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BET Bromodomain Inhibition Potentiates Radiosensitivity in models of H3K27-Altered Diffuse Midline Glioma

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Conflict of interest statement
The authors have declared that no conflict of interest exists.
ABSTRACT

Diffuse midline glioma (DMG) H3K27-altered is one of the devastating childhood cancers. Radiation therapy remains the only effective treatment yet provides a 5-year survival rate of only 1%. Several clinical trials have attempted to enhance radiation anti-tumor activity using radiosensitizing agents, although none have been successful. Given this, there is a critical need for identifying effective therapeutics to enhance radiation sensitivity for the treatment of DMG. Using high-throughput radiosensitivity screening, we identified bromo- and extra-terminal domain (BET) protein inhibitors as potent radiosensitizers in DMG cells. Genetic and pharmacologic inhibition of BET bromodomain activity reduced DMG cell proliferation and enhanced radiation-induced DNA damage by inhibiting DNA repair pathways. RNA-seq and CUT & RUN showed that BET bromodomain inhibitors regulate the expression of DNA repair genes mediated by H3K27 acetylation at enhancers. BET bromodomain inhibitors enhanced DMG radiation-response in patient-derived xenografts as well as genetically engineered mouse models. Together, our results highlight BET bromodomain inhibitors as radiosensitizer and provide a rationale for developing combination therapy with radiation for the treatment of DMG.
Introduction

Diffuse midline gliomas (DMGs) with H3K27M mutation (histone H3 lysine 27 replaced with methionine) are diffusely infiltrating glial neoplasms affecting midline structures of the central nervous system (CNS) (1). DMG is one of the devastating childhood tumors, with a median survival of 9 to 12 months from diagnosis (2). Factors that contribute to the dismal prognosis include the infiltrative nature and anatomic location of the tumor within the pons which precludes surgical resection. The identification of effective therapies has been extremely challenging, with over 250 clinical trials involving different combinations of chemotherapeutic agents commonly used in adult glioma proving ineffective in treating DMG (3). Fractionated focal radiation to a total dose of 54–60 Gy over a six-week period remains the only standard treatment modality that can provide transient symptom relief and a delay in tumor progression in about 70-80% of patients. However, radiation-treated children with DMG show evidence of disease progression within the first year of completing radiation therapy (4, 5). Given this reality, the identification of efficacious therapeutic agents that enhance the anti-tumor effects of radiation is desperately needed for improving treatment outcomes for this patient population.

In contrast to adult gliomas, DMG is uniquely dependent on the H3K27M mutation for its initiation and maintenance (6–9). H3K27M mutation occurs in H3F3A and HIST1H3B/C genes encoding histone H3 variants H3.3 and H3.1, respectively, in as much as 80% of DMGs and is associated with a shorter survival among patients with DMG (6, 7, 10). We and others have identified a key functional consequence of H3K27M mutation: mutant protein sequestration of the polycomb repressive complex 2 (PRC2) methyltransferase resulting in functional inactivation of PRC2 (8, 9, 11, 12). This inactivation leads to a global reduction of H3K27 di-methylation (K27me2) and tri-methylation (K27me3) that, in turn, leads to extensive transcriptional reprogramming of mutant cells and promotes a stem-cell like, therapy-resistant phenotype.

While decreasing H3K27 methylation, K27M mutation also increases K27 acetylation (K27ac), which is necessary for bromo- and extra-terminal domain (BET) protein transcriptional activation through RNA polymerase II (13-15). We have previously profiled the epigenome of H3K27M-mutant DMG cells
and found that K27M mutant co-localized with K27ac (15). Heterotypic H3K27M-K27ac nucleosomes co-localized with BET bromodomain protein 2 and 4 (BRD2 and BRD4) at actively transcribed gene loci to activate transcription in DMG (15). Highly selective BET bromodomain inhibitors, such as JQ1 or I-BET (16-19), block the binding between BRD and acetylated histones to suppress gene transcription, representing a promising therapeutic strategy for treating DMG (13–15, 20). Indeed, targeted BET bromodomain activity using JQ1 inhibited tumor growth and extended the survival of animal bearing H3K27M-mutant DMG patient-derived xenografts (PDXs) (15). Because of their promising anti-tumor activity, BET bromodomain inhibitors are being tested in several clinical trials with cancer patients including myeloma (NCT03068351), leukemia (NCT02158858), prostate cancer (NCT02711956), and other advanced solid cancers (NCT01587703, NCT02419417) including pediatric brain cancer (NCT03936465). However, how small molecule inhibitors, such as those targeting BET bromodomain activity, interact with radiation has not been explored in DMG.

Through an unbiased high-throughput radiosensitivity screen, we found the BET bromodomain inhibitors as potent radiosensitizers of H3K27M-mutant DMG cells. The depletion of BRD using short hairpin RNAs (shRNAs) and single-guide RNAs (sgRNAs), and BET bromodomain inhibition using small molecule inhibitors, reduced DMG cell proliferation and enhanced radiation-induced DNA damage by inhibiting DNA repair pathways. Moreover, BET bromodomain inhibition down-regulated the expression of DNA repair genes associated with H3K27ac occupancy and enhanced DMG radiation-response in vitro and in vivo. Together, these results highlight BET bromodomain inhibiton as a potential radiosensitization and provide a rationale for developing combination therapy with radiation for the treatment of this deadly pediatric brain cancer.

RESULTS

BET bromodomain inhibitors are identified as potent radiosensitizers by high-throughput drug screening.
We first performed an unbiased high-throughput radiosensitivity screen in H3.3 wild type (WT) and K27M-mutant DMG neurosphere cells using total of 2,880 compounds including 1,280 FDA-approved drugs and 1,600 clinical compounds (mainly small molecule inhibitors of epigenetic processes) in the presence or absence of 10 Gy irradiation. Radiosensitizing effects were quantified by cell death number using confocal image analysis combined with Hoechst nuclear staining and propidium iodide (PI) DNA staining (Figure 1a). We identified several clinical grade BET bromodomain inhibitors as potent radiosensitizers in the screen (Supplemental Table 1), which increased cell death in combination with radiation (Figure 1b, Supplemental Figure 1). H3.3 K27M-mutant DMG neurosphere cells were more sensitive to BET bromodomain inhibitors in combination with radiation than H3.3 WT DMG neurosphere cells (Supplemental Figure 1). These BET bromodomain inhibitors were subsequently validated for their anti-proliferative effects in DMG cells. AZD5153 and molibresib (I-BET762) in combination with radiation showed strong additive cytotoxic effects relative to each monotherapy (white dot line, Figure 1c). However, methotrexate and temozolomide, which has been using in combination with radiation in adult glioblastoma (GBM), did not show additive radiosensitizing effects nor monotherapy cytotoxic effects in DMG cells. Our results are consistent with the results from clinical trials, which show that DMG transiently responds to the combination of temozolomide and radiation, but with no survival benefit from the combination therapy (21).

Targeted inhibition of BET bromodomain activity reduces cell proliferation and induces apoptosis in K27M-mutant DMG cells.

To address whether BET bromodomain activity is required for K27M-mutant DMG cell growth, we studied the effects of depletion of BRD proteins (BRD2, 3, 4) on DMG cell proliferation using CRISPR-Cas9 knockout (KO) (Supplemental Figure 2). KO effects were confirmed at the protein level (Supplemental Figure 2a), and the effects of BRDs depletion on cell proliferation were analyzed by the MTS assay (Supplemental Figure 2b). BRD4 depletion reduced H3K27ac and reciprocally increased
H3K27me3 protein expression, whereas the depletion of BRD2 and 3 did not effect on the expression levels of H3K27me3 (Supplemental Figure 2a). In addition, only BRD4 depletion suppressed the growth of DMG cells (Supplemental Figure 2b). We further analyzed the effects of BRD4 depletion on DMG cell growth (Figure 2 and Supplemental Figure 3). BRD4 KO and shRNA knockdown (KD) were confirmed at the protein level (Figure 2a, Supplemental Figure 3a), and the effects of BRD4 depletion on cell proliferation were analyzed by the MTS assay (Figure 2b, Supplemental Figure 3b), colony formation assays (Figure 2c, Supplemental Figure 3c), and BrdU incorporation assay (Figure 2d) in two K27M-mutant DMG cell lines (SF8628 and DIPG007). BRD4 depletion significantly reduced DMG cell growth relative to scramble control (Figure 2b, Supplemental Figure 3b). BRD4 depletion also suppressed colony formation in DMG cells (Figure 2c, Supplemental Figure 3c). The BrdU positive cell population in S-phase was decreased by BRD4 depletion in DMG cells (Figure 2d). These results indicate that BRD4 activity is required for DMG cells proliferation and provide possibility of a rational therapeutic target in DMG.

