Published in final edited form as:

Surgery. 2015 September ; 158(3): 819–826. doi:10.1016/j.surg.2015.04.017.

BET INHIBITION BLOCKS TUMOR PROGRESSION AND PROMOTES DIFFERENTIATION IN NEUROBLASTOMA

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Abstract

BACKGROUND—*MYCN* amplification is a key molecular hallmark of high-risk neuroblastoma. Previously considered an “undruggable” target, *MYCN* transcription can be disrupted by inhibiting the bromodomain and extraterminal (BET) domain family of proteins that epigenetically regulates *MYCN* transcription. JQ1 is a potent small molecule BET inhibitor that has been shown to induce cell cycle arrest and to initiate apoptosis in neuroblastoma. Here, we sought to validate the anti-tumorigenic effects of JQ1 in neuroblastoma and to evaluate whether blocking N-myc expression with JQ1 promotes neural differentiation.

METHODS—We determined the effects of JQ1 treatment on human neuroblastoma *in vitro* cell growth in both monolayer and sphere-forming conditions. Subcutaneous neuroblastoma xenografts were used for *in vivo* study. Western blotting and immunohistochemistry were performed to evaluate for neural differentiation and stem cell markers.

RESULTS—JQ1 treatment blocked neuroblastoma cell growth in both monolayer and sphere-forming conditions; JQ1 also attenuated neuroblastoma xenograft growth. Neurofilament expression was enhanced with JQ1 treatment, indicating that JQ1 induces neuronal differentiation. Sphere forming conditions resulted in increased expression of stem cell markers; these were reversed with JQ1 treatment.

CONCLUSIONS—BET inhibition attenuates neuroblastoma progression and promotes neural differentiation, providing insight into clinical applications of BET inhibitors in the treatment of patients with neuroblastoma.

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Presented at the 10th Annual Academic Surgical Congress in Las Vegas, NV, February 3-5, 2015

Keywords

JQ1; Neuroblastoma; Sphere; N-myc

INTRODUCTION

Neuroblastoma is the most common extracranial solid tumor in infants and children, arising from neural crest precursors of the sympathetic nervous systems.¹ Clinical heterogeneity is a unique feature of neuroblastoma with some tumors spontaneously regressing, while others are refractory to even the most aggressive therapies. Risk-group categorization for neuroblastoma was developed to predict disease response to therapy and to treat patients based on tumor biology. The prognosis for children with high-risk disease remains dismal with 50% overall survival.¹ Key features of high-risk disease include age at diagnosis >18 months, tumor stage, and *MYCN* amplification.¹ *MYCN* is amplified in approximately 30% of all high-risk neuroblastomas. Increased expression of N-myc drives neuroblastoma proliferation, thus making it an attractive target for therapeutic intervention. However, transcription factor of oncoproteins, such as N-myc, have historically been resistant to conventional drug development strategies due to a lack of high affinity binding sites for small molecules.² Recent drug discovery strategies have attempted to subvert these limitations by targeting epigenetic regulators of oncoprotein transcription factors.²

The bromodomain and extraterminal (BET) proteins are a family of epigenetic modifiers that have recently been shown to regulate *MYC* expression.³ BETs feature bromodomains that selectively recognize histone acetylated lysine residues and serve as co-activators that regulate gene expression.⁴ Small molecule BET inhibitors have been identified and shown to block *MYC* expression in a variety of cancers, including multiple myeloma, acute myeloid leukemia, and lymphoma.^{3, 5, 6} JQ1, a novel thieno-triazolo-1,4-diazepine, is a potent BET inhibitor whose efficacy in neuroblastoma was recently investigated by Puissant et al.⁷ They described that JQ1 inhibits N-myc expression, arrests cell proliferation, and induces apoptosis *in vitro* in neuroblastomas and suppressed tumorigenesis *in vivo*.⁷

