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BET bromodomain proteins are required for glioblastoma cell proliferation

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Keywords: glioblastoma, epigenetics, bromodomain, stem cells, histones, histone acetylation mimics, temozolomide

Abbreviations: GBM, glioblastoma; BET, bromodomain and extra terminal domain; TMZ, temozolomide

Epigenetic proteins have recently emerged as novel anticancer targets. Among these, bromodomain and extra terminal domain (BET) proteins recognize lysine-acetylated histones, thereby regulating gene expression. Newly described small molecules that inhibit BET proteins BRD2, BRD3, and BRD4 reduce proliferation of NUT (nuclear protein in testis)-midline carcinoma, multiple myeloma, and leukemia cells in vitro and in vivo. These findings prompted us to determine whether BET proteins may be therapeutic targets in the most common primary adult brain tumor, glioblastoma (GBM). We performed NanoString analysis of GBM tumor samples and controls to identify novel therapeutic targets. Several cell proliferation assays of GBM cell lines and stem cells were used to analyze the efficacy of the drug I-BET151 relative to temozolomide (TMZ) or cell cycle inhibitors. Lastly, we performed xenograft experiments to determine the efficacy of I-BET151 in vivo. We demonstrate that BRD2 and BRD4 RNA are significantly overexpressed in GBM, suggesting that BET protein inhibition may be an effective means of reducing GBM cell proliferation. Disruption of BRD4 expression in glioblastoma cells reduced cell cycle progression. Similarly, treatment with the BET protein inhibitor I-BET151 reduced GBM cell proliferation in vitro and in vivo. I-BET151 treatment enriched cells at the G1/S cell cycle transition. Importantly, I-BET151 is as potent at inhibiting GBM cell proliferation as TMZ, the current chemotherapy treatment administered to GBM patients. Since I-BET151 inhibits GBM cell proliferation by arresting cell cycle progression, we propose that BET protein inhibition may be a viable therapeutic option for GBM patients suffering from TMZ resistant tumors.

Introduction

Epigenetic signaling pathways regulate gene expression without altering DNA sequences.1,2 This is achieved, in part, by adding acetyl groups to lysine residues on histones H2A, H2B, H3, and H4, thereby modulating chromatin structure and gene expression.1,2 Histone acetyltransferases (HATs) attach acetyl groups to histones while histone deacetylases (HDACs) remove acetyl moieties.1,2 Coupled to HAT and HDAC activity, bromodomain reader proteins bind acetylated histones and recruit transcriptional complexes, therefore representing an important link between histones and transcription.1,2 Among the 46 known bromodomain proteins, the bromodomain and extra terminal domain (BET) proteins BRD2, BRD3, BRDT, and BRD4 bind the super elongation complex (SEC) and the polymerase associated factor complex (PAFp) in certain biological contexts.3 In addition, BRD4 recruits the positive transcription elongation complex P-TEFb.4,5 Several recent studies have uncovered roles for the BET proteins by designing small molecules that act as histone mimics, thereby displacing BET proteins from acetylated histones and the transcriptional machinery.3,6-10 BET protein displacement from chromatin is associated with decreases in transcription of lipopolysaccharide (LPS)-inducible genes and oncogenes, and is therefore attractive therapeutically for the treatment of inflammation or cancer.1,2 Small molecules that antagonize BET protein binding to acetylated histones have shown efficacy in mouse models of inflammation, NUT-midline carcinoma, mixed lineage leukemia (MLL) fusion, multiple myeloma, high-risk acute lymphoblastic leukemia, and lung adenocarcinoma.3,6-9,11 These small molecules (JQ1, GSK525762A [I-BET] and I-BET151) exhibit remarkable specificity for the BET bromodomain proteins BRD2, BRD3, BRD4, and BRDT over other bromodomain proteins and epigenetic enzymes.3,6-9,11 Consistent with this specificity, GSK525762A is currently being tested in clinical trials for the treatment of NUT-midline carcinoma (NCT01587703). Collectively, these studies suggest that BET bromodomain small molecule antagonists may be effective in treating aggressive cancers.