We and others have shown that targeting BET bromodomain activity using JQ1 inhibitor resulted in potent anti-tumor activity in patient-derived DMG cells and PDX models (15, 20). Despite with promising anti-tumor activity, JQ1 has not been translated to clinical trials due to its short half-life (16, 18, 22, 23, 24, 25). Thus, we expanded our findings to test the efficacy of clinical grade BET bromodomain inhibitors including Molibresib (I-BET762), PLX51107, BMS-986158, and AZD5153 in SF8628 DMG cells (Figure 3a). Of those, AZD5153 and BMS-986158 showed potent growth inhibition of SF8628 DMG cell with 50% growth inhibition (IC50) values at 0.41 μM and 0.69 μM, respectively, which were similar with the IC50 value of JQ1 (0.50 μM) (Figure 3a). AZD5153 and BMS-986158 have been used in phase 1 clinical trials for malignant solid tumors including pediatric brain cancer and blood cancer (AZD5153: NCT03205176, NCT03527147, NCT03013998, BMS-986158: NCT03936465, NCT04817007, NCT02419417, NCT05372354). However, the pre-clinical efficacy of BMS-986158 was disappointing in orthotopic (brainstem) DMG PDX models (Supplemental Figure 3d) due to its poor penetrance across the blood-brain barrier (BBB) with a brain penetration ratio of 5.02 ± 1.32 % (Supplemental Table 2). AZD5153 showed a brain penetration ratio of 12.9 ± 1.25 %, higher than that of BMS-986158. Thus, we
tested the efficacy of AZD5153 for cytotoxicity and radiosensitivity in DMG in vitro and in vivo. AZD5153 treatments induced dose-dependent inhibition of cell growth in five K27M-mutant DMG cell lines as well as human astrocytes expressing K27M H3F3A transgene (Astro-KM) cell, with IC50 values of 0.41 μM (SF8628), 0.053 μM (DIPG007), 0.022 μM (SU-DIPG36), 0.063 μM (SU-DIPG4), 0.013 μM [genetically engineered mouse model (GEMM)-DMG], and 0.020 μM (Astro-KM) (Figure 3b). Normal human astrocyte (NHA) and Astro-WT cells showed less sensitivity to AZD5153 with IC50 values of 1.35 μM and 1.47 μM, respectively (Figure 3b). IC50 values of AZD5153 also induced time-dependent inhibition of DMG cell growth (Figure 3c) and reduced colony formation in DMG cells (Figure 3d).

BET bromodomain inhibition sensitizes DMG cells to radiation.

To verify the radiosensitizing effect of BET bromodomain inhibition in K27M-mutant DMG cells, clonogenic survival assay was conducted in three DMG cell lines (SF8628, DIPG007, GEMM-DMG). Cells were treated with AZD5153 (Figure 4a), JQ1 (Supplemental Figure 4a), and depletion of BRD4 with shBRD4 and sgBRD4 (Supplemental Figure 4b), concurrently with ionizing radiation (IR). To quantify the radiosensitizing effect, we calculated a dose enhancement factor (DEF) which represents the ratio of the dose with IR alone, divided by the dose with IR and BRD4 inhibition at 10% survival. If the DEF is greater than one, the BRD4 inhibition will be functioning as radiosensitizer. AZD5153 treatment showed a radiation-enhancing effect with DEFs of 1.22 (SF8628), 1.32 (DIPG007), and 1.10 (GEMM-DMG) (Figure 4a). JQ1 has similar effects on radiation response in DMG cells with DEFs of 1.25 (SF8628), 1.40 (DIPG007), and 1.14 (GEMM-DMG) (Supplemental Figure 4a). shBRD4 KD and sgBRD4 KO also increase the radiation response of DMG cells with DEFs of 1.22 (shBRD4-484 and -487, SF8628), 1.34 (shBRD4-484 and -487, DIPG007), and 1.18 (sgBRD4-1, SF8628), 1.20 (sgBRD4-2, SF8628), 1.28 (sgBRD4-1 and -2, DIPG007) (Supplemental Figure 4b). Furthermore, we conducted BrdU incorporation (Figure 4b), apoptosis (Figure 4c), senescence (Supplemental Figure 5a, b), and sphere formation (Supplemental Figure 5c, d) assays. AZD5153 treatment resulted in a decreased BrdU positive S phase cell population relative to control (Figure 4b). Combination treatment of AZD5153 + IR further decreased S
phase cell population when compared to AZD5153 alone (Figure 4b). The Annexin V apoptosis assay showed that either AZD5153 or IR monotherapy increased Annexin positive cells compared to control (Figure 4c). Combination treatment of AZD5153 + IR increased Annexin V positive cells, outperforming each monotherapy. The beta-galactosidase assay revealed increasing senescence-associated beta-galactosidase staining in the DMG cells treated with either AZD5153 or IR monotherapy (Supplemental Figure 5a). Combination treatment of AZD5153 and IR further increased beta-galactosidase positive DMG cells. The cell size is known to be associated with senescence. To quantify the cell size, DMG cells were gated for G1 DNA content and sorted with the side scatter parameter (SCC) using flow cytometry (Supplemental Figure 5b). As similar results with beta-galactosidase staining, combination treatment of AZD5153 and IR further increase the cell size relative to either monotherapy. Combination treatment also reduced self-renewal activity (Supplemental Figure 5c) and neurosphere formation in compared to either monotherapy (Supplemental Figure 5d). These results suggest that, when compared to monotherapy, combination treatment of AZD5153 + IR further increased the radiosensitivity in DMG cells by decreasing the cell population of radioresistant S phase and stemness, and increasing apoptosis and senescence in DMG cells.

**BET bromodomain inhibition down-regulates the genes involved in DNA repair and cell cycle in K27M-mutant DMG cells.**

We have previously shown that JQ1 treatment causes a change in the expression of the genes that promote tumor growth in K27M-mutant DMG (15). In our current RNA-seq analysis, we performed unsupervised principal component analysis of SF8628 DMG cells treated with DMSO and BET bromodomain inhibitors (AZD5153, JQ1) for 24 and 48 hours. We found a global gene expression shift in AZD5153 treated DMG cells compared to DMSO treated samples (Figure 5a). We compared the RNA-seq data between the samples treated with DMSO and AZD5153 in combination with previous RNA-seq data in the samples treated with JQ1. The differentially expressed genes are highly correlated between the samples treated with JQ1 and AZD5153 (Figure 5b), including 3301 up-regulated and 3591 down-regulated...
genes in response to the BET bromodomain inhibitor. Gene Set Enrichment Analysis (GSEA) (Figure 5c, d, Supplemental Figure 6a, b) and Gene Ontology (GO) pathway analysis (Figure 6a, b, Supplemental Figure 6c, d) showed that cell cycle (e.g., CDK6, CDCA7, and UHRF1) and DNA double-strand breaks (DSBs) repair pathways (e.g., BRCA1, RAD51, XRCC1, XRCC4, and POLQ) were among the most significantly down-regulated in the BET bromodomain inhibitor treatment. AZD5153 and JQ1 treatments also up-regulated gene pathways involved in autophagy (e.g., ATGA4, MAPILC3B) and catabolism pathways including glycolysis and protein/macromolecule catabolic pathways (e.g., SIRT1, MTOR) (Figure 5c, d, Supplemental Figure 6a, b). The senescence-associated genes, CDKN1A and HMGA1, were upregulated by AZD5153 treatment (Supplemental Figure 7a, b). However, CDKN2A was downregulated by AZD5153 treatment. This could be due to increase H3K27me3 which repressed the PRC2 targets including CDKN2A (Supplemental Figure 7a, b).

