated 6-well plates in the presence of 8 μg/mL polybrene after spinoculation at 800 × g, 32°C for 30 minutes. Cells were then selected in culture media containing 2 μg/mL puromycin for at least 48 hours.

**Xenograft implantation**

Five-week-old female immunodeficient NOD/SCID/IL2r-γnull (NSG) mice (The Jackson Laboratory; Strain #005557) were used for generating xenograft mouse models. Tumor cells were prepared by suspending 2 × 10^7 MCC cells in 80 μL of media + 120 μL of Matrigel (BD Biosciences; catalog no. #354248) and inoculated on right rear flanks. Palpable tumor growth appeared within 3 to 5 days of inoculation, and treatment per protocol began when tumors reached approximately 100 mm^3 in volume.

**In vivo drug study**

Tumor-bearing mice were randomized into treatment and control groups (n ≥ 7 for each condition) and began receiving i.p. injection administration of vehicle (10% 2-hydroxypropyl-β-cyclodextrin in water) or 50 mg/kg/d JQ1 for 3 weeks duration. Mice were monitored daily, tumor xenografts were measured with digital calipers, and tumor volume was calculated as L^2 × W/2, where L is length and W is width. All animal experiments were done under a protocol approved by the University Institutional Animal Care and Use Committee. In accordance with institutional guidelines on animal care, experimental endpoints were determined by one of the following: (i) completion of 21-day treatment course, or (ii) attainment of tumor burden exceeding 2 cm in any dimension, or (iii) further complications affecting animal welfare. Upon reaching experimental endpoints, mice were humanely euthanized, and tumors were excised and dissected for characterization and mechanistic studies.

**Statistical analysis**

All the measurements were made in triplicate, and all values are represented as mean ± SD. Statistical analysis was performed with the Student t test or one-way ANOVA; *, a P value of <0.05 was considered statistically significant.

**Results**

**c-Myc overexpression is common in MCC tumors and primary MCC cell lines**

c-Myc is a transcription factor that not only regulates the expression of many genes crucial for cell proliferation and differentiation, it is also one of the most prevalent oncogenes deregulated in human cancers (13, 38). Interestingly, an array-CGH study revealed amplification of a region harboring L-Myc in MCC (10). Thus, we were compelled to examine c-Myc expression in MCC fresh tumors. We found that 87.5% (14/16) of MCCs overexpressed c-Myc as compared with normal skin by immunoblotting (Fig. 1A). Our findings confirm a recent publication, suggesting that MCV small T antigen contributes to c-Myc overexpression (11). To see whether c-Myc overexpression correlates with MCV status, DNAs were extracted from MCC fresh tumors and MCV was detected as described previously (36). As shown in Fig. 1B, six MCC samples that were negative for MCV also possessed c-Myc overexpression. Intriguingly, two MCV–positive MCC (Tumor-10 and Tumor-15) failed to demonstrate c-Myc amplification. Thus, c-Myc overexpression in
MCC tumors was independent of MCV status in our study. Next, we assessed c-Myc expression in three primary MCC cell lines established in our laboratory. MCC-2, MCC-3, and MCC-5 cell lines have been described previously (37, 39). Primary MCC cells grow in clusters in culture and display large, round to oval, vesicular nuclei with scant cytoplasm that are characteristic of MCC (Supplementary Fig. S1). As shown in Fig. 1C, both MCC-3 and MCC-5 overexpressed c-Myc at the mRNA and protein levels, but discernible expression of c-Myc was detected in MCC-2 cells. We next wanted to determine the cell growth properties among these three primary MCC cell lines. Cell proliferation was assessed by cell counting manually. As shown in Fig. 1D, MCC-3 and MCC-5 cells with c-Myc overexpression possessed higher cell proliferation as compared with MCC-2 cells. Therefore, we have demonstrated that c-Myc overexpression is common in MCC tumors and it is independent of MCV. Moreover, primary MCC cells with c-Myc overexpression carry a higher proliferation rate.

