Radiosensitization by Histone H3 Demethylase Inhibition in Diffuse Intrinsic Pontine Glioma

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Abstract

Purpose: Radiotherapy (RT) has long been and remains the only treatment option for diffuse intrinsic pontine glioma (DIPG). However, all patients show evidence of disease progression within months of completing RT. No further clinical benefit has been achieved using alternative radiation strategies. Here, we tested the hypothesis that histone demethylase inhibition by GSK-J4 enhances radiation-induced DNA damage, making it a potential radiosensitizer in the treatment of DIPG.

Experimental Design: We evaluated the effects of GSK-J4 on genes associated with DNA double-strand break (DSB) repair in DIPG cells by RNA sequence, ATAC sequence, and quantitative real-time PCR. Radiation-induced DNA DSB repair was analyzed by immunocytochemistry of DSB markers γH2AX and 53BP1, DNA-repair assay, and cell-cycle distribution. Clonogenic survival assay was used to determine the effect of GSK-J4 on radiation response of DIPG cells. In vivo response to radiation monotherapy and combination therapy of RT and GSK-J4 was evaluated in patient-derived DIPG xenografts.

Results: GSK-J4 significantly reduced the expression of DNA DSB repair genes and DNA accessibility in DIPG cells. GSK-J4 sustained high levels of γH2AX and 53BP1 in irradiated DIPG cells, thereby inhibiting DNA DSB repair through homologous recombination pathway. GSK-J4 reduced clonogenic survival and enhanced radiation effect in DIPG cells. In vivo studies revealed increased survival of animals treated with combination therapy of RT and GSK-J4 compared with either monotherapy.

Conclusions: Together, these results highlight GSK-J4 as a potential radiosensitizer and provide a rationale for developing combination therapy with radiation in the treatment of DIPG.

Introduction

Diffuse midline glioma (DMG) with H3K27M mutation, resulting in substitution of lysine 27 with methionine (K27M) in the genes encoding histone H3 protein, is a newly defined entity that comprises all diffusely infiltrating astrocytic neoplasms affecting midline structures of the central nervous system (1). The pontine category of DMG, diffuse intrinsic pontine glioma (DIPG), mainly affects children of 4 to 7 years of age, presenting with cranial nerve deficits, balance issues, and other brainstem signs and symptoms (1–3). DIPGs are among the most devastating childhood tumors with a median survival of 9 to 12 months from diagnosis. Investigations into curative therapies have been extremely challenging, and over 250 clinical trials involving different combinations of chemotherapeutic agents commonly used in glioma treatment have demonstrated no response in DIPG (3). As their predilection for the brainstem precludes surgical resection, fractionated focal radiotherapy to a total dose of 54 to 60 gray (Gy) over a 6-week period remains the only treatment modality that can alleviate symptoms. This provides a “honeymoon period” of temporary symptomatic relief and a delay in tumor progression in about 70% to 80% of DIPG patients. No further clinical benefit has been achieved using alternative radiation strategies or combination therapies with radiation sensitizers (4, 5). Unfortunately, no effective salvage...
In addition to its antitumor activity, GSK-J4 resulted in significant changes in K27M DIPG cell transcriptional programs, demonstrating potent antitumor activity, and acts to restore K27 methylation in K27M DIPG cells, while previously shown that the JMJD3 demethylase inhibitor GSK-J4 cation of effective treatment outcomes for this patient population.

Establishment of SF8628 and SF9427 cell cultures from surgical specimens, and tumor cell modification for expression of firefly luciferase for in vivo bioluminescence imaging have been described (10–13). The DIPG-007 (K27M DIPG) cell line was kindly provided by Dr. Angel Montero Cardabos (Hospital Sant Joan de Déu, Barcelona, Spain). Human astrocytes expressing wild-type (Astro WT) or K27M H3F3A transgene (Astro KM) have been previously described (7, 10). GBM43 cell lines were established and propagated as subcutaneous xenografts, as previously described (10, 12). 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 nonessential amino acids (11140-050) from Thermo Fisher. SF9427 and DIPG-007 cell lines were grown adherently in tumor stem medium (TSM) base with 5% FBS. 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 nonessential amino acids (11140-050), GlutaMAX-I supplement (35050-061), and antibiotic-antimycotic (15240-069), B-27 supplement minus vitamin A (12587-010) from Thermo Fisher, 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 (STEM-Accord Biotech; 100-146). PDGF-A and PDGF-B (Shenandoah Biotech; 100-16 and 100-18), and 0.2% heparin (STEM-Accord Biotech; 100-146).