BET bromodomain inhibition is known to suppress gene expression by dissociating BRD from the active chromatin mark, histone H3K27ac (26). We have shown that genomic occupancy of H3K27ac and BRD is required for enhancer activity and gene expression in DMG cells (15). To determine the effects of BET bromodomain inhibition on gene expression associated with H3K27ac occupancy, we performed CUT & RUN followed by next generation sequencing in DMG cells treated with AZD5153 (Figure 7). The CUT & RUN data showed that the majority of H3K27ac enrichments were found in introns (1st Intron: 13.47%, other intron: 28.97%) and intergenic regions (38.8%) (pie chart, Figure 7a). Meta plot and heatmaps showed the enrichments of H3K27ac signal near the previously defined enhancer regions in DMG cells (Figure 7b). AZD5153 treatment dramatically reduced H3K27ac occupancy at enhancer regions. To investigate the enrichment of transcription factors among the H3K27ac DNA binding sites, we used the DiffBind R package to determine differential peaks between DMSO and AZD5153 treated samples (FDR < 0.05). We performed simple enrichment motif analysis in SF8628 DMG cells and found a significant enrichment of DNA sequencing motifs involving neuronal developmental transcriptional factors such as LHX1-3 and HOX13 (e-value < 0.05, Supplemental Table 3). Interestingly, the H3K27ac peaks of two representative DNA repair genes, BRCA1 and RAD51, are diminished at enhancer regions in the SF8628 cells treated with
AZD5153 (Figure 7c). The expression of these DNA repair genes was significantly downregulated in the AZD5153 treated samples in RNA-seq analysis (Figure 5b, c). The senescence-associated gene expression was not associated with H3K27ac occupation (Supplemental Figure 7c). Taken together, our results suggest that BET bromodomain inhibition promotes a transcriptionally silent chromatin state by reducing H3K27ac occupancy and represses the expression of the genes involving DNA DSB repair in K27M-mutant DMG cells.

BET bromodomain inhibition enhances radiation-induced DNA damage.

We next analyzed the effects of BET bromodomain inhibition on radiation-induced DNA damage and repair pathways in DMG cells. Fluorescence immunocytochemistry of the DNA DSB marker γH2AX and repair marker 53BP1 were examined to quantify the extent of DNA damage and repair in irradiated SF8628 DMG cells either in the presence or absence of BET bromodomain inhibitors, AZD5153 (Figure 8a) and JQ1 (Supplemental Figure 4c). γH2AX and 53BP1 foci increased one hour following IR, indicating increased DNA DSB damage and repair by IR. At 24 hours after IR, γH2AX and 53BP1 foci were largely reduced in those cells due to successful repair upon DNA damage. However, irradiated DMG cells treated with BET bromodomain inhibitors sustained high levels of γH2AX at 24 hours compared to cells treated with IR alone while 53BP1 foci were decreased (Figure 8a, Supplemental Figure 4c). Similarly, comet assays showed that IR increased comet tail formation in SF8628 and DIPG007 DMG cell lines, indicating increased unrepaired DNA damage (Figure 8b). The DNA damage further increased comet tail formation in irradiated DMG cells treated with AZD5153 compared to the cells treated with IR alone. These results suggest that BET bromodomain inhibition may contribute to the DNA repair process to enhance radiation-induced DNA damage. Western blotting showed that AZD5153 treatment decreased the expression of DNA repair markers: BRCA1, RAD51, and XRCC1 in DMG cell lines (Figure 9a). H3K27ac was also decreased by AZD5153 in a dose dependent manner (Figure 9a). Radiation-induced γH2X expression peaked at 1 hour following radiation (Figure 9b, Supplemental Figure 4d). The expression of BRCA1, RAD51, and RAD50 were also induced by radiation and peaked at 3-6 hours following radiation.
Figure 9b. BET bromodomain inhibitors extended radiation-induced γH2X expression over 6 hours following radiation (Figure 9b, Supplemental Figure 4d). In contrast, the expression of BRCA1, RAD51, and RAD50 were decreased by BET bromodomain inhibitors over the time of radiation. These results suggest that BET bromodomain inhibition extends radiation-induced DNA damage signaling by suppressing the DNA repair pathway.

DNA damage is repaired by two major pathways; homologous recombination (HR) and nonhomologous end-joining (NHEJ) repair (27, 28). HR repairs DNA DSB during S and G2 phases and provides a template for error free repair. In contrast, NHEJ is active throughout the cell cycle and directly involves ligation of DNA ends without homology. To analyze the DNA damage repair pathways in DMG cells, we transfected green fluorescent protein (GFP)-reconstitution reporter cassettes for HR and NHEJ (29) into SF8628 DMG cells in the presence or absence of AZD5153. AZD5153 treatment reduced DNA repair ability through both HR and NHEJ DNA repair pathways (Figure 9c), which is consistent with RNA-seq results showing that AZD5153 down-regulated the genes involved in both HR and NHEJ repair pathways (Figure 5). Collectively, our results indicate that BET bromodomain inhibition increased radiation-induced DNA damage by inhibiting HR and/or NHEJ DNA repair pathway in K27M-mutated DMG cells.

**BET bromodomain inhibitors enhance radiation anti-tumor effects in patient-derived and genetically engineered DMG animal models.**

Based on the radiosensitizing effect of BET bromodomain inhibition on the growth of K27M-mutant DMG cells, we hypothesized that BET bromodomain inhibition increases radiation anti-tumor activity and the survival benefit in DMG mouse models. To address this, the mice were implanted with SF8628 or GEMM-DMG cells into the pons and treated with AZD5153 (50mg/kg) or JQ1 (30mg/kg) for 2 weeks in the presence or absence of radiation at total dose of 9 Gy (1.5 Gy per day for 3 days a week for 2 weeks) (Figure 10a). BET bromodomain inhibitor monotherapy inhibited tumor growth and extend survival of mice with SF8628 DMG PDX (Figure 10b, c) as well as GEMM-DMG models (Supplemental Figure 8).
Similarly, radiation monotherapy provided a significant therapeutic benefit (Figure 10b, c, Supplemental Figure 8). We found that combination treatment of AZD5153 and radiation therapy (RT) significantly prolonged animal survival (Figure 10b, Supplemental Figure 8). Similarly, we found the combination treatment with JQ1 and radiation showed a significant survival benefit (Figure 10c). These in vivo efficacy studies included euthanizing the mice at the end of treatment to obtain brainstem tumor samples to analyze tumor cell proliferation (Ki-67, Figure 10d), apoptosis (TUNEL, Figure 10d), senescence (p21, p16, Supplemental Figure 9), and migration [normal human nuclear antigen (NHA), Supplemental Figure 9, bottom]. Analysis of intra-tumor Ki-67 staining showed all therapies significantly reduced SF8628 DMG cell proliferation relative to the control group (Figure 10d, top). There were significantly less Ki-67 positive cells in the samples treated with combination therapy compared to either monotherapy. TUNEL staining results showed the highest proportion of positive cells were in tumors derived from mice receiving combination therapy of AZD5153 and radiation relative to either monotherapy (Figure 10d, bottom). No TUNEL positivity was evident in normal brain surrounding tumor in mice receiving any of the combination treatments. Senescence marker, p21 staining showed increase positive cells in tumors treated with AZD5153 and in combination with radiation (Supplemental Figure 9, top). However, p16 positive cells were decreased by the treatment (Supplemental Figure 9, middle). This could be due to downregulate the expression of CDKN2A gene which codes p16 protein. NHA staining revealed decreasing NHA positive cell in the tumor treated with either AZD5153 or radiation (Supplemental Figure 9, bottom). Combination treatment further decreased NHA positive cells relative to each monotherapy.

Discussion

DMG is one of the most devastating childhood cancers with a limited response to radiation therapy, resulting in dismal prognosis with a median overall survival of less than 12 months. There is a critical need for new therapeutics that enhance the radiation effect for the treatment of DMG. Here, we identified BET bromodomain inhibitors as potent radiosensitizers in DMG using unbiased high throughput radiosensitivity library screening (Figure 11a). High-throughput screening (HTS) is a useful tool for identifying the
candidate compounds from a large chemical library (30). We successfully integrated the HTS with neurosphere-based assays using automated fluorescent live-cell imaging in the presence or absence of radiation and identified several clinical grade BET bromodomain inhibitors as top candidates for radiosensitization (Figure 1, Supplemental Figure 1, Supplemental Table 1). In this assay, we used propidium iodide (PI) to detect dead cell population. However, PI staining may not capture long-term mechanism of radiation-induced cell death. Further evaluation of proliferative cell death caused by radiation, such as mitotic catastrophe (31), would be needed to understand the mechanism of radiation-induced cell death.

BET bromodomain inhibitors disrupt the binding between acetylated histone and BRD proteins and inhibit active transcription, leading to enhancing radiation effect in DMG (Figure 1b) (14–16). K27M-mutant DMG cells are vulnerable to BET bromodomain inhibition due to the transcriptional dysregulation resulting from the mutation (14, 15, 20, 32). We demonstrated that BET bromodomain inhibition, using shRNA or sgRNA mediated BRD depletion (Figure 2, Supplemental Figure 2, 3) and treatment of small molecule inhibitors (AZD5153 and JQ1) (Figure 3), suppressed the growth of human and mouse K27M-mutant DMG cells. Importantly, BET bromodomain inhibition in combination with radiation further increased radiosensitivity of DMG cells by reducing the radioresistant S phase cell population and stemness, and increasing apoptosis and senescence (Figure 4, Supplemental Figure 5).