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BET protein inhibition might be a possible therapy against the most common primary adult brain cancer, glioblastoma (GBM).\textsuperscript{13,14} Prognosis for GBM patients is poor with median survival of approximately 13 mo for those patients who had complete surgical resection, and even lower for those where surgery is contraindicated.\textsuperscript{13,14} Current treatment regimens include radiotherapy in combination with the DNA alkylating agent temozolomide (TMZ).\textsuperscript{13,14} However, TMZ resistance is nearly universal, and therefore novel small molecule inhibitors of GBM cell proliferation are needed.\textsuperscript{13,14} Further, recent studies have demonstrated that GBM stem cells are resistant to TMZ therapy and promote tumor recurrence\textsuperscript{15} and, therefore, small molecules that effectively eliminate GBM stem cells are highly desirable.

We demonstrate that small molecule inhibitors of BET proteins are possible therapies for GBM patients since they inhibit proliferation of GBM cells. Treatment of GBM cells with I-BET151 potently inhibits proliferation. I-BET151 treatment arrested U87MG cells in G1/S, thus providing mechanistic insight into how I-BET151 may reduce cell proliferation in vivo. I-BET151 treatment also inhibits growth of U87MG xenografts in immunocompromised mice, suggesting that I-BET151 analogs that cross the blood brain barrier may be effective as single agents or in combination therapy for GBM treatment.

**Results**

**BRD2 and BRD4 are significantly elevated in glioblastoma tumors**

To identify possible therapeutic targets in GBM, we used NanoString Technology to determine the expression of 40 bromodomain proteins in 27 GBM tumors relative to 9 control samples isolated from epilepsy patients. We utilized the NanoString nCounter system to assess gene expression quantitatively.\textsuperscript{16} As shown in Figure 1A–C and Table 1, the RNA levels of ten bromodomain-containing proteins were differentially expressed in GBM tumors as determined by Bonferroni corrected P values. Because Bonferroni multiple comparison correction can often result in false negatives, a Benjamini-Hochberg multiple comparisons correction was also used to identify significant genes, using a stringent false discovery rate (FDR) of 1%.\textsuperscript{17}

Genes exhibiting significant expression changes tended to cluster together based on sequence similarity (Fig. 1C). Two members of the BET family of bromodomains, BRD2 and BRD4, were significantly increased in glioblastoma, as their expression was 1.8-fold higher in GBM samples relative to control. Further, when the BRD proteins were ranked by P value, BRD4 was the third most significantly elevated RNA relative to all other RNAs encoding bromodomain proteins (Fig. 1A and B; Table 1). Collectively, these studies suggested that BRD2 and BRD4 might be attractive therapeutic targets since they are elevated in GBM tumors relative to control tissue.

**BET bromodomain protein inhibition reduces glioblastoma cell proliferation**

Since BRD4 was elevated in GBM tumors relative to controls and has been implicated in promoting proliferation of multiple cancer cell lines, we asked whether disrupting its activity affected U87MG cell proliferation. We reduced BRD4 expression utilizing a well-characterized siRNA and measured BRD4 mRNA levels via qRT-PCR analysis. As seen in Figure 2A, BRD4 mRNA levels were significantly decreased in cells transfected with siRNA targeting BRD4 relative to control siRNA-transfected cells (P < 0.001). BRD4 siRNA treated cells contained lower cellular ATP (P < 0.001) as measured by a CellTiter-Glo assay (Fig. 2B).

Further, U87MG cells containing lower BRD4 proliferated less than control-transfected cells (P < 0.001), as measured by an EdU incorporation assay (Fig. 2C).

To determine whether pharmacological inhibition of BRD2, BRD3, and BRD4 similarly reduced U87MG ATP levels and cell proliferation, we utilized the small molecule inhibitor I-BET151. I-BET151 treatment dose-dependently reduced cellular levels of ATP as measured by a CellTiter-Glo assay (Fig. 3A and B). The mean IC\textsubscript{50} value for I-BET151 in a CellTiter-Glo assay was 1.05 ± 0.18 μM at 48 h and 0.572 ± 0.048 μM at 72 h (Fig. 3A and B). Slightly higher IC\textsubscript{50} values were observed for I-BET151 on glioblastoma cell lines A172 and SW1783 as well as patient derived glioblastoma stem cells (approximately 1.28 ± 0.23 μM, 2.68 ± 0.45 μM, and 1.12 ± 0.23 μM, respectively, Figs. S1 and S2). Further, I-BET151 was as potent as TMZ and different cell cycle inhibitors in reducing cellular ATP levels in U87MG cells (Fig. S1). The reduction in ATP levels was accompanied by inhibition of cell proliferation since I-BET151 treatment reduced EdU incorporation in U87MG and Patient-derived cells (Fig. 3C and D; Fig. S7).