Materials and Methods

Translational Relevance

Diffuse intrinsic pontine glioma (DIPG) is one of the most devastating childhood cancers that limited response to radiotherapy (RT), resulting in median overall survival of approximately 10 months. There is a critical need for new therapeutics that enhance the radiation effect while enabling the use of lower dose of radiation to limit long-term side effects in young patients. DIPG pathogenesis involves histone H3 gene mutations and reduction of global H3K27 methylation that promotes tumor growth. We found that the JMJD3 demethylase inhibitor, GSK-J4, which restores H3K27 methylation, decreases expression of the genes involved in DNA damage repair, and enhances radiation-induced DNA damage by inhibiting homologous recombination repair, mediated by chromatin modification. The combination of GSK-J4 and RT, in human DIPG xenografts, had substantial growth inhibition and survival benefit, compared with either alone, supporting the possible use of GSK-J4 to increase RT antitumor effect while using lower doses of radiation.

Materials and Methods

Cell sources and propagation

Primary pediatric human glioma cell lines SF8628 (K27M DIPG) and SF9427 [H3 wild-type glioblastoma (GBM)] were obtained from the University of California, San Francisco medical center, and in accord with an institutionally approved protocol.

RNA seq was performed using the NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs; NEB E7490) and NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs; E7530) according to the manufacturer’s instructions. Briefly, first-strand cDNA was obtained from DNase 1-treated RNA samples from SF8628 and DIPG-007 cells treated with dimethyl sulfoxide (DMSO; Sigma D2650) for 0 hours (untreated) and 48 hours or GSK-J4 (6 μmol/L, R&D Systems; 4594) for 6, 24, 48, and 72 hours (two replicates per time point). Second-strand cDNA synthesis was performed followed by purification using 1.8 × Agencourt AMPure XP Beads (Beckman Coulter A63881). The cDNA library was end prepared, followed by immediate adapter ligation and ligation reaction purification. Following PCR enrichment and reaction purification, the library quality was assessed on a Bioanalyzer (Agilent High Sensitivity Chip). We used STAR 2.4 to align the RNA-sequencing samples to the reference genome (hg19) and counted the number of reads mapping to each gene in the ensemble GRCh37 gene model. Differential expression between the different groups was performed through the use of DESeq2 (14). Hierarchical clustering of the resulting matrix was done in R using heatmap2.

Quantitative real-time PCR (qPCR)

Six-well tissue culture plates were seeded with 10,000 cells. The cells were allowed to adhere for 16 hours and then treated with DMSO or 6 μmol/L of GSK-J4 for 72 hours, followed by radiation at 6 Gy. Radiation was delivered by gamma irradiator. Cells were collected at 6 hours following radiation. Total RNA was extracted using the RNAeasy Mini kit (Qiagen; 74104), and 2 μg of RNA was