DNA damage is thought to be the most important consequence of radiation effect, and the genetic alterations of DNA repair pathways are frequently detected in pediatric high-grade glioma including DMG (6, 10, 33, 34). We and others have demonstrated that the majority of DNA DSBs caused by radiation are repaired within 24 hours of completing radiation (35-37). Thus, DNA repair is a key factor in radiosensitivity and can be a therapeutic target to enhance the radiation anti-tumor activity in K27M-mutant DMG. Our gene expression profiling of K27M-mutant DMG cells treated with BET bromodomain inhibitors (AZD5153 and JQ1) revealed significant decreases in transcripts from the genes involved in DNA repair pathways for both HR and NHEJ, including BRCA1, RAD51, XRCC1, and XRCC4 (Figure 5, 6,
Supplemental Figure 6). To advance understanding of the transcriptional regulation in DNA repair gene pathways by BET bromodomain inhibition, we mapped genome-wide occupancy of H3K27ac in K27M-mutant DMG cells using CUT & RUN (Figure 7). We have previously profiled the epigenome of K27M-mutant DMG cells and shown that K27M mutation associates with increased H3K27ac and the heterotypic H3K27M-K27ac nucleosomes colocalize with BET BRD2 and BRD4 at the loci of actively transcribed genes (15). We analyzed the specific loci with H3K27M-K27ac occupation from the previous study (15) and found that BET bromodomain inhibition diminished a genome wide distribution of H3K27ac at enhancer regions including two representative DNA repair genes, BRCA1 and RAD51 (Figure 7). DNA damage induces cellular senescence (38). We found senescence-associated genes, CDKN1A and HMGAI, were upregulated by BET bromodomain inhibition (Supplemental Figure 7a, b). However, CDKN2A was downregulated by BET bromodomain inhibition. There was no association between H3K27ac occupation and the senescence-associated gene expression (Supplemental Figure 7c). It is possible that senescence-associated genes are controlled by different epigenetic regulation such as H3K27me3. Indeed, BET bromodomain inhibition reciprocally increase H3K27me3 (Supplemental Figure 2a), which results in silencing PRC2 target genes such as CDKN2A. Our results indicated that BET bromodomain inhibitor downregulates the genes involved in DNA damage repair mediated by H3K27ac at enhancers, provides a basis for the possibility of BET bromodomain inhibitor acting as radiation enhancers in DMG (Figure 7). In fact, BET bromodomain inhibitors, AZD5153 and JQ1, inhibited HR and NHEJ DNA repair pathways and prolonged the radiation-induced DNA damage in K27-mutant DMG cells (Figure 8, 9, Supplemental Figure 4).

The molecular mechanisms of BET bromodomain inhibition in transcription and chromatin machinery for DNA damage repair in DMG are not fully understood. Upon binding to the chromatin, BET BRDs are known to function in the assembly of complexes that facilitate chromatin accessibility to transcription factors allowing for the recruitment of RNA polymerases II (RNAPII) (39-42). In particular, BRD4 is required for subsequent progression of RNAPII through hyperacetylated nucleosomes during
transcription elongation through interactions of its bromodomains with acetylated histones in order to prevent transcriptional stalling (40-42). Michael Yaffe’s group demonstrated that deregulated transcription following inhibition or loss of BRD4 in cancer cells leads to the accumulation of RNA:DNA hybrids (R-loops) and collisions with the replication machinery causing replication stress, DNA damage, and apoptotic cell death during S phase (43). We observed that BET bromodomain inhibition decreases the cell population of radioresistant S phase, diminished self-renewal activity, results in increased apoptotic cell death and cellular senescence (Figure 4, Supplemental Figure 5). In the study of H3K27me3-deficient medulloblastoma cells (44), JQ1 inhibition sensitized medulloblastoma cells to radiation by enhancing the apoptotic response through suppression of Bcl-xL and upregulation of Bim. Loss of H3K27me3 caused an epigenetic switch from H3K27me3 to H3K27ac at specific genomic loci, altering the transcriptional profile, which associated with a radioresistant phenotype in H3K27me3-deficient medulloblastoma. Stemness is a key characteristic of radioresistance in glioma (45). BET bromodomain inhibition may sensitize H3K27me3-deficient tumors to radiation by reducing radioresistance phenotype and enhancing apoptotic response and cellular senescence. We will further investigate the role of BET bromodomain inhibition in the transcription machinery associated with histone modification of H3K27me3 and H3K27ac for understanding radiation-induced DNA damage response in DMG.

Consistent with in vitro experiments, our animal studies demonstrated that the combination therapy of BET bromodomain inhibitor and radiation showed growth inhibition and increase survival benefit in human and murine DMG mouse models, compared to either therapy alone (Figure 10). The survival improvement of the combination therapy yet provides modest. One limitation of in vivo efficacy of BET bromodomain inhibitors is a poor brain penetration (Supplemental Table 2). To increase the drug concentration in the brain, we would further investigate new drug delivery systems such as disrupting the BBB using focused ultrasound (46, 47) or bypassing the BBB using convection-enhanced delivery (48) and intranasal delivery (49). Nevertheless, our findings support the possible use of BET bromodomain inhibitor to increase radiation anti-tumor effect for the treatment of DMG.
METHODS

Sex as a biological variable

Our study examined six-week-old female athymic mice (rnu/rnu genotype, BALB/c background). The animals were purchased from Envigo and housed under aseptic conditions. The animals are well established and used to develop diffuse midline glioma (DMG) patient-derived xenografts (PDXs) in our published studies (15, 35, 37, 50, 51). There are no reported sex differences among DMG patients.

Xenograft studies

Mice were injected with 1 μL of SF8628 or GEMM-DMG cell suspension (100,000 cells/μL) into the pontine tegmentum at a depth of 5 mm from the inner base of the skull as previously described (15, 35, 37, 51, 52). For the efficacy study of AZD5153 and radiation, animals were randomized into four treatment groups: 1) vehicle control (0.5% hydroxy methylcellulose, 0.1% Tween 80 for AZD5153, 1% DMSO for JQ1, n=11), 2) ADZ5153 (oral gavage of 50 mg/kg, n=11) or JQ1 (intraperitoneal injection of 30 mg/kg, n=11) treatment for 5 times a week for two consecutive weeks, 3) radiation monotherapy (1.5 Gy, 3 times a week for two consecutive weeks for a total dose of 9 Gy, n=10 for AZD5153 study, n=11 for JQ1 study), 4) combination therapy of AZD5153 or JQ1 and radiation (n=10 for combination with AZD, n=11 for combination with JQ1). Biweekly bioluminescence imaging was used to monitor tumor growth and response to therapy as previously described (15, 35, 36, 37, 50, 51). Mice were monitored daily and euthanized at endpoints which included irreversible neurological deficit or body condition score less than 2. All animal protocols were approved by the Northwestern University Institutional Animal Care and Use Committee.