Although the use of pro-differentiating agent (i.e., retinoic acid) has become a part of standard therapy for neuroblastoma, the molecular mechanisms have not been fully explored. N-myc is a key driver of neuroblastoma proliferation and blocks neuronal differentiation.⁸ As such, we hypothesized that inhibition of N-myc with the small molecule BET inhibitor JQ1 would block neuroblastoma progression and induce differentiation. Establishing neuroblastoma cells as neurospheres in serum-free media has been touted as an *in vitro* selection process that alters the cancer differentiation profile and increases the expression of stem cell markers. Furthermore, rearing neuroblastomas as neurospheres has been shown to enrich for tumor-initiating capacity of *in vitro* culture methods.⁹

Here, we have validated the anti-tumor effects of JQ1 by demonstrating that JQ1 inhibits N-myc expression and blocks neuroblastoma growth as both monolayers and neurospheres and our *in vivo* model of primary tumor formation. Moreover, we have demonstrated that JQ1 enhances the expression of key differentiation markers, suggesting that JQ1 may act, in part, by promoting neuroblastoma differentiation. These findings provide further preclinical

evidence of the efficacy of JQ1 in neuroblastoma and insight into the mechanism by which JQ1 inhibits neuroblastoma growth.

MATERIALS AND METHODS

Materials

Antibodies against N-myc, CD133, Sox2 were purchased from Cell Signaling (Beverly, MA). Antibodies against ZNF423, Nestin, and vimentin were purchased from Abcam (Cambridge, MA). Antibody against NF-M and NF-L was from Novus (Littleton, CO). Nanog and secondary antibodies against mouse and rabbit IgG were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against β -actin and CD57 were from Sigma-Aldrich (St. Louis, MO). JQ1 was synthesized by Dr. Jun Qi and kindly provided by Dr. James Bradner (Dana-Farber Cancer Institute, Boston, MA). Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies, Inc. (Santa Clara, CA). Agarose (SeaPlaque®) was from Lonza Inc. (Allendale, NJ).

Cell culture and tumor sphere formation

Human neuroblastoma cell lines, BE(2)-C, IMR-32, BE(2)-M17, SK-N-SH, and SK-N-DZ were purchased from ATCC (Manassas, VA). JF is a primary neuroblastoma cell line established from resected tumor that was a gift from Dr. Jason M. Shohet (Baylor College of Medicine, Houston, TX). Monolayer cells were maintained in RPMI 1640 media with L-glutamine (Cellgro Mediatech, Inc. Herndon, VA) supplemented with 10% FBS. Cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. For tumor sphere assays, 1×10^3 cells were plated on ultra-low attachment culture dishes and grown in neurobasal media supplemented with B27 serum substitute, 20 ng/mL epidermal growth factor and 20 ng/mL basic fibroblast growth factor (Invitrogen, Carlsbad, CA). Tumor spheres were imaged using a bright-field microscope and then stained with 0.05% crystal violet for quantification. Limiting dilution analysis was completed to quantify tumor-forming capacity with and without JQ1 (1 μ M). Serial dilutions were performed to attain estimated cell-plating doses of 500, 250, 125, 62, 31, 16, 8 and 4 cells/well (6 wells/group) in 100 μ l of sphere-promoting media on an ultra-low attachment 96-well plate (Corning, Manassas, VA). After 4 days incubation, each well was scored for presence of sphere formation. The frequency of sphere forming cells in each group was estimated from a plot of the estimated cells/well and the fraction of negative wells (those without sphere formation) on a log scale. This analysis was performed online using the Walter and Eliza Hall Institute, Bioinformatics Division (accessed at <http://bioinf.wehi.edu.au/software/elda/>).¹⁰

Cell viability and anchorage-independent growth

To assess for cellular proliferation, a modified MTT assay was used. Cells were seeded onto 96-well plates (3×10^3 cells/well) in RPMI 1640 culture media containing 10% FBS and grown for up to 4 days. Cell viability was assessed using CCK-8 kit daily. The values, corresponding to the number of viable cells, were read at OD450 with the FlexStation 3 Microplate Reader (Molecular Devices, Sunnyvale, CA). For anchorage-independent growth assay, cells were trypsinized and resuspended in RPMI 1640 media containing 0.4% agarose and 7.5% FBS. Transfected BE(2)-C cells were overlaid onto a bottom layer of solidified

0.8% agarose in RPMI 1640 media containing 5% FBS (3×10^3 cells/well) in 12-well plates and incubated for 3 weeks. Colonies were stained with 0.05% crystal violet dissolved in 70% methanol, photographed, and quantified.