Since I-BET151 treatment reduced proliferation of U87MG cells, we tested whether it affected cell cycle transition. To test this directly, we performed propidium iodide analysis (PI-FACS) and found that I-BET151 treatment increased the number of U87MG cells in the G1 phase of the cell cycle. I-BET151 treated cells also contained lower percentage of S phase cells, suggesting that BET bromodomain proteins control G1 progression (Fig. 4).

BET bromodomain protein control of cell cycle progression may reduce U87MG cell cycle transit from G1 to S phase given the increased number of G1 cells observed after I-BET151 treatment. To test this directly, we performed FUCCI live cell imaging analysis of U87MG cells in the presence of I-BET151 or DMSO control. The FUCCI system has been utilized to measure cell cycle progression in vitro and in vivo.\textsuperscript{18} It utilizes fluorescent degradation reporters, which mark G1 (red), S/G2/M (green), or the G1/S transition (yellow). As shown in Figure 5A and B, treatment of U87GM cells with I-BET151 increased the number of G1/S (yellow) cells relative to control over time. By contrast, the percentage of S/G2/M (green) cells decreased upon I-BET151 treatment (Fig. 5A and B; Fig. S3). Collectively, these studies suggest that I-BET151 treatment arrests cells at the G1/S transition.

I-BET151 treatment reduces tumor size of U87MG xenografts

I-BET151 inhibition of cell proliferation in U87MG cells suggests that it may be an effective means of inhibiting GBM tumor growth in vivo. To test this directly, we injected immunocompromised mice subcutaneously with U87MG
cells followed by I-BET151 (10 mg/kg; i.p. daily) or saline injection. As shown in Figure 6, saline injected animals had much larger tumors than the I-BET151 injected counterparts ($P < 0.05$), suggesting that I-BET151 reduced proliferation of U87MG cells in vivo. Importantly, I-BET151 was as effective as TMZ at inhibiting tumor growth in vivo. Further, I-BET151 appears to be well tolerated by animals, since no difference in weight was observed in saline or I-BET151 treated mice (Fig. S4).

**Discussion**

Our studies identified the BET bromodomain proteins as possible therapeutic targets in GBM. We demonstrated that the BET bromodomain proteins BRD2 and BRD4 are significantly elevated in GBM tumors relative to controls. Based on these findings, we tested whether siRNA mediated depletion of BRD4 in U87MG cells significantly reduced proliferation. BRD4 depletion reduced U87MG cell proliferation as measured by an EdU incorporation assay. Similarly, treatment with the BET bromodomain inhibitor I-BET151 reduced EdU incorporation in U87MG cells, suggesting the I-BET151 effects on cell proliferation are at least partly mediated through BRD4 inhibition. I-BET151 treatment arrests U87MG cells at the G1/S transition as revealed by FACS and FUCCI analysis. These in vitro studies suggested that I-BET151 treatment might be a possible means of reducing GBM cell proliferation in vivo. Indeed, I-BET151
### Table 1. Relative Expression of Bromodomain Proteins in glioblastoma (GBM) and control (CTR) samples

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Genes with $P < 0.001$ (Bonferroni correction) are shown in bold. In italics are genes that passed a Benjamini-Hochberg post-test with a false discovery threshold of 1%. Asterisk next to the gene name indicates that gene was below the threshold for background, and was therefore deemed to be undetected in the samples.
shown to inhibit BRD2, BRD3, and BRD4 activity. We have responsible for growth inhibition in vivo. I-BET151 has been growth in a flank mouse model.