Cell sources and propagation

SF8628 (H3.3K27M DMG) was obtained from the University of California San Francisco (UCSF) medical center, and in accord with an institutionally approved protocol. Establishment of SF8628 cell culture from surgical specimens, and tumor cell modification for expression of firefly luciferase for in vivo
bioluminescence imaging, have been described (15, 35, 37, 50, 51). DIPG007 (H3.3K27M DMG) cell line was kindly provided by Dr. Angel Montero Carcaboso (Hospital Sant Joan de Déu, Barcelona, Spain). SU-DIPG4 (H3.1K27M DMG) and SU-DIPG36 (H3.1K27M DMG) cell lines were kindly provided by Dr. Michelle Monje (Stanford University, Stanford, CA). Normal human astrocyte (NHA), human astrocytes expressing wild-type (Astro-WT), and K27M H3F3A transgene (Astro-KM) have been previously described (8, 51). The SF8628 and human astrocyte cells were propagated as monolayers in complete medium consisting of Dulbecco’s Modified Eagle’s medium (DMEM, 11965092) supplemented with 10% fetal bovine serum (FBS, A31604–02) and non-essential amino acids (11140–050) from ThermoFisher. DIPG007, SU-DIPG4, SU-DIPG36 cell lines were grown in tumor stem medium (TSM) as neurosphere culture or with 5% FBS as adherent culture. TSM base was prepared using the following: neurobasal-A medium (10888–022), DMEM/F-12 medium (11330–032), HEPES buffer (15630–080), sodium pyruvate (11360–070), MEM non-essential amino acids (11140–050), GlutaMAX-I supplement (35050–061), antibiotic-antimycotic (15240–096), B-27 supplement minus vitamin A (12587–010) from ThermoFisher, EGF and FGF (Shenandoah Biotech, 100–26 and 100–146), PDGF-A and PDGF-B (Shenandoah Biotech, 100–16 and 100–18), and 0.2% heparin (STEMCELL Technologies, 07980). H3.3K27M-mutant neurosphere cells were derived from a genetically engineered mouse model of DMG (GEMM-DMG) (Ntv-a; p53fl/fl; PDGFB; H3.3K27M; Cr) (48). GEMM cells were cultured under neurosphere conditions in DMEM supplemented with 10% proliferation supplement (Stem Cell Technologies), 1% Pen–Strep (Invitrogen), 20 ng/mL human basic FGF (Invitrogen), 10 ng/mL human EGF, and 2 μg/mL heparin. Short tandem repeat (STR), using the Powerplex16HS System (Promega DC2101), were obtained to confirm the identity of the cell lines. All cells were cultured in an incubator at 37°C in a humidified atmosphere containing 95% O2 and 5% CO2 and were mycoplasma-free at the time of testing with a Mycoplasma Detection Kit (InvivoGen).

**short hairpin RNAs and single-guide RNA treatments**

BRD4 and scrambled control short hairpin RNAs (shRNAs) [BRD4 shRNAs: V3THS_378004,
V3THS_326487, V3THS_326484, Control shRNA: RHS4346, Dharmacon IDs, Lafayette, CO were used to generate lentivirus and infected tumor cells according to the manufacturer’s instructions. At 24 hours post-lentiviral infection, cells were selected using 2 μg/mL puromycin for 5 days prior to in vitro assays.

We also generated single-guide RNAs (sgRNAs) to knockout of BRD2, BRD3, and BRD4 expression. sgRNA for ROSA26 gene was used as control (Supporting Data Values). The lentiCRISPRv2 vector (a gift from F. Zhang, Addgene plasmid #52961) was digested with BsmBI and inserted the sgRNAs were inserted into the vector (53). The ligation reactions were transfected into Stbl3 cells. Positive clones were confirmed with sanger sequence. These plasmids were co-transfected into HEK293T with psPAX2, pMD2.G with PEI reagents (Polysciences 23966). Supernatants containing virus particles were collected at 48 and 72 hours and infected DMG cells. After 48 hours of lentiviral infection, cells were selected with 2 μg/ml puromycin for 5 days prior to the in vitro assay.

Clonogenic survival assay

Six-well tissue culture plates were seeded with 400–10,000 cells and allowed to adhere for 12 hours. The modified cells with BRD4 shRNAs or sgRNAs, or unmodified cells treated with 50 nM AZD5153 or 50-100 nM JQ1 alone were irradiated at dose of 0.5, 1, 2, 3, 4, 6, and 8 Gy. Radiation was delivered by gamma irradiator. Cells were incubated at 37°C for two weeks after which colonies were counted following staining with 0.05% crystal violet. Plating efficiencies were calculated as the ratio of the number of colonies formed to the number of cells seeded. Colonies of >50 cells were used to indicate surviving fractions. Surviving fractions were calculated as the plating efficiency of treated cells divided by the plating efficiency of control cells. Dose enhancement factors (DEFs) were calculated the ratio of the dose with radiation alone, divided by the dose with radiation and BRD4 inhibition at 10% survival.

DNA repair assays

Green fluorescent protein (GFP)-reconstitution reporter cassettes for detection of homologous recombination (HR) and non-homologous end joining (NHEJ) have been previously reported (29, 34).
Plasmids containing HR or NHEJ reporter cassettes were linearized and transfected into cells to measure HR or NHEJ as a function of GFP expression. Transfections were performed using Lipofectamine 2000 (ThermoFisher 11668027). Cells with integrated reporter constructs were selected by adding 1mg/mL geneticin (ThermoFisher 10131–035). HR or NHEJ cassette expressing cells were treated with 1 μM of AZD5153 for 72 hours, then transfected with a mixture of 5 μg ISceI expressing plasmid and 2 μg pDsRed2-N1 (Clonetech 632406). Four days following transfection, cells were harvested, suspended in PBS and placed on ice. Cells were then analyzed on a FACS LSR Fortessa. Cells expressing either GFP, pDsRed2-N1, or no fluorescent protein were used as calibration controls. Data were analyzed using FlowJo software (FlowJo, LLC). DNA repair efficiency was determined as a ratio of GFP+ / DsRed+ cells normalized to 100% of vehicle control (DMSO).

Comet assay

Cells were treated with 500 nM of AZD5153 or 0.5% DMSO followed by 4Gy irradiation, and alkaline comet assays were performed (54). Briefly, 10,000 cells were resuspended in 75 μl of 0.5% (w/v) low-melting-point agarose and pipetted on slides precoated with 1.5% (w/v) normal-melting-point agarose. Coverslips were placed on top to spread the cell suspension evenly, and the slides were incubated on ice for 10 minutes. Next, the slides were slowly immersed into ice-cold, freshly made lysis buffer for 1 hour. After removal from the lysis buffer, the slides were placed into an electrophoresis tank filled with alkaline buffer (4 °C) for 20 minutes and electrophoresis was performed for 20 minutes at 300 mA. Slides were then removed, and drops of neutralization buffer were added three times. Finally, the slides were stained with propidium iodide (20 μg/ml). Images were observed under a fluorescence microscope. The DNA damage in >50 cells for each experimental condition was quantified by determining the tail moment, a function of both the tail length and the intensity of the DNA in the tail relative to the total DNA, by using OpenComet Score software (55).

RNA sequencing and analysis
Cells were cultured as described above and treated with vehicle or 1 μM AZD5153 for 24 and 48 hours. RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Paired-end (150bp) FASTQ files for the AZD5153-treated and DMSO-treated samples were obtained from Novogene and read quality and absence of adapter sequences were verified using FastQC. Single-end (50bp) FASTQ files for JQ1-treated and DMSO-treated samples were obtained from NCBI GEO (GSE78801, SRA SRP071040). FASTQ files were aligned to the hg38 genome using RNA-STAR (56) and aligned reads were counted using HTSeq-count (57). HTSeq-count files were imported into R (https://www.r-project.org/) and differential expression analysis was performed with the DESeq2 package (58) using default settings. For the combined JQ1 and AZD5153 analysis, the samples were normalized to stably expressed genes to reduce batch effect (ENSG00000085978, ENSG00000103275, ENSG00000110442, ENSG00000157764, ENSG00000169951, ENSG00000170832, ENSG00000235859). DESeq2 normalized reads were imported into GSEA v4.2.3 (59) and GSEA was run using MSigDB v2023.1 with the following parameters: permutations = 1000, permutation type = gene set, enrichment statistic = weighted, gene ranking metric = signal2noise, max size = 500, min size = 15, normalization mode = meandiv. Gene Ontology analysis was performed using ShinyGO 0.76.2 (60). Principal component analysis was conducted in R using the pca3d package and heatmaps were generated using the ComplexHeatmap R package (61). Enriched genes from GO or GSEA were used to generate violin plots. Violin and volcano plots were generated in R using ggplot2.