**BET inhibitor JQ1 abolishes c-Myc expression and represses primary MCC cell proliferation**

Targeting c-Myc by the BET inhibitor JQ1 has demonstrated efficient suppression of c-Myc expression as well as antitumor activity in many types of human cancer both in vitro and in vivo (28, 32). We therefore decided to examine the effects of growth inhibition by JQ1 in MCC cells. On the basis of the published studies, there is a wide range of IC₅₀ of JQ1 used (200 nmol/L–5 μmol/L; refs. 25, 31). However, IC₅₀ for most hematopoietic tumor cell lines are between 500 and 1,000 nmol/L and the duration of treatment was between 3 and 5 days. Thus, we decided to test JQ1 at a series of concentration between 200 and 800 nmol/L and at 24, 48, and 72 hours. In agreement with other published findings, a significant reduction of c-Myc expression was found after JQ1 treatment in both MCC-3 and MCC-5 cells (Fig. 2A). A time- and dose-dependent inhibition of MCC cell proliferation was observed after JQ1 treatment as determined by CCK-8 assay and manual counting (Fig. 2B).
Consistent with our hypothesis, a greater inhibition was found in MCC-3 and MCC-5 cells with c-Myc overexpression. Interestingly, a growth inhibition of MCC-2 cells was found with a higher concentration of JQ1 (800 nmol/L; Supplementary Fig. S4A). After 72 hours treatment and at the lowest tested dosage of JQ1 (200 nmol/L), we observed approximately 20% of growth inhibition in MCC-3 and MCC-5, whereas MCC-2 cells were almost entirely unaffected. At the highest dosage of JQ1 (800 nmol/L) after 72 hours treatment, we observed >50% of growth inhibition in MCC-3 and MCC-5 as compared with 10% inhibition in MCC-2 cells (Fig. 2B and Supplementary Fig. S4A). Although we observed suppression of cell growth in MCC cells without c-Myc overexpression by JQ1, in this study, we decided to focus on the biologic effects of c-Myc inhibition in MCC cell lines (MCC-3 and MCC-5) possessing c-Myc amplification. Because a 50% inhibition of growth was seen in both MCC cell lines at 800 nmol/L concentrations of JQ1 after 72 hours treatment, we chose JQ1 at the concentration of 800 nmol/L for all studies carried out in this article. To evaluate the effect of long-term inhibition by JQ1, colony formation assay was performed. Similar to the results above, JQ1 (800 nmol/L) significantly decreased the number of colony formation in MCC cells compared with that of controls.

Figure 2. The BET protein inhibitor JQ1 reduces c-Myc expression and attenuates primary MCC cell proliferation. A, decreased c-Myc expression in MCC-3 and MCC-5 treated with JQ1 (800 nmol/L) for 72 hours by qRT-PCR and immunoblotting. The mRNA expression of target genes was normalized to that of MRPS2 and a value of 1.0 was assigned to the mRNA expression of target genes in the control group (means ± SEM; **, P < 0.01 vs. control); β-actin was used as a loading control for immunoblotting. B, MCC-3 and MCC-5 cells are sensitive to JQ1 inhibition. MCC cell lines (MCC-3 and MCC-5) were cultured with JQ1 at different concentrations (200, 400, and 800 nmol/L) for 24, 48, 72, and 120 hours, respectively (means ± SEM; *, P < 0.05; **, P < 0.01; ***, P < 0.001 vs. control). C, decreased colony formation in MCC-3 and MCC-5 cells treated with JQ1 (800 nmol/L) compared with that of DMSO control. A total of 3,000 cells were mixed with methylcellulose medium with JQ1 (800 nmol/L) or DMSO and the mixture was plated in each 35-mm dish for 21 days at 37 °C (means ± SEM; ***, P < 0.001 vs. control); scale bars, 200 μm.

JQ1 Suppresses MCC Xenograft Tumor Growth
Our data suggest that BET inhibition has potent antiproliferative effects in MCC cells with c-Myc overexpression in vitro.