Western blotting

Cells were collected using cell lysis buffer with 1 mM PMSF and denatured samples were prepared for immunoblotting. Nonspecific binding sites were blocked with 5% milk in TBS-T (120 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.05% Tween 20) for 2 h at room temperature or overnight at 4°C. Target proteins were detected by using rabbit or mouse anti-human antibodies (1:500–2000 dilution) overnight at 4°C. Anti-rabbit or anti-mouse secondary antibodies conjugated with HRP were incubated for 1 h and visualized using an enhanced chemiluminescence system. Equal loading and transfer were confirmed with β -actin.

Immunohistochemistry

Four-micrometer-thick tissue sections were prepared and placed on charged slides. After paraffin removal, the sections were rehydrated and placed in heated citrate buffer for 25 min. Paraffin-embedded sections were stained with monoclonal antibodies for anti-vimentin (1:250 dilutions, Cell Signaling) or anti-NF-L (1:500 dilutions, EMD Millipore Corporation) overnight at 4°C using DAKO EnVision Kit (Dako Corp., Carpinteria, CA). After 4 washes with PBS, the sections were incubated for 30 min with secondary antibody labeled with peroxidase. Lastly, tissue specimens were covered with substrate-chromogen solution (1 ml) added with liquid DAB (20 μ l), counterstained with hematoxylin and observed under light microscopy (400X). For negative controls, primary antibody was omitted from the above protocol.

In vivo tumor study

Male athymic nude mice (4–6 weeks old) were maintained as described.¹¹ All studies were approved by the Vanderbilt University Institutional Animal Care and Use Committee and were conducted in accordance with guidelines issued by the NIH. BE(2)-C cells (1×10^6) in 50 μ l of HBSS were subcutaneously injected into the bilateral flanks using a 26-gauge needle as described.¹² Ten days after tumor inoculation, mice with established tumors were divided into two groups ($n = 5$ per group): control (vehicle: 10% DMSO in 5% dextrose) or JQ1 (50 mg/kg/day) group. Vehicle or JQ1 was administered intraperitoneally on a daily basis. Tumor volume was assessed as previously described¹³ and body weights were recorded weekly. At sacrifice, tumors were excised, weighed, and fixed in formalin for further analyses.

Statistical analysis

Student's paired *t* test was used for comparisons between the treatment groups *in vitro* and xenograft experiments were analyzed as described.^{11, 12} Data represent the means $\pm$ SEM. A *p* value of <0.05 was considered significant.

RESULTS

JQ1 treatment decreased neuroblastoma cell proliferation and inhibited N-myc expression

We first characterized the effects of JQ1 on neuroblastoma cell growth and survival *in vitro* using established cell lines reared as monolayers in serum-enriched media. After plating cells on 96-well plates with serum-supplemented media, attached monolayer cells were treated with JQ1 at 1 μ M and harvested over a time course (1–4 days). Significant decreases in cell viability were observed with JQ1 treatment in all neuroblastoma cell lines examined (Fig. 1A). As such, proliferation was halted in both *MYCN* amplified, BE(2)-C, IMR, JF, BE(2)-M17, SK-N-DZ, as well as *MYCN* non-amplified, SK-N-SH, neuroblastoma cell lines. JQ1 treatment also decreased N-myc protein levels as measured by Western blotting. Specifically, N-myc protein expression was suppressed with JQ1 treatment in all of our experimental *MYCN*-amplified cell lines (Fig. 1B). N-myc protein level was undetectable in SK-N-SH, a *MYCN* non-amplified cell line. These findings validate those previously reported by Puissant *et al.*⁷ that JQ1 effectively inhibits neuroblastoma cell growth *in vitro* and blocks N-myc protein expression.