**Cleavage Under Targets and Release Using Nuclease (CUT & RUN) assay**

CUT & RUN was performed as previously described (62, 63). Cells were treated with 500 nM of AZD5153 or 0.5% DMSO for 48 hours, and 500,000 cells were harvested and fixed with freshly prepared 0.5% PFA for 2 minutes. After stopping the cross-linking using 500 μL 2.5 M glycine, cells were washed 3 times with digitonin wash buffer (20 mmol/L HEPES, pH 7.4, 150 mmol/L NaCl, 0.5 mmol/L spermidine, protein inhibitor cocktail, 0.02% digitonin, 0.05% SDS, and 1% Triton X-100) and bound to Concaavalin A–coated beads prewashed with binding buffer. Cells on beads were then incubated with antibodies on a
nutator overnight at 4°C [1:500 for H3K27 acetylation (H3K27ac) antibody (Cell Signaling 8173)] After overnight incubation, cells were washed 3 times with 0.02% digitonin wash buffer, resuspended in 100 μL digitonin buffer containing Protein A-Micrococcal Nuclease fusion protein (pA-MNase) and second antibody complex assemblies (pA-MNase and second antibodies were preassembled at a 2:1 ratio in 50% glycerol at 4°C for 1 hour) and nutated at 4°C for 1 hour. After pA-MNase–second antibody binding, cells were washed 3 times with digitonin wash buffer for 5 minutes. Finally, cells were resuspended in 100 μL icecold digitonin buffer in a heating block precooled in the ice water bath. Digestion of the chromatin was initiated by the addition of 2 mmol/L CaCl2 in the tube and lasted for 60 minutes at 0°C. The digestion was stopped by addition of 100 μL 2× STOP buffer (340 mmol/L NaCl, 20 mmol/L EDTA, 4 mmol/L EGTA, 0.02% digitonin, 100 μg/mL RNase A, and glycogen 50 μg/mL). Digested DNA was released by incubation at 37°C for 30 minutes, and the supernatant was collected. Then, 5 μL proteinase K and 200 μL 2× elution buffer [20 mmol/L Tris-HCl (pH = 8.0), 300 mmol/L NaCl, 20 mmol/L EDTA, 2% SDS, 10 mmol/L DTT] was added to each sample for reverse cross-linking at 65°C overnight. DNA was extracted by phenol–chloroform and dissolved in 12 μL low EDTA TE buffer. Libraries were prepared by the ACCEL-NGS 1S plus DNA library kit, and samples were sequenced using an Illumina NextSeq 500 platform. Paired-end (100bp) FASTQ files were obtained from MedGenome. Adapter and quality trimming, alignment, normalization (CPM), and peak calling (SEACR) were performed using the nf-core cutandrun pipeline (64). Consensus peaks were imported into R and differential analysis was conducted using DiffBind v3.6.5. Peak annotation and generation of pie charts to visualize genomic peak locations were performed using ChIPseeker v1.22.1 (65). The peaks shown in Figure 6b were identified from a previous publication (15).

FASTQ files for two H3K27M ChIP-seq samples and corresponding control samples were obtained from GEO series GSE78801 and were processed using the nf-core chipseq pipeline with default parameters. Consensus peaks were filtered to include the top 5000 enriched regions. These regions were used to generate heatmaps and metaplots for AZD5153- and DMSO-treated samples using computeMatrix (referencePoint option), plotHeatmap, and plotProfile from deeptools v3.5.1 (66).
Statistics

Survival plots were generated and analyzed using the Kaplan-Meier method and Graph-Pad Prism v9.5 software (GraphPad Software, San Diego, CA). Differences between survival plots were estimated using a log-rank test with Holm adjustment. For other analyses, one-way ANOVA was applied for multiple group comparison with a post-hoc Tukey’s test and a two-tailed unpaired t-test for comparison in 2 groups using the Prism software.

Study approval

All animal protocols were approved by the Northwestern University Institutional Animal Care and Use Committee.

Data availability

All data are available from the corresponding author and are provided in the Supplemental Supporting Data Values file. RNA sequencing and CUT & RUN sequencing data were deposited and available in Gene expression omnibus (GSE236598).

AUTHOR CONTRIBUTIONS

J.W., M.C., N.T., N.M., Z.Z., O.J.B., and R.H. designed the study. J.W. performed the majority of the experiments and J.W. and R.H. wrote the manuscript. J.W., T.S., E.U., K.A., Y.I., O.J.B., and R.H. performed and analyzed the in vivo experiments. J.W. and Y.M. performed CUT & RUN. M.C. and M.J.G performed and analyzed the high-throughput drug screening. S.G. performed all bioinformatics analyses and provided interpretation of the data. J.W., T.S., E.U., S.K., and N.T. performed and analyzed the apoptosis assay and interpreted the data. J.W., T.S., E.U., and Y.I. performed and analyzed the immunohistochemistry studies. O.J.B provided clinical supervision in the interpretation of data. Z.Z., M.N., O.J.B. and R.H. provided supervision in the interpretation of data. All authors commented on the manuscript and approved the included data.
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REFERENCES


43. Lam FC et al. BRD4 prevents the accumulation of R-loops and protects against transcription-replication collision events and DNA damage. Nat Commun. 2020;11(1):4083.


Figure 1.

A

Transmitted light  Hoechst nuclear stain  Spheroid track
PI dead cell stain  Hoechst/PI overlay

B

Neurosphere death (norm. to untreated)

No radiation  10Gy

No Radiation

10 Gy Radiation

C

Methotrexate

Normalized cytotoxicity

No drug  0.02μM  0.06μM  0.2μM  0.6μM  2μM  6μM

Normalized cytotoxicity

AZD5153

10Gy + Drug  Drug only

Normalized cytotoxicity

Temozolomide

Molibresib (I-BET762)
**Figure 1. High-throughput drug screening with radiation identified BET bromodomain inhibitors as radiosensitizers in DMG cells.** Tumor cells isolated from GEMM-DMG (Ntv-a; p53fl/fI; PDGFB; H3.3K27M; Cre) were cultured ex vivo as neurospheres and used to drug screen for radiosensitizers. (a) Representative image of neurospheres in transmitted light, Hoechst staining (nuclear), PI staining (dead cell), and Hoechst/PI overlay. Evaluation of the number, area, and dead cell intensity of neurospheres. (b) A library of 1,280 FDA approved drugs and 1,600 clinical candidates was screened in the presence or absence of 10 Gy radiation. Left: Compounds to the right of the dark green diagonal were indicated as radiosensitized neurospheres ≥ 3σ beyond the additive drug with 10 Gy radiation. Compounds to the right of the light green diagonal indicated as the neurospheres for additive drug with 10 Gy radiation. Right: Representative images of neurospheres treated with BET bromodomain inhibitor (BRDi) in the presence or absence of 10 Gy radiation. (c) Radiosensitizing effect (orange) and cytotoxic effect (gray) with Methotrexate, AZD5153, Temozolomide, and Molibresib (I-BET762).
Figure 2.

A

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B

SF8628 and DIPG007 normalized OD490 over days post treatment: [Graphs showing differences between control and sgBRD4-1, sgBRD4-2, sgBRD4-3 treatments with statistical significance indicated with asterisks.]

C

BRD4 KO

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sgBRD4

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Figure 2. BRD4 depletion suppressed cell growth in DMG cells. (a) Western blotting results showing sgRNAs-mediated depletion of BRD4 expression (sgBRD4-1, 2, 3) in SF8628 cells (left) and DIPG007 (right). (b) Cell growth plot showing anti-proliferative effects of sgBRD4-1, 2, 3 in SF8628 cells and DIPG007. The plot represents the absorbance quantification [optical density (OD), \( \lambda = 490 \) nm] measured each day (left). Values shown are the average (mean ± SEM) from triplicate samples for each condition as day 1 normalized. Dot plot representation of OD 490 values on day 5 (right). Statistical analysis was performed using one-way ANOVA comparisons: SF8628, sgBRD4-1 ***\( P = 0.0001 \); sgBRD4-2 ***\( P = 0.0002 \); sgBRD4-3 ****\( P < 0.0001 \); DIPG007, sgBRD4-1 *\( P = 0.0261 \); sgBRD4-2 *\( P = 0.0113 \); sgBRD4-3 **\( P = 0.0033 \). n = 3 (c) BRD4 depletion for colony forming effect on cells. Bar graph representation of colony numbers in DMG cells (right). Values shown are the average (mean ± SEM) from triplicate samples for each condition. One-way ANOVA comparisons between the control and BRD4 depletion: **** indicates \( P < 0.0001 \); sgBRD4-2 ***\( P = 0.0004 \). (d) BrdU incorporation assay of BRD4 depletion effects. Cells were pulsed with 10\( \mu \)M BrdU for one hour and treated with Alexa Fluor 488 BrdU antibody and 7-Aminoactinomycin D (7-ADD) (left). Bar graph representation of BrdU-positive cell numbers (right). Values shown are the average (mean ± SEM) from duplicate samples for each incubation condition. S phase cell population were analyzed with one-way ANOVA comparisons of each BRD4 sgRNA: **** indicates \( P < 0.0001 \); SF8628, sgBRD4-1 **\( P = 0.0017 \); sgBRD4-2 **\( P = 0.0050 \); sgRNA3 **\( P = 0.0072 \); DIPG007, sgBRD4-1 ***\( P = 0.0001 \).
Figure 3.