**JQ1-induced cell-cycle arrest is via upregulation of p21, p27, and p57 in MCC cell lines**

To further investigate the growth inhibitory mechanisms of JQ1 on MCC, we evaluated the effects of JQ1 on cell-cycle progression and apoptosis by flow cytometry. Both MCC-3 and MCC-5 cells treated with JQ1 at 800 nmol/L for 72 hours were collected and subjected to BrdUrd cell-cycle or Annexin V/PI analysis, respectively. Consistent with the antiproliferative effects of JQ1, a pronounced decrease of cells in S phase with a concomitant increase in cells in G0–G1 phase was observed in treated groups as compared with that in the control groups (Fig. 3A), suggesting a cell-cycle arrest at the G0–G1 phase. Further assessment of the involvement of the cell-cycle target genes, downregulation of cell-cycle promoter gene cyclin D1, and upregulation of negative cell-cycle regulator genes p21, p27, and p57 was detected at the RNA and protein levels after JQ1 treatment in both MCC-3 and MCC-5 cells (Fig. 3B). To further elucidate which cell-cycle–negative regulatory proteins contribute to cell-cycle arrest by JQ1, we have successfully abolished expression of p21, p27, or p57 by shRNA in MCC cells, respectively (Fig. 4A and Supplementary Fig. S4B). Interestingly, individual knockdown of p21, p27, or p57 expression partially rescued JQ1-induced cell-cycle arrest to a similar degree, which suggested that JQ1-induced suppressed proliferation is mainly due to upregulation of p21, p27, and p57 (Fig. 4B and C). Of note, JQ1 failed to induce apoptosis in MCC cells, as demonstrated by Annexin V study (Supplementary Fig. S2A and S2B). MCC cells treated with staurosporine (1 μmol/L) for 4 hours served as positive controls.

c-Myc knockdown is sufficient to recapitulate the antitumor effect of JQ1 in MCC cells

To confirm whether c-Myc is the major effector of JQ1 inhibition in MCC cells, we next depleted c-Myc expression by shRNA followed by JQ1 treatment. Successful knockdown of c-Myc expression by shRNA in MCC cells is shown in Fig. 5A and B. Similarly, c-Myc knockdown caused a significant reduction of cells in S phase that was associated with G1 arrest (Fig. 5C and D). Cells in S phase reduced from 21% to 2.2% in MCC-3 cells, and from 19.2% to 1.3% in MCC-5 cells. Moreover, there was no additive effect by JQ1 treatment in c-Myc knockdown cells, suggesting that JQ1-induced cell-cycle arrest was mediated by c-Myc. Although we cannot exclude the involvement of additional cellular targets, the strong concordance between phenotypes induced by c-Myc knockdown and JQ1 supports the
notion that c-Myc is the major target of JQ1 in MCC with c-Myc amplification.

In addition to c-Myc, we also examined other transcription factors, such as Notch and NFκB and c-Jun, which have been suggested to be regulated by BET brodomain proteins in other cell types (40, 41). c-Jun expression is extremely low in both MCC-3 and MCC-5 cells (data not shown). In contrast with suppressed c-Myc expression upon JQ1 treatment,
Notch and NFκB expressions are increased upon JQ1 treatment (Supplementary Fig. S4C), indicating that these molecules are less likely involved in JQ1-induced cell-cycle arrest.

cDNA microarray combined with chromatin immunoprecipitation have revealed that Myc regulates all aspects of protein synthesis, including downstream of the mTOR pathway (12). Moreover, a recent study suggests that mTOR-dependent phosphorylation of the eukaryotic translation initiation factor 4E-binding protein-1 (4E-BP1) is found to be required for Myc-driven hematologic cancers (42). Therefore,
we decided to examine whether the mTOR pathway is down-stream of c-Myc in MCC cells. Interestingly, both p-mTOR and p-4E-BP1 were unchanged after JQ1 treatment, suggesting that 4E-BP1 is not regulated by c-Myc in MCC-3 and MCC-5 cells (Supplementary Fig. S3A).

**JQ1 impaired MCC xenograft tumor growth in vivo**

We proceeded to test the impact of JQ1 on MCC cells in our xenograft MCC mouse models. To strengthen our hypothesis that c-Myc was the major downstream effector of JQ1 in MCC cells, we included MCC-2 cells without c-Myc amplification as controls. MCC-2, MCC-3, and MCC-5 cells (2 × 10^5 cells from each cell line) were mixed with Matrigel (BD Biosciences; 80 μL of media + 120 μL of Matrigel), respectively. Tumor cells were inoculated subcutaneously into the rear flanks of NSG mice. When xenograft tumors approached approximately 100 mm^3 in volume (or 7 mm in diameter), treatment was started with as per the standard treatment protocol as previously published (31). NSG mice bearing xenograft tumors began to receive i.p. administration of 50 mg/kg/d JQ1 or vehicle for a 3-week duration. NSG mice bearing MCC-5 xenograft tumors were sacrificed after completion of 21-day treatment. However, NSG mice bearing MCC-3 tumors were terminated at day 19 because control tumors were reaching 2 cm in dimension. Mice treated with JQ1 had no obvious signs of toxicity (based on body weight, food and water intake, activity, and general exam). As shown in Fig. 6A, xenograft tumors receiving JQ1 treatment showed great reductions of tumor volume as compared with xenograft tumors receiving vehicle. Histologically, xenograft tumor cells demonstrated large, round to oval, vesicular nuclei with scant cytoplasm, which is characteristic of MCC (Fig. 6A). As measured by mean ± SEM, JQ1 significantly attenuated xenograft tumor growth.