Further studies are required to identify the I-BET151 target(s) responsible for growth inhibition in vivo. I-BET151 has been shown to inhibit BRD2, BRD3, and BRD4 activity. We have shown that BRD4 depletion reduces U87MG cell proliferation and thus is one of the likely targets affected by I-BET151 in vitro and in vivo. BRD2 has an established cell cycle role and a recent study found that depletion of either BRD2 or BRD4 but not BRD3 reduces glioblastoma cell proliferation. We compared the effects of BRD3 and BRD2 knockdown to BRD4 depletion and we confirmed that BRD3 is not essential for U87MG proliferation (Fig. S8). However, further studies are required to determine the relative contribution of BRD2 and BRD4 to GBM cell proliferation since these two proteins are likely to have overlapping or independent functions and targets in proliferation and inflammation.

Perhaps similar to what was performed for various kinases, mouse models can be developed that harbor I-BET151 insensitive versions of BRD2 or BRD4 to fully validate these targets in GBM.

An alternative means of validating BRD2 and BRD4 as targets in GBM is the use of different chemical scaffolds, which inhibit BRD2 and BRD4 activity. Indeed, JQ1 contains a different chemical scaffold from I-BET151 and inhibits growth of GBM cells. Since JQ1 is brain penetrant, it is an important tool compound for studying the role of BET bromodomain proteins in vivo.

It is unlikely that I-BET151 is brain penetrant and thus it is unclear if it can also be used for in vivo validation of BET bromodomain proteins as targets in GBM. However, based on its high polar surface area (93 A²), I-BET151 is less likely to be robustly brain penetrant, which is further supported by its low permeability and moderate efflux in MDCK-MDR1 cells (unpublished observations). Thus, chemical optimization is likely required to generate brain penetrant I-BET151 analogs.

It will be interesting to determine whether these I-BET151 analogs inhibit progression of GBM cell proliferation from G1 into S phase since our studies suggest this is a major means through which I-BET151 inhibits cell cycle progression. We find that I-BET151 induces accumulation of U87MG cells in G1 as revealed by FACS analysis. This is likely true for multiple BET bromodomain inhibitors since JQ1 induced G1 arrest in U87MG cells. Our studies extend these findings to suggest that BET bromodomain inhibition induces accumulation of cells at the G1/S transition. Fucci analysis revealed that U87MG cells treated with I-BET151 accumulate at G1/S. This was confirmed in HeLa cells, which arrested at G1/S upon I-BET151 treatment as revealed after synchronisation and FACS analysis (unpublished observations). Thus, I-BET151 induction of G1/S arrest may be broadly applicable to multiple cell types where BET bromodomain proteins are required for entry into S phase from G1.

BET bromodomain control of the G1/S transition may be related to a newly discovered role for these proteins in transcriptional modulation at super enhancers. BRD4 was found to be associated with super-enhancers that control transcriptional modulation at super enhancers. BRD4 was be related to a newly discovered role for these proteins in B. BET bromodomain inhibition induces accumulation of cells at the G1/S transition. Fucci analysis revealed that U87MG cells treated with I-BET151 accumulate at G1/S. This was confirmed in HeLa cells, which arrested at G1/S upon I-BET151 treatment as revealed after synchronisation and FACS analysis (unpublished observations). Thus, I-BET151 induction of G1/S arrest may be broadly applicable to multiple cell types where BET bromodomain proteins are required for entry into S phase from G1.

BET bromodomain control of the G1/S transition may be related to a newly discovered role for these proteins in transcriptional modulation at super enhancers. BRD4 was found to be associated with super-enhancers that control expression of oncogenes required for multiple myeloma or GBM cell proliferation. Interestingly, treatment with JQ1 reduced BRD4 binding to super enhancers within 6 h, which is within the time frame we began observing statistically significant differences between I-BET151 and DMSO treated cells by Fucci analysis (Fig. S3). Future studies are required to determine whether the cell cycle inhibition we observe at G1/S is related to BET bromodomain proteins’ role as transcriptional modulators at super-enhancers.