A

Cell viability (%) vs Concentration (μM)

Molobisib
PLX5117
BMS986158
AZD5153
JQ-1

B

Cell viability (%) vs AZD5153 (μM)

SF8628
DIPG007
SU-DIPG36
SU-DIPG4
GEMM-DMG
ASTRO-KM
ASTRO-WT
NHA

C

SF8628
DIPG007
SU-DIPG36
SU-DIPG4
GEMM-DMG

Days post treatment vs OD490

Control
AZD5153

D

SF8628
DIPG007
SU-DIPG36
SU-DIPG4
GEMM-DMG

AZD5153

% Colonies vs Control

****
*
Figure 3. BET bromodomain inhibitors suppressed cell growth in DMG cells. (a) Cell growth plot showing anti-proliferative effects of clinical grade BET bromodomain inhibitors (Molibresib, PLX5117, BMS986158, AZD5153, JQ-1) at 0-4 µM in SF8628 cells. Values shown are the average (mean ± SD) from triplicate samples for each incubation condition. (b) Graph showing proliferation response of DMG cells (SF8628, DIPG007, SU-DIPG36, SU-DIPG4, GEMM-DMG), Astro-KM, Astro-WT, and NHA cells to increasing concentration of AZD5153. Values shown are the average (mean ± SEM) from triplicate samples for each incubation condition. (c) Cell growth plot showing proliferation response to IC₅₀ values of AZD5153 of SF8628, DIPG-007, SU-DIPG36, SU-DIPG4, GEMM-DMG cells at each time point. The plot represents an optical density (OD) value at 490 nm. Values shown are the average (mean ± SEM) from triplicate samples for each condition. Dot plot representation of OD 490 values on day 5. Statistical analysis was performed using a two-tailed unpaired t-test: SF8628, **P = 0.0029; DIPG007, ****P < 0.0001; SU-DIPG36, **P = 0.0034; SU-DIPG4, *P = 0.0040; GEMM-DMG, **P = 0.0025. (d) Colony forming effect on cells treated with IC₅₀ values of AZD5153. Bar graph representation of colony numbers in the DMG cells treated with DMSO (0.5%) or IC₅₀ values of AZD5153. Values shown are the average (mean ± SEM) from triplicate samples for each condition. Unpaired t-test values for comparisons between the absence and presence of AZD5153 (n=3): SF8628, ****P < 0.0001; DIPG007, *P = 0.0109; SU-DIPG36, **P = 0.0038; SU-DIPG4, **P = 0.0037; GEMM-DMG, ***P = 0.0002.
Figure 4.

A

SF8628

DIPG007

GEMM-DMG

Surviving Fraction

0.01

0.1

1

Dose (Gy)

DEF = 1.22

DEF = 1.32

DEF = 1.10

IR

AZD5153+IR

IR

AZD5153+IR

IR

AZD5153+IR

B

SF8628

DIPG007

GEMM-DMG

BroU

7-ADD

Control

AZD5153

IR

AZD+IR

C

SF8628

DIPG007

GEMM-DMG

Annexin

PI

Control

AZD5153

IR

AZD+IR
Figure 4. BET bromodomain inhibition increased radioresponse and apoptosis in DMG cells. (a) Clonogenic survivals for K27M-mutant DMG cells (SF8628, DIPG007, GEMM-DMG) treated with AZD5153 (50nM for SF8628 and DIPG007, 10nM for GEMM-DMG) for 12 hours before ionized radiation (IR). Survival fractions, shown as mean ± SEM based on averages from triplicate samples, were normalized to plating efficiency. Dose Enhancement Factor (DEF) was calculated at 10% survival level. (b) Effects of AZD5153 and IR on cell proliferation using BrdU incorporation assay. Cells were treated with 500 nM AZD5153 in the presence or absence of 4Gy IR for 48 hours, pulsed with 10 μM BrdU for one hour, then, analyzed by flow cytometry. Cell sorting scatter plots for vehicle control (0.5% DMSO), AZD5153, and IR treated cells are shown (left), with graphs showing S-phase composition (right). One-way ANOVA comparisons between each treatment (n=3): **** indicates $P < 0.0001$; control vs AZD5153, ***$P = 0.0005$ (SF8628), **$P = 0.0065$ (GEMM-DMG); AZD5153 vs IR + AZD5153, **$P = 0.0020$ (SF8628), ***$P = 0.0002$ (GEMM-DMG). (c) Annexin V analysis of AZD5153 apoptosis effects. Cells were treated with vehicle control (0.05% DMSO) or 1 μM AZD5153 concurrently with and without 6 Gy IR. Cells were collected after 48 hours and treated with Alexa Fluor 488 Annexin V and flow sorted. Bar graph representation of Annexin V-positive cell numbers (right). One-way ANOVA comparisons of each treatment (n=3): **** indicates $P < 0.0001$; control vs AZD5153, *$P = 0.0115$ (SF8628), **$P = 0.0076$ (DIPG007), **$P = 0.0030$ (GEMM-DMG); control vs IR, *$P = 0.0124$ (SF8628), **$P = 0.0014$ (DIPG007), *$P = 0.0245$ (GEMM-DMG), AZD5153 vs AZD5153 + IR, **$P = 0.0027$ (SF8628), ***$P = 0.0029$ (DIPG007), *$P = 0.0177$ (GEMM-DMG), IR vs. AZD5153 + IR, **$P = 0.0027$ (SF8628), **$P = 0.0029$ (DIPG007), **$P = 0.0022$ (GEMM-DMG).
Figure 5. BET bromodomain inhibition altered gene expression in DMG cells. (a) Principal component analysis (PCA) of RNA-seq in SF8628 DMG cells treated with 1 µM AZD5153 and 0.5% DMSO (triplicates each time point), or 300 nM JQ1 and 0.5% DMSO (duplicates each time point), for 24 and 48 hours. (b) Heatmap generated from RNA-seq data, showing differentially expressed genes (padj < 0.05) in SF8628 DMG cells treated with 1 µM AZD5153 and 0.5% DMSO (triplicates each time point), or 300 nM JQ1 and 0.5% DMSO (duplicates each time point), for 24 and 48 hours. Black lines within vertical bars to the left indicate genes involved in DNA repair and cell cycle pathways. (c) Volcano plot of SF8628 DMG cells treated with 1 µM AZD5153. AZD5153-treated samples are shown as colored dots by associated pathways (x-axis: log₂ Fold Change; y-axis: –log₁₀ padj values). (d) GSEA pathway analysis in AZD5153 treated SF8628 DMG cells. Significantly down-regulated (FDR < 0.001, upper panel) and up-regulated (Macroautophagy: FDR < 0.001, Glycolysis: FDR = 0.021, lower panels) pathways.
Figure 6.

A

- **Down Regulated Genes**
  - Cell cycle proc.
  - Cell cycle
  - Mitotic cell cycle proc.
  - Mitotic cell cycle
  - Cell division
  - DNA metabolic proc.
  - Cytoskeleton organization
  - Chromosome organization
  - Cellular response to DNA damage stimulus
  - DNA replication
  - DNA repair
  - Spindle organization
  - Chromosome segregation
  - Circulatory system development
  - Tube development
  - Reg. of cell cycle phase transition
  - Sister chromatid segregation
  - Double-strand break repair
  - Tube morphogenesis
  - Reg. of cell cycle proc.

- **Up Regulated Genes**
  - Intracellular transport
  - Protein transport
  - Establishment of protein localization
  - Cellular protein localization
  - Cellular macromolecule localization
  - Processes utilizing autophagic mechanisms
  - Autophagy
  - Macrophagy
  - Organonitrogen compound catabolic proc.
  - Plasma mem bounded cell projection org
  - Macromolecule catabolic proc.
  - Cell projection organization
  - Intracellular protein transport
  - Cytosolic transport
  - Organelle localization
  - RDNA heterochromatin assembly
  - Nucleolar chromatin organization
  - Protein catabolic proc.
  - Nucleosome assembly
  - Organonitrogen compound biosynthetic pro

B

- **GO Cell Cycle**
  - Treatment: D650, AZ261/03
  - Normalized expression over time (24h, 48h)

- **GO DNA Repair**
  - Treatment: D650, AZ261/03
  - Normalized expression over time (24h, 48h)

- **GO Autophagy**
  - Treatment: D650, AZ261/03
  - Normalized expression over time (24h, 48h)