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reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad; 17088890). Real-time detection and quantification of cDNA were performed with the iCycler instrument (Bio-Rad; 1861096). qPCR was conducted using SYBR@GreenER qPCR Supermixes (Bio-Rad; 1725270). Fluorescence data were collected at annealing stages, and real-time analysis was performed with iCycler iQ Optical System Software V3.0a. Gene expressions were normalized with that of GAPDH endogenous gene control. All experiments were repeated at least three times. For data analysis, the fold change in the target gene relative to the GAPDH was determined by fold change = 2^(-ΔΔCT), where ΔΔCT = ΔCTtarget – ΔCTGAPDH and ΔCT = ΔCTtreatment – ΔCTcontrol. Forward (F) and reverse (R) primers used for qPCR were 5'-GGCGTGAACCTCACAGAT-3' (F) and 5'-TCTTCTCGGTGTTGCTCTC-3' (R) for PCNA, 5'-CAGCCCTACAGCAAGGAATC-3' (F) and 5'-GGTGTGACTGGGATCTCCT-3' (R) for XRCC1, 5'-GGTGCAGAGCTACGAGAAGG-3' (F) and 5'-ATGAAGTCCCCTTCTGTAGC-3' (R) for POLE1, 5'-CCTGGCCITTFTGAAGTCTCC-3' (F) and 5'-CTGCCCACTGCTGAAAAT-3' (R) for FANCA, and 5'-CCACGACACACCTGTACCC-3' (F) and 5'-GCCATGACTGTGGTCAATGAG-3' (R) for GAPDH. All primers were designed using Primer 3 (http://frodo.wi.mit.edu/primer3/).

ATAC-sequence library preparation and analysis

We submitted 80,000 cells for ATAC sequence according to the manufacturer's instructions (Active Motif). On average, 35 million paired-end 42 base pair (bp) sequencing reads per sample were generated by Illumina sequencing (using NextSeq 500). The paired-end reads were mapped to the reference genome (hg19) using the bowtie2 algorithm. The overall alignment rate was over 99%. We used MACS1.4 to call the binding peaks (15). Normalized sample count matrix was used as input for DESeq2 (14) to call differential binding peaks between GSK-J4 (6 µM) and DMSO treated samples. We assigned the peaks according to the nearest genes using R-ChIPseeker package and separated the peaks into two broad categories: namely, TSS-associated peaks, including peaks within promoter or 5'UTR, and enhancer-associated peaks, including peaks within distal, downstream, 3'UTR region. TSS/Enhancer peaks with at least 10 reads support were used for GSEA analysis (16). GSEA analysis was applied in weighted mode against the gene sets collection in MSigDB (v5.1). Gene sets with over 5,000 genes or less than 10 genes were excluded for further analysis. Each gene set was permuted 1,000 times to calculate P value and FDR values.

Immuno blotting

Cells were cultured as described above and treated with 6 µmol/L of GSK-J4 for 72 hours. Control cells and treated cells were irradiated with 6 Gy and cell pellets were collected at different time points of 1, 3, 6, 24, and 48 hours after radiation. Total cell lysate was collected from asynchronously proliferating cells in lysis buffer (Thermo Fisher; 89900) supplemented with 1% protease (Thermo Fisher; 78430) and 1% phosphatase (Thermo Fisher; 78420) inhibitor cocktails, resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and then immunoblotted using standard techniques. Transfer was conducted using 1 × NU-Page transfer buffer (Thermo Fisher; NP0006-1) with 10% methanol. The protein samples were transfected into Immu no-Blot PVDF membranes (Bio-Rad; 1620177) for 90 minutes at 40 V for phospho-histone H2A.X (γH2AX) and β-actin or 120 minutes at 30 V for phospho-Rad50 (pRad50) and Rad51. The membranes were blocked for 1 hour at room temperature in 5% skim milk in Tris-HCl buffered saline with 0.05% tween 20 (TBST), and then probed with primary antibodies (1:1,000 dilution) for overnight at 4°C, washed three times in TBST, incubated with horseradish peroxidase-conjugated secondary antibody (1:2,000 dilution), followed by washing in TBST and then in phosphate-buffered saline (PBS), and signals visualized by enhanced chemiluminescence (ECL) using 1:1 supernignal west pico plus substrate (Thermo Fisher; 34577) for pRad50 and Rad51, and 1:1 Pierce ECL. Western blotting substrate (Thermo Fisher; 32106) for γH2AX. The primary antibodies phospho-Rad50 (Ser635, #14223), Rad51 (D4B10, #8675), phospho-Histone H2AX (Ser139, 20E3, #9718), and β-actin (13E5, #4970) and secondary antibody (anti-Rabbit IgG, #7074P2) were obtained from Cell Signaling Technology.