BET bromodomain protein control of super enhancers is thought to be cancer type dependent. For instance, JQ1 treatment reduced BRD4 occupancy at the C-MYC enhancer in multiple myeloma but not GBM cells. This agrees with a recent
study where \textit{C-MYC} levels were unchanged after JQ1 treatment of GBM cells.\textsuperscript{22} Our studies suggest that \textit{C-MYC} levels do not decrease after I-BET151 treatment (Fig. S5A). Thus, it does not appear that BET bromodomain proteins regulate \textit{C-MYC} levels in GBM cells. Similarly, we did not observe upregulation of Hexim1, an inhibitory component of the positive transcription elongation factor b (P-TEFb) complex, which was increased after JQ1 in T-cells.\textsuperscript{26} To determine if it was similarly upregulated in GBM cells, we tested \textit{Hexim1} expression after 24 h of I-BET151 treatment. However, we found significant downregulation of \textit{Hexim1} after I-BET151 treatment (Fig. S9D). This may suggest that \textit{Hexim1} is differentially regulated in GBM cells relative to T-cells.

To identify possible transcriptional targets of BET bromodomain proteins in GBM cells, we treated U87MG, A172, and GBM stem cells with either 500 nM I-BET151 or DMSO for 24 h and performed Affymetrix array analysis (Fig. S5B). As shown in Figures S5 and S6, some RNAs were significantly altered upon I-BET151 treatment. However, we were unable to find common RNAs that could be directly linked to GBM cell proliferation. Perhaps BET bromodomain proteins regulate a network of proteins involved in proliferation and only by performing mathematical modeling can we discover their role in controlling the G1/S transition.\textsuperscript{27}

A recent study demonstrated that the RNA and protein levels of the cyclin-dependent-kinase inhibitor p21\textsuperscript{CIP1} increase after JQ1 treatment.\textsuperscript{22} Importantly, p21\textsuperscript{CIP1} downregulation reduced growth inhibition after JQ1 treatment, suggesting that p21\textsuperscript{CIP1} is an important downstream target of BET bromodomain proteins.\textsuperscript{22} Consistent with these findings, we observed a significant increase of p21\textsuperscript{CIP1} in both mRNA and protein after I-BET151 treatment (Fig. S9A and B). p21\textsuperscript{CIP1} upregulation after BET bromodomain protein inhibition is consistent with our FACS and FUCCI analysis, which showed an increase of cells in G1 after I-BET151 treatment. p21\textsuperscript{CIP1} protein levels are thought to be high during G1 to maintain low cyclin-dependent-kinase activity. During the G1/S transition, p21\textsuperscript{CIP1} levels decrease to allow S phase entry. Thus, BET bromodomain proteins may reduce p21\textsuperscript{CIP1} levels during late G1/S to promote S phase entry and cell cycle progression. By contrast, BET bromodomain inhibitors may induce p21\textsuperscript{CIP1} levels, thus blocking cells at the G1/S transition. This model predicts that BET bromodomain inhibition is analogous to cyclin-dependent-kinase attenuation during the G1/S transition. It will be important to determine whether BET bromodomain inhibitors synergize with cyclin-dependent-kinase inhibitors in vitro and in vivo. We did not observe apoptosis after I-BET151 treatment. This may be due to the fact that we were concentrating on short treatments (24–72 h) and were mainly observing cell cycle arrest. Our FUCCI analysis visualized the cells for 24 h and we did not observe cell death within this time frame. This was also true for our PI-FACS analysis. However, after 72 h of treatment we began to observe reduction of an anti-apoptotic
BCL2, which may eventually lead to apoptosis (Fig. S9). Finally, it will also be essential to determine whether patients who develop resistance to cyclin-dependent-kinase inhibitors are responsive to BET bromodomain inhibition. Future studies are required to fully explore the potential of bromodomain protein inhibition in combination therapies for the treatment of GBM.

Materials and Methods

Tissue specimen collection
Glioblastoma samples and relative controls (epilepsy) specimens were provided by the Florida Center for Brain Tumor Research (IRB project # 134-2006).

NanoString nCounter assay
Total RNA samples were submitted to the University of Miami Oncogenomics Core Facility for analysis using the NanoString nCounter gene expression system (Nanostring Technologies). Detailed methods for NanoString have been described elsewhere. Briefly, two 100 base pair sequences complementary to each mRNA of interest were constructed. These probes consisted of one capture and one reporter probe specific to 40 human mRNA targets. The reaction was multiplexed so that all probes were simultaneously hybridized to 100 ng of total mRNA in one sample. The tripartite capture-reporter-target complexes were affinity purified and immobilized to a streptavidin-coated cartridge via a biotin tag on the capture probe. Each sample lane contained positive and negative controls to be used to adjust for systemic variability and to estimate non-specific background signal, respectively. A color-coded sequence unique to the reporter probe for each target RNA was read using an automated imager after elongation, alignment, and immobilization of the molecule using electrophoresis.