JQ1 induced neuronal differentiation *in vitro*

It has been speculated that neuroblastoma derives from neural crest stem cells that fail to undergo differentiation during development in infants and children. Therefore, one clinical treatment strategy is to induce differentiation with agents such as retinoic acid.¹⁴ Studies have demonstrated that JQ1 treatment causes cell cycle arrest and apoptosis.⁷ We examined whether JQ1 induces neuroblastoma cell differentiation and found morphologic changes in *MYCN* amplified BE(2)-C cell after JQ1 treatment. Specifically, BE(2)-C cells treated with JQ1 adopted a polarized phenotype with longer neurites (Fig. 2A). Furthermore, we investigated the effect of JQ1 on the expression of neural differentiation markers. The protein expression of ZNF423 and NF-M, two known markers of neuroblastoma differentiation, were increased at 72 h after JQ1 treatment (Fig. 2B). Taken together, our results demonstrate that JQ1 induced cell morphology changes consistent with neural differentiation, and this was validated by increased expression of known neural differentiation markers. Our findings demonstrate that JQ1 treatment enhances differentiation of neuroblastoma cells grown under standard *in vitro* conditions.

JQ1 inhibited neurosphere formation and attenuated stem cell markers

We further characterized the *in vitro* effects of JQ1 treatment on BE(2)-C cells reared in serum-free media and ultra-low attachment culture dishes that permit formation of neurospheres. Rearing cells as neurospheres has been shown to alter the differentiation profile of neuroblastomas, favoring the expression of stem cell markers and enriching for tumor-initiating cells.⁹ First, neurospheres were grown in ultra-low attachment plates using serum-free media, and sphere formation was quantified at one week. Our results showed that JQ1 treatment significantly reduced both tumor sphere size (Fig. 3A) and number in a dose-dependent manner (Fig. 3B).

To better quantify the effects of JQ1 on sphere-forming capacity, we utilized the principle of limiting dilution analysis to assess the sphere-forming capacity of BE(2)-C cells reared in

the presence and absence of JQ1. Ninety-six well plates were scored for sphere formation after 4 days incubation. Treatment with JQ1 (1 μ M) induced a three-fold decrease in sphere forming capacity of BE(2)-C measured after 4 days of incubation (Fig. 3C). Taken together, these findings demonstrate that JQ1 alters the sphere-forming potential of BE(2)-C cells.

Rearing neuroblastomas as neurospheres alters their differentiation profile and enriches for the expression stem cell markers. We next set out to determine whether JQ1 inhibited the expression of these stem cells markers in BE(2)-C spheres, as evidence that JQ1 promotes a more differentiated protein expression profile. First, we compared the protein levels of CD133, vimentin, CD57, Nanog, Nestin, and Sox2, by Western blotting between tumor spheres and parental cells. Expression of the neural stem cell markers CD133, CD57, Nanog, and Nestin were enhanced in our tumor spheres in comparison to parental monolayer cells. However, Sox2 expression levels were not increased in BE(2)-C spheres. JQ1 treatment strikingly decreased protein levels of all tested stem cell markers (CD133, vimentin, CD57, Nanog, Nestin, and Sox2) in tumor spheres (Fig. 3D).¹⁵⁻¹⁹ These findings demonstrate that JQ1 blocks stem cell marker expression in BE(2)-C cells grown under sphere-promoting conditions.

JQ1 inhibited anchorage-independent colony growth

Anchorage-independent growth is one of the hallmarks of cell transformation. In order to evaluate the effect of JQ1 on tumorigenesis, we examined soft agar colony growth as an *in vitro* surrogate for malignant phenotype. We used both BE(2)-C cells reared as either monolayers or spheres to assess anchorage-independent growth colony forming capacity in semisolid media. The parental cells were trypsinized and sphere cells were dissociated, and single cells were cultured in semisolid agar plate in RPMI media with or without JQ1 addition for 3 weeks. Our results showed that sphere cells formed 40% more colonies than parental cells (Fig. 4), suggesting that rearing cells in sphere-forming conditions selects for a more aggressive phenotype. These findings are consistent with recent reports that rearing neuroblastomas in sphere-forming conditions enhances their tumor-initiating capacity.⁹ Importantly, JQ1 treatment significantly decreased the cell colony size and number for both spheres and monolayer cells (Fig. 4). Overall, these results provide a functional measure of anchorage-independence, demonstrating that JQ1 alters the aggressiveness of neuroblastomas reared both as serum-free spheres or attached in serum-supplemented conditions.