DNA-repair assay

Green fluorescent protein (GFP)-reconstitution reporter cassettes for detection of homologous recombination (HR) and nonhomologous end-joining (NHEJ) have been previously reported (17). 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 (Thermo Fisher 11668027). Cells with integrated reporter constructs were selected by adding 1 mg/mL geneticin (Thermo Fisher 10131-035). HR or NHEJ cassette expressing cells were treated with 6 µmol/L of GSK-J4 for 72 hours, then transfected with a mixture of 5 µg Escl 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 FACS LSFortessa. Cells expressing either GFP, pDsRed2-N1, or no fluorescent protein were used as calibration controls. Data were analyzed using Flowlo software (Flowlo). DNA-repair efficiency was determined as a ratio of GFP+/DsRed+ cells normalized to 100% of vehicle control (DMSO).

Immunocytochemistry

Cells (50,000) were seeded into 8-well chamber slides and incubated with GSK-J4 (6 µmol/L) for 72 hours and then irradiated with 6 Gy. Four percent paraformaldehyde (PFA; Polysciences; 18814) was used to fix tumor cells on cover slips at 1, 6, and 24 hours after radiation. Slips were then rinsed in PBS and blocked with 0.3% Triton X-100 and 5% normal goat serum (Cell Signaling Technology; 5425) in PBS for 1 hour at room temperature. Coverslips were incubated overnight at 4°C with γH2AX (Ser139, 20E3; Cell Signaling Technology; 9718) or 53BP1 (Cell Signaling Technology; 4937) antibody at 1:250 dilution or RAD51 (Abcam ab63801) antibody at 1:100 dilution in PBS containing 0.3% Triton X-100 and 5% normal goat serum followed by 15-minute incubation at room temperature in anti-rabbit antibody Alexa Fluor 488 (Cell Signaling Technology; 4412) or anti-rabbit antibody Alexa Fluor 555 (Cell Signaling Technology; 4413) at 1:1,000 dilution in PBS with 5% normal goat serum for single reporter labeling. The coverslips were rinsed in PBS 4 times, and nuclei were stained by incubating the coverslips at room temperature in the dark with 1:500 4',6-diamidino-2-phenylindole (DAPI; Cell Signaling Technology; 8961) diluted in PBS, followed by additional rinses in PBS and sterile water. The cover slips were then mounted on glass slides using Vectashield (Vector Laboratories; H-1200) and analyzed with a Carl Zeiss
Axioimager 2 microscope (Zeiss). Foci were counted in 100 cells per treatment condition.

**Clonogenic survival assay**

Six-well tissue culture plates were seeded with 400 to 10,000 cells. The cells were allowed to adhere for 16 hours and treated with GSK-J4 alone at concentrations of 0, 1, and 2 μmol/L for 72 hours, and 2.5 μmol/L of GSK-J4 for 24 or 72 hours, either before or after radiation at doses of 0.5, 1, 2, 3, 4, 6, 8, and 10 Gy. Radiation was delivered by gamma irradiator. Cells were incubated at 37°C for 3 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 ratio of the number of colonies formed to control. At 24 hours after RT, cells were treated with RT and GSK-J4. GSK-J4 treatments were initiated 72 hours before RT (GSK-J4 + RT). Vehicle only (DMSO) was used as a control. At 24 hours after RT, cells were fixed in 80% ethanol and stained with propidium iodide. Cells were then subjected to flow-cytometric analysis using a BD FACS Calibur instrument, and data were analyzed using FlowJo 8.8 software.