- **GO Catabolism**
  - Treatment: D650, AZ261/03
  - Normalized expression over time (24h, 48h)
Figure 6. BET bromodomain inhibition altered gene sets in biological pathways in DMG cells. (a) GO enrichment analysis of top 20 down-regulated pathways (upper panel) and up-regulated pathways (lower panel) in SF8628 DMG cells treated with 1 μM AZD5153. (b) Violin plots to compare the expression of the four gene signatures across conditions (upper left: cell cycle; upper right: DNA repair; lower left: autophagy, lower right: catabolism). Unpaired t-test values for comparisons each treatment: **** indicates $P < 0.0001$; Autophagy, ****$P = 0.00025$ for 24 hours, ****$P = 0.00015$ for 48 hours; Catabolism, ****$P = 0.00057$ for 24 hours, ****$P = 0.00032$ for 48 hours.
Figure 7. BET bromodomain inhibition altered genome-wide H3K37ac occupancy and transcription in DMG cells. CUT & RUN was performed using H3K27ac antibody in SF8628 DMG cells treated with 1 µM AZD5153 or 0.5% DMSO for 48 hours. (a) Pie charts showing the distributions of H3K27ac across the DMG genome. (b) Heatmaps showing H3K27ac occupancy in DMSO (left panel) vs. AZD5153 (right panel) treatment. Meta plots above indicate corresponding H3K27ac occupancy. Each plot is centered on the summit of the average occupancy and extended 5 kb upstream and downstream (-5 kb and +5 kb, respectively). Corresponding gene expression at the H3K27ac binding sites generated from RNA-seq are shown to the right. (c) Gene annotation tracks showing H3K27ac occupancy and gene expression for *BRCA1* (upper) and *RAD51* (lower) locus. The enhancer region was highlighted with a square in each gene.
Figure 8.

A

<table>
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<th>Control</th>
<th>AZD5153</th>
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<th>IR 24h</th>
<th>AZD + IR 24h</th>
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Average number of positive foci:

- γH2AX: ns, * for AZD5153
- 53BP1: ns, ** for AZD5153

B

- SF8628:
  - ![Image](image11.png)

- DIPG007:
  - ![Image](image12.png)
**Figure 8. BET bromodomain inhibition enhanced radiation-induced DNA damage in DMG cells.** (a) Effect of AZD5153 (1 μM) on γH2AX and 53BP1 foci formation in 6 Gy irradiated SF8628 DMG cells. Left: Representative images of nuclei from each treatment, showing γH2AX (upper) and 53BP1 (lower) foci. Right: Graph showing average number of γH2AX and 53BP1 foci/nucleus. Values shown are the average (mean ± SEM) from triplicate samples. One-way ANOVA comparisons between treatments: γH2AX, *p = 0.0368 between IR vs. AZD5153 + IR at 24 hours. 53BP1, **p = 0.0043 between IR vs. AZD5153 + IR at 24 hours. (b) Representative images of alkaline comet assay in SF8628 (left) and DIPG007 (right) DMG cells treated with AZD5153 following by IR. Bar graph showing value (mean ± SEM) from triplicate samples for each treatment for DNA damage grade sore in 50 cells (right). One-way ANOVA comparisons between treatments: **** indicates P < 0.0001; SF8628, control vs. IR, **p = 0.0010; AZD5153 vs. IR, **p = 0.0012; IR vs. AZD5153 + IR, **p = 0.0095. DIPG007, control vs. IR, **p = 0.0069; control vs. AZD5153 + IR, ***p = 0.0002; AZD5153 vs. IR, *p = 0.0117, AZD5153 vs. AZD5153 + IR, ***p = 0.0003; IR vs. AZD5153 + IR, *p = 0.0398.
Figure 9.

A

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B

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<tr>
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<tr>
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<td>GAPDH</td>
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C

HR

NHEJ

Control

AZD5153

GFP

Specimen 001 HR-005 (P)
Specimen 001 NHEJ-005 (P)

HR efficiency (\% control)

NHEJ efficiency (\% control)

***

****
Figure 9. BET bromodomain inhibition induced DNA damage and suppressed DNA repair in DMG cells. (a) Western blotting showing the effect of AZD5153 (0-10 µM) on expression of DNA repair marker: BRCA1, RAD51, and XRCC1, DNA damage maker: γH2AX, and H3K27ac, GAPDH, and total H3. (b) Western blot showing effects of AZD5153 (5 µM) on expression change over time after 6 Gy IR in SF8628 DMG cells. (c) DNA repair assay showing effect of AZD5153 (500 µM) on HR and NHEJ pathways in SF8628 DMG cells. Flow plots represent fluorescence signals from HR and NHEJ reporter cassettes. Repair efficiency represents the ratio of GFP+ to DsRed+ cells normalized to 100% of vehicle control (0.5% DMSO). Value (mean ± SEM) shown are based on averages from quadruplicate samples. Unpaired t-test values for comparisons between control and AZD5153 samples: ***P = 0.0004 (HR), ****P < 0.0001 (NHEJ).
Figure 10.

A

Cell Implantation

Bioluminescence Image

Day 0

RT

BRD inhibitor

Day 34 → 48

B

D53

Control

AZD5153

RT

RT+AZD

Percent survival (%)

0 50 60 70 80 90 100

Days after implantation

C

D49

Control

JQ1

IR

IR+JQ1

Percent survival (%)

0 50 60 70 80 80

Days after implantation

D

AZD5153

RT

Ki-67

TUNEL

Control

AZD

RT

AZD+RT
Figure 10. BET bromodomain inhibition enhanced radiation anti-tumor activity in DMG PDX models. (a) Experimental design for in vivo efficacy study of BET bromodomain (BRD) inhibitor in combination with radiation therapy (RT) in DMG animal models. (b, c) Mice with SF8628 PDXs were randomized to four treatment groups: control (DMSO, n=11), AZD5153 (50mg/kg) or JQ1 (30mg/kg) alone (n=12), RT alone (n=10 for AZD study, n=11 for JQ1 study), and AZD5153 + RT (n=10) or JQ1 + RT (n=11). Left: Dot plot representation of bioluminescence values on day 53 (AZD study) and day 49 (JQ1 study). One-way ANOVA comparisons between treatments: AZD study, control vs. AZD5153, \( *P = 0.0329 \); control vs. RT, \( **P = 0.0038 \); control vs. AZD5153 + RT, \( **P = 0.0016 \). JQ1 study, control vs. RT, \( *P = 0.0128 \); control vs. JQ1 + RT, \( **P = 0.0083 \). Middle: Tumor bioluminescence overlay images. Right: Corresponding survival plots for each treatment. Statistical analysis using a log-rank test: **** indicates \( P < 0.0001 \); AZD study, control vs. AZD5153, \( ***P = 0.0007 \); control vs. RT, \( ***P = 0.0002 \); RT vs. AZD5153 + RT, \( **P = 0.0072 \). JQ1 study, control vs. RT, \( **P = 0.0038 \); JQ1 vs. JQ1 + RT, \( ***P = 0.0006 \); RT vs. JQ1 + RT, \( *P = 0.0136 \). (d) Ki-67 and TUNEL staining for intracranial tumor at the end of treatment. Value (mean ± SEM) representing the average of positive cells in four high-powered fields in three tumor samples (n=3, right). One-way ANOVA comparisons between treatments: Ki-67, control vs. AZD5153, \( **P = 0.0042 \); control vs. RT, \( **P = 0.0068 \); control vs. AZD5153 + RT, \( **P = 0.0002 \); RT vs. AZD5153 + RT, \( *P = 0.0355 \). TUNEL, control vs. RT, \( **P = 0.0077 \); control vs. AZD5153 + RT, \( ****P < 0.0001 \); AZD5153 vs. AZD5153 + RT, \( ***P = 0.0001 \); RT vs. AZD5153 + RT, \( **P = 0.0024 \).
Figure 11.

A

High-throughput drug screening

Drug treatment with radiation → Evaluation
Sphere number
Sphere area
Dead cell intensity
BRDi (BET inhibitors)

Epigenetic regulation of DNA repair genes

control

enhancer

RAD51, BRCA1

BRDi

enhancer

RAD51, BRCA1

B

Epigenetic inhibition of DNA repair genes

Histone H3
acetylation

BRDs

RAD51

BRCA1

BRDi

BET inhibitor

Enhanced Radiation
Figure 11. Working model. (a) High-throughput drug screening. BET bromodomain inhibitors (BRDi) were identified as radiosensitizers using high-throughput drug screening in the DMG cells treated with radiation. BRDi decreased H3K37ac occupancy at enhancer regions, which leads to suppressed transcription involving DNA repair in DMG cells. (b) Epigenetic inhibition of DNA repair genes. BRDi disrupts the interaction between acetylated histone (Ac) and BRDs to inhibit active transcription for the genes involving radiation-induced DNA damage repair, results in enhancing radiation effect in DMG.