![Figure 6](https://example.com/fig6.png)

**Figure 6.** Effect of JQ1 on MCC xenograft growth in vivo. A, representative examples of xenograft tumors treated with JQ1 or vehicle and characteristic histology features of MCC in xenograft tumors. B, comparison of tumor volumes ± SEM from tumor-bearing NSG mice treated with JQ1 or vehicles. NSG mice bearing MCC-2, MCC-3, and MCC-5 xenograft tumors were treated with JQ1 at 50 mg/kg/d JQ1 by i.p. injection between 18 and 21 days. C, immunoblotting of MCC-3 and MCC-5 xenograft tumor tissues with the indicated antibodies; β-actin was used as a loading control.
growth (more than a 4-fold reduction in MCC-3 xenograft tumors and a 3-fold reduction in MCC-5 xenograft tumors; Fig. 6B). This was accompanied by prolonged event-free survival as tumor in the treatment group never attained 600 and 1,100 mm³ in MCC-3 and MCC-5 tumors, respectively. Consistent with our central hypothesis, MCC-3 cells with highest c-Myc expression correlates with the greatest reduction (>4-fold) in MCC-3 xenograft tumor volume. Conversely, MCC-2 xenograft tumors lacking c-Myc amplification are less sensitive to JQ1 (>1-fold reduction).

By RT-PCR analysis, xenograft tumors showed consistent expression pattern of MCC markers, such as cytokeratin 18, 19, 20, synaptophysin, neurospecific enolase, and Merkel cell-specific transcription factor Math-1 as expressed in MCC cells (Supplementary Fig. S3B). Immunoblotting analysis further confirmed suppressed c-Myc expression and upregulation of p21, p27, and p57 in the treatment groups (Fig. 6C). As expected, decreased cell proliferation as determined by IHC staining of Ki67, was found in the xenograft tumors in the treatment group as compared with those in the control group (Fig. 7). Similarly, increased numbers of p21-, p27-, and p57-positive cells were found in xenograft tumors in the treatment group by IHC (Fig. 7). Although our in vivo data are promising, it is generated in NSG mice with profound immune deficiency. In addition, JQ1 can potentially interact with several immune-related pathways. Nevertheless, our data provide initial evidence that the BET protein inhibitor JQ1 might be applicable in the clinic for MCC.

Discussion

MCC is an aggressive skin cancer. Standard treatment is surgery followed by radiotherapy for local and regional disease or chemotherapy for distant metastasis (43). Despite standard treatment, one third of patients will eventually develop distant metastases, for which currently there is no cure. Therefore, molecular events driving MCC pathogenesis need to be further defined to benefit heterogeneous patient populations. In search of receptor tyrosine kinase involvement in MCC tumorigenesis (providing a rationale for the use of targeted molecular therapies), studies have found variable expression of c-kit, VEGFs, PDGFalpha, and PDGFbeta in MCC compared with normal skin (44–46). Although a hyper-activated PI3K/Akt/mTOR pathway is reported in MCC (8, 9, 39), the etiology of this aberrant pathway activation is still elusive. Because c-Myc represents a unifying molecular feature in many hematopoietic malignancies (12) and amplification of L-Myc is reported in MCC (10), we were intrigued to study c-Myc. Interestingly, while we were conducting this study, an article published by Kwun and colleagues (11) suggests that MCV small T antigen stabilizes c-Myc expression. However, the role of c-Myc overexpression in MCC pathogenesis still needs further elucidation. Consistent with the central role of Myc in the pathogenesis of human cancer, we are the first to report that c-Myc activation is common in MCC.