Data analysis was conducted using nSolver software (NanoString Technologies). To correct for systemic variability, each sample lane was normalized to the average signal of the positive spiked-in controls. To control for differences between sample input, each lane was then normalized to a reference gene (SMYD3 was chosen based on stable expression between glioblastoma and control samples). Finally, to determine whether expression levels were above background noise, the mean of all the negative spiked-in controls was added to three times the standard deviation. Any target exhibiting a mean signal below this background threshold in both glioblastoma and control samples was considered to be undetected. A t-test with Bonferroni corrected familywise error rate (P < 0.001) was used to identify genes with significant expression changes between control and glioblastoma samples. A Benjamini-Hochberg analysis was also used to identify significant genes, allowing for a false discovery rate of 1%.

Cell lines
U87MG, A172, and SW1783 glioblastoma cell lines were purchased from ATCC and cultured in the recommended media at 37 °C and 5% CO₂. Patient’s cell line UM20 was cultured in DMEM/F12 plus 5% FBS and non-essential amino acids (NEAA, Invitrogen). Patient’s cancer stem cell lines Glio1 and Glio3 were cultured in DMEM/F12 (3:1) (Invitrogen) supplemented with 20 ng/ml EGF (Epidermal Growth Factor), 20 ng/ml bFGF.
positive cells was calculated with the Bio-application software “Target Activation” as ratio of nuclei to EdU-positive cells. For each sample, 25 fields were counted in order to cover the entire surface of the well.

**Propidium Iodide-FACS**

An amount of 1 × 10^5 U87MG cells were plated into two wells of a six well plate. Twenty-four hours later, 500nM of I-BET151 or DMSO was added to each well and processed for PI-FACS as previously described. GraphPad prism was used to analyze the FACS data. Two-way ANOVA was used to determine significance (**P < 0.001, n = 3).**

**Fluorescence ubiquitination cell cycle indicator (FUCCI) analysis**

U87MG cells were plated at a low confluency (25–30%) and transduced with geminin-GFP and Cdt1-RFP expressing virus (Premo™ FUCCI Cell Cycle Sensor BacMam 2.0, Invitrogen) for 16 h following the manufacturer’s instructions. These two constructs are ubiquitinated by specific ubiquitin E3 ligases targeting them to the proteasome for degradation. These E3 ligases are temporarily regulated during the cell cycle. As a result, the nuclei of the cells progressing through the cell cycle varied in color: the nuclei of cells in G1 phase were labeled with the red fluorescent protein (RFP). The nuclei of cells in S through M phases were labeled with the green fluorescent protein (GFP). As the cells transitioned between phases G1 and S, they expressed both RFP and GFP, appearing yellow after image co-localization. Cells lacking color were transitioning between M and G1.

Media containing 500 nM I-BET151 or DMSO was added to the transduced cells. Cells were then immediately placed in a microscope incubator chamber and images were captured every hour for 24 h (Confocal LSM7, Leica). Time series images were analyzed with the Fiji software obtaining the number of cells in each cell cycle phase. Three independent experiments were performed. Using GraphPad Prism, data were analyzed by Two-way repeated measures ANOVA, followed by Bonferroni
Post hoc comparison of all the means to determine significance 

\[ (**P < 0.001; *P < 0.01; *P < 0.05; n = 3) \]

**Compounds**

I-BET151 (GSK1210151A) was synthesized to > 99.5% purity as described in Dawson et al.\(^3\) by Reagents 4 Research LLC. Temozolomide was purchased from Tocris Bioscience (Catalog Number 2706). BKM-120 (Catalog Number S2247), GDC-0941 (S1065), MLN8237 (S1133), MK-2206 (S1078), and PD0320991 were purchased from SelleckChem.