JQ1 inhibited *in vivo* tumor growth and promoted differentiation

Given our *in vitro* findings that JQ1 blocks neuroblastoma growth by promoting differentiation, we next examined the effect of JQ1 treatment on neuroblastoma subcutaneous xenografts. After establishing subcutaneous xenografts with BE(2)-C cells, athymic nude mice were randomized to receive either JQ1 (50 mg/kg) or vehicle (control) daily. Tumor volume was measured twice weekly, and the xenografts were observed daily until sacrifice. Smaller tumor volumes were noted after 7 days of treatment with JQ1, and this became statistically significant by treatment day 10 (Fig. 5A, B). At sacrifice, tumor weight in mice receiving JQ1 was significantly less in comparison to vehicle alone (Fig. 5C). Body weight of mice treated with JQ1 was similar to control group indicating that JQ1

treatment caused no gross toxic side effects. Our results are consistent with previous studies and confirm that JQ1 inhibits neuroblastoma growth in an *in vivo* murine model.

We next examined the expression of the neural crest stem cell marker, vimentin, and neural differentiation marker, neurofilament-L (NF-L), in tumor sections by immunohistochemistry staining. Our results showed that JQ1 treatment blocked vimentin expression (Fig. 5D; *upper panels*) while markedly enhancing NF-L staining in tumor sections (Fig. 5D; *bottom panels*). These results support the findings of our *in vitro* sphere and monolayer conditions demonstrating enhanced expression of neural differentiation markers with JQ1 treatment.

DISCUSSION

Recent studies have identified that recurrent somatic mutations are rare in high-risk neuroblastoma, indicating that epigenetic modifications may play a role in neuroblastoma tumorigenesis and progression.²⁰ Epigenetic aberrations are an increasingly recognized feature of cancer cells, where chromatin regulatory machinery is exploited to enforce oncogenic gene expression programs.²¹ Understanding cancer-specific mechanisms of epigenetic regulators in neuroblastoma may lead to the development of novel therapeutic agents to prevent tumor progression and subsequently improve outcomes for patients.

Members of the MYC family of proteins are among the most common amplified or mutated oncoproteins in cancers, including neuroblastoma.²² However, it has been a challenge to develop drugs that inhibit this oncogenic transcription factor.² BET inhibitors, such as JQ1, are now recognized as one of the first drugs to inhibit this elusive cancer target and have been shown to inhibit tumor progression in multiple different cancer models by modulating MYC expression.^{3, 6} In neuroblastoma, JQ1 has generated considerable excitement based as its ability to block transcription of MYCN, a key driver of neuroblastoma tumorigenesis whose amplification status is a marker of poor prognosis.

Here we sought to validate the existing literature supporting the preclinical efficacy of JQ1 in neuroblastoma using by *in vitro* and *in vivo* models of proliferation, aggressiveness and tumor formation. The purported mechanism of JQ1 is induction of cell cycle arrest and initiation of apoptosis. However, we have demonstrated that JQ1 also alters the protein expression profiles towards a more differentiated state in two *in vitro* conditions and one *in vivo* model. Specifically, JQ1 promoted neurofilament and ZNF423 expression in monolayer cells and blocked the expression of several purported stem cell markers that were enriched by rearing cells in serum-free, low-attachment sphere conditions. We have also demonstrated that JQ1 treatment enhanced neurofilament protein expression in our subcutaneous flank tumors with reciprocal downregulation of the mesenchymal marker, vimentin. Taken together, our findings suggest that the anti-tumorigenic effects of JQ1 are, at least in part, by promoting differentiation of neuroblastoma towards a neural fate.