Although c-Myc is one of the most deregulated oncogenes in human cancers, a therapeutic approach to target c-Myc
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has remained elusive. Like many transcriptional targets, the lack of a ligand-binding domain creates an obstacle toward direct inhibition (47). BETs are transcriptional regulators that epigenetically control gene expression, which are key in cell proliferation, cell-cycle progression, and apoptosis (22, 23). Dysfunction of BET proteins has been associated with the development of aggressive tumors, such as NUT midline carcinomas (26, 48). Recently developed selective BET protein inhibitors are attractive because of promising antitumor activity demonstrated in diverse preclinical models, such as multiple myeloma (31), hematologic malignancies (21, 24, 25, 27, 28, 32), glioblastoma (30), lung cancer (29, 33), and medulloblastoma (49). The efficacy of these compounds has been attributed mainly to their ability to suppress c-Myc expression as well as downstream targets. On the basis of these prior studies, BET inhibitors would be expected to have activity primarily against MCC possessing higher expression of c-Myc at the transcription level. In agreement with this notion, we have uncovered G1 cell-cycle arrest by JQ1 in MCC cells with c-Myc amplification, particularly via suppressed c-Myc expression, downregulation of cyclin D1, and upregulation of cell-cycle inhibitors such as p21, p27, and p57 in JQ1-treated MCC cells. Moreover, knockdown of p21, p27, and p57 partially reverts JQ1-induced G0–G1 arrest in MCC cells. Interestingly, individual knockdown of p21, p27, and p57 exhibits the same extent of neutralization of JQ1 effect in MCC cells. Although JQ1 fails to confer cell death in MCC cells, this is unlikely due to the lower concentration used (800 nmol/L as compared with 1 μmol/L used in studies using models of hematopoietic malignancies). Thus, BET inhibition may have synergistic effects with targeted proapoptotic agents (e.g., ABT-737 and YM155) in the clinical setting.

Of note, several reports have demonstrated off-target effects on genes or signaling pathways by JQ1 in addition to c-Myc, such as TYRO3, prosurvival gene BIRC5/survivin, NFκB target gene BIRC3, and JAK/STAT pathway gene IL7R. Because of the strong concordance between the phenotypes by c-Myc knockdown and JQ1, it is most likely that c-Myc is the major gene involved in JQ1-induced cell-cycle arrest in MCC cells with c-Myc overexpression. Because expression of Notch1 and NFκB is increased in both MCC-3 and MCC-5 cells upon JQ1 treatment, they are less likely to be involved in JQ1-induced cell-cycle arrest. Importantly, the greatest suppression of xenograft tumor by JQ1 is evident in MCC-3 cells with the highest c-Myc expression. Of note, MCC-2 cells without c-Myc amplification also confers JQ1 sensitivity at a higher concentration, suggesting alternative mechanisms in growth inhibition in MCC cells without c-Myc overexpression. Intriguingly, unlike previous publications demonstrating that the mTOR pathway and c-Myc converge at 4E-BP1, phosphorylation of 4E-BP1 are not regulated by JQ1 in our experimental system (42).

It is controversial to whether BET protein levels are altered when cells are subjected to JQ1 (31). In our study, BRD4 expression at the protein level is unchanged in both MCC-3 and MCC-5 cells after JQ1 treatment, which is consistent with the theory that JQ1 competitively displaces BRD4 from chromatin without changing its level (data not shown). Interestingly, BRD4 expression is found to be higher in metastatic melanoma (26). It is debatable whether knockdown of BRD4 is able to phenocopy the antitumor effects of BET inhibitors in different cell types. Therefore, the concomitant displacement of other BET proteins may broaden the oncosuppression effects of these small-molecule compounds despite the fact that BRD4 is the key BET protein, which has been studied extensively so far. It would be compelling to explore the biologic consequence of complete abrogation of BET proteins not only in MCC with c-Myc amplification but also in MCC lacking Myc amplification.

It is evident that epigenetic modification affects cancer initiation and progression. Of note, new small-molecule drugs modifying the epigenetic landscape of tumors have improved disease overall survival as well as potentiate the cytotoxic effects of chemotherapy (30). Here, we have demonstrated that epigenetic inhibition of c-Myc by JQ1 retards tumor growth in xenograft MCC mouse models with no obvious toxicity, which establishes the feasibility of c-Myc inhibition by BET protein inhibitors within an acceptable therapeutic window of tolerability as a single agent or in combination with other therapies in holding disease progression in check. However, BET protein inhibitors may be most effective when used in combination with agents possessing cytotoxicity in the clinical setting for MCC. Insights provided by our study identify BET protein inhibitors as rational therapeutic options and warrant further exploration in MCC with and without pathologic c-Myc activation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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