**BRD2, BRD3 and BRD4 knockdown**

U87MG cells were plated and transfected the same day with Lipofectamine (Life Technologies) and 50 nM siRNA (Silencer Select validated siRNA for BRD4 ID#s23901 and Negative Control ID#4390843) according to the manufacturer’s instructions. Cells were transfected a second time 2 d after plating and RNA extracted 5 d after plating. U87MG were transfected with 25 nM of siRNAs targeting either BRD2 (IDs66245) or BRD3 (IDs15544). Cells were then lysed with Trizol (Life Technologies), total RNA extracted with chloroform, and the RNA was further purified with the RNeasy Mini Kit (Qiagen). cDNA was synthesized (cat#18080-400, Life Technologies) and real-time qPCR performed with Taqman probes for BRD4 or actin. The amount of target gene expression was calculated in relation to a reference gene using \( \Delta \Delta \text{Ct} \) analysis (2-\[\Delta \Delta \text{Ct} \text{ reference}\]). Error bars represent the standard deviation from the mean for three independent experiments (**P < 0.001 as determined by the Student t test; n = 3).

**UM20 cell line**

Glioblastoma tissue was washed with PBS, minced, and incubated in 0.1% trypsin and 0.04% DNase in Hank’s balanced salt solution (HBSS) for 45 min at 37 °C while rotating. Cells were then passed through a 70 μm and 40 μm filter and plated. They were grown as a monolayer in DMEM/F12 (1:1) containing non-essential amino acids (NEAA) supplemented with 5% FBS. They were grown as a monolayer in DMEM/F12 (1:1) containing non-essential amino acids (NEAA) supplemented with 5% FBS.

**Patient derived GBM stem cells (Gliol1 and Gliol3)**

Tumors were minced in PBS and digested in Hanks balanced salt solution (HBSS) containing 0.1% trypsin/EDTA and 0.2 mg/ml DNase I (Roche) for 30 min at 37 °C. Cells were serially passed through 70 then 40 μm filters, pelleted and incubated with red cell lysis buffer (Sigma) to remove red blood cells. Cells were washed in DMEM and plated in DMEM/F12 (3:1) (Life Technologies) supplemented with 20 ng/ml EGF, 20 ng/ml bFGF, and 2%Gem1Neuroplex and 1% anti-mycotic/anti-biotic (Gemini Bio-Products; 300-110P, 300-112P, 400161-010, 400101). Neurospheres were dissociated with Accutase (Invitrogen; A110501) and one-half of the media was replaced with PBS, trypsinized, counted, and re-suspended in Advanced DMEM/F12 (Invitrogen, cat# 12634) at a concentration of 5 × 10⁶ per mL. NU-Foxn1nu mice (Charles River Laboratories [n = 17]) were injected subcutaneously in the right flank with 200 μl of the cell solution (3 × 10⁶ cells per mouse). Mice were weighed once a week. Tumor sizes were measured with digital calipers every two to three days, recorded, and used for tumor volume calculations (WxLxH). Treatment with I-BET151 began 7 d after subcutaneous injection. The mice were evenly grouped with reference to tumor volume at the beginning of treatment. Eight mice were injected with I-BET151 and nine mice were injected with saline. I-BET151 was dissolved in 5% Tween80 (Sigma-Aldrich, cat# P1754, 500 mL), 5% DMSO (Sigma-Aldrich, cat# 34869), and 90% saline (G Biosciences, cat# 786-561). Intraperitoneal injections (IP) were administered daily at a concentration of 10 mg/kg for 21 d.

**Array gene ST**

U87MG, A172, T98G, LN18, Patient Glioblastoma Stem Cell 1 (Glio1), Patient Glioblastoma Stem Cell 3 (Glio3) were plated and subsequently treated with DMSO or I-BET151 at a concentration of 500 nM for 24h. RNA was extracted (Qiagen kit), cDNA synthesized (Life technology), and hybridized to Affymetrix Gene 1.0 ST as previously described.\(^29\)

**Array data analysis**

The Human Gene ST 1.0 array interrogates 36,079 annotated RefSeq (build 36) transcripts (Gene ST 2.0 covers 40,716 transcripts RefSeq build 51). The probe set intensities were quantified using the Affymetrix Scanners using GeneChip Command Console (AGCC) and analyzed with RMA normalization using Genome Console software (Affymetrix).

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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