Intriguingly, JQ1 halted proliferation in our non MYCN-amplified SK-N-SH cell line, demonstrating that the anti-tumorigenic effects of BET inhibitors in neuroblastoma are not exclusively attributable to blocking N-myc expression. These findings are consistent with previous trials demonstrated the efficacy of IBET-726 in non MYCN-amplified SK-N-SH

and SH-SY5Y cell lines.²³ Repression of the anti-apoptotic gene BCL2 was reported to be a non-MYC target of BET inhibition in these cell lines. Taken together, our findings merit further investigations into identifying alternative aberrant signaling pathways that may be targeted by this family of emerging anticancer therapeutics.

To our knowledge, this is the first evidence of BET inhibitors inducing neuronal differentiation in neuroblastoma, highlighting a novel mechanism by which BET inhibitors may yield therapeutic benefit. Interestingly, ZNF423 expression has previously been identified as a critical mediator of retinoic acid-mediated neuroblastoma growth inhibition and differentiation.²⁴ As such, our findings that JQ1 enhances ZNF423 expression invite further inquiry into the potential synergistic role of JQ1 and retinoic acid in mediating neuroblastoma differentiation. HDAC inhibition was recently demonstrated to enhance neuroblastoma differentiation.²⁵ Our investigation provides further evidence to highlight the therapeutic potential of targeting epigenetic regulators to promote neuroblastoma differentiation. Overall, preclinical trials, including our own, have demonstrated the pleiotropic effects of JQ1 on neuroblastoma apoptosis, growth arrest, and differentiation and provide guidance for therapeutic strategies for the use of BET inhibitors in the treatment of high-risk neuroblastoma.

ACKNOWLEDGMENTS

This work was supported by a grant R01 DK61470 from the National Institutes of Health and the Rally Foundation for Cancer Research.

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Figure 1. JQ1 decreased neuroblastoma cell viability

(A) JQ1 treatment (1 μM) decreased cell viability of all human neuroblastoma cell lines examined, BE(2)-C, IMR-32, JF, BE(2)-M17, SK-N-SH, and SK-N-DZ (mean ± SEM; $*=p < 0.05$ vs. CON). Cell viability was measured over a four-day period using CCK-8 assay. (B) JQ1 treatment (1 μM) decreased N-myc protein levels in all neuroblastoma cell lines examined except in *MYCN*-non-amplified SK-N-SH cells. β-actin was probed for protein loading control.

Figure 2. JQ1 induced neuroblastoma cell differentiation in BE(2)-C cells

(A) Human neuroblastoma BE(2)-C cells were plated as monolayer cells in serum-supplemented media and treated with JQ1 (1 μ M) for 3 days. JQ1 treatment (*bottom panel*) induced neuroblastoma cellular differentiation characterized by a polar appearance with longer, outbranching neurites. (B) JQ1 treatment increased the expression of neural differentiation markers, ZNF423 and NF-M, as determined by immunoblotting. β -actin served as our loading control.

Figure 4. JQ1 blocked anchorage-independent neuroblastoma colony growth

(A) BE(2)-C parental monolayer and BE(2)-C sphere cells were cultured in soft agar for 3 weeks in media supplemented with JQ1 (0.5 μ M; *bottom panel*). Representative images of colony formed in soft agar (scale bar = 100 μ m). (B) The colony formation was assessed and quantitated (mean $\pm$ SEM; * $=p < 0.05$ vs. CON).

Figure 5. JQ1 inhibited tumor growth *in vivo*

(A) Subcutaneous neuroblastoma xenografts were established with BE(2)-C cells injected in the scapular regions of athymic nude mice. A representative photo is shown at 12 days post-treatment demonstrating tumor growth inhibition with JQ1 (*right panel*). (B) Tumor volume of xenografts of mice treated with daily injections (i.p.) of JQ1 (50 mg/kg/day) or vehicle (CON; 10% DMSO in 5% dextrose). Data are presented as the means $\pm$ SEM ($n=5$ /group, $*=p < 0.05$ vs. CON). (C) Tumor explant and total body weights were measured at sacrifice, 12 days post-treatment with JQ1 (50 mg/kg/day; $n=5$ /group, $*=p < 0.05$ vs. CON). (D) Representative immunohistochemistry of tumor sections from JQ1 and control group stained with anti-mouse vimentin and NF-L antibodies (*brown stain*; scale bar = 20 μ m).