**Cell-cycle analysis**

GSK-J4 effects on cell-cycle distributions were determined by treating cells with 6 μmol/L of GSK-J4 for 72 hours and/or followed by radiation (6 Gy). DIPG cells (1.0 × 10⁶) were plated into 10-cm dishes and allowed to adhere overnight. Samples were then treated with GSK-J4 only, radiation (RT) only, or with RT and GSK-J4. GSK-J4 treatments were initiated 72 hours before RT (GSK-J4 + RT). Vehicle only (DMSO) was used as a control. At 24 hours after RT, cells were fixed in 80% ethanol and stained with propidium iodide. Cells were then subjected to flow-cytometric analysis using a BD FACS Calibur instrument, and data were analyzed using FlowJo 8.8 software.

**Xenograft studies**

Six-week-old female athymic mice (nu/nu genotype, BALB/c background) were purchased from Envigo and housed under aseptic conditions. Pontine injection of tumor cells was performed as previously described (10, 13). Each mouse was injected with 1 μL SF8628 DIPG cell suspension (100,000 cells/μL) into the pontine tegmentum at a depth of 5 mm from the inner base of the skull. Animals were randomized into four treatment groups: (i) control (DMSO, n = 9); (ii) GSK-J4 monotherapy (intraperitoneal injection of 100 mg/kg of GSK-J4 for 10 consecutive days, n = 9); (iii) radiation monotherapy (0.5 Gy, 3 times a week for 2 consecutive weeks for a total dose of 6 Gy, n = 9); and (iv) GSK-J4 and radiation combination therapy (n = 7). Biweekly bioluminescence imaging was used to monitor tumor growth and response to therapy as previously described (10, 13). Mice were monitored daily and euthanized at endpoint, which included irreversible neurologic deficit or body condition score less than 2.

**IHC**

Brains were collected from the mice at 3 hours following completion of the last treatment (n = 2 for each treatment). Paraformaldehyde-fixed brains were paraffin-embedded and sectioned (10 μm) for hematoxylin and eosiin (H&E). IHC was performed using an automated processor (DISCOVERY ULTRA; Ventana Medical Systems, Inc.). The primary antibodies used for IHC are as follows: PCNA (PC10, ab29, abcam, dilution 1:10,000), XRCC1 (33-2-5, ab1838, abcam, dilution 1:50), PolD1 (A304-005A, Bethyl, dilution 1:500), JMJD3 (AP1022A, ABDENT, dilution 1:40), H3K27me3 (C36B11, #9733, Cell Signaling Technology, dilution 1:200) and Ki67 (2 μg/mL, Ventana Inc.). To assay apoptotic response to treatment, TUNEL staining was performed using the DeadEnd Colorimetric TUNEL system (Promega) according to the manufacturer's protocol. All images were taken at 40× magnification.

**Statistical analysis**

Survival plots were generated and analyzed using the Kaplan–Meier method and GraphPad Prism v7.0 software (GraphPad Software). Differences between survival plots were estimated using a log-rank test with Holm adjustment (18, 19). For all other analyses, a two-tailed unpaired t test was applied using the Prism software.

**Study approval**

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

**Results**

**Effect of GSK-J4 on genes associated with DNA damage repair in K27M DIPG cells**

We have previously shown that GSK-J4 treatment causes a change in the expression of the genes that promote tumor growth in K27M DIPG (10). In our current RNA-seq analysis, we performed unsupervised principal component analysis of multiple samples derived from two K27M DIPG cell lines (SF8628 and DIPG-007) including DMSO collected at 0 and 48 hours and GSK-J4–treated samples collected at 0, 6, 24, 48, 72 hours following treatment. We found global gene-expression shift in GSK-J4–treated DIPG cells compared with DMSO treated samples (Supplementary Fig. S1A). In addition, we compared the RNA-seq data between SF8628 and DIPG-007 cell lines. The direction of gene-expression change between these two DIPG cell lines was highly correlated (2-sided Pearson correlation test P < 0.001, ***), suggesting the observed gene-expression changes are shared among DIPG cell lines (Supplementary Fig. S1B). We performed GSEA pathway analysis and found that DNA DSB repair pathways were among the most significantly downregulated in both SF8628 and DIPG-007 DIPG cell lines upon GSK-J4 treatment (Fig. 1A and B and Supplementary Table S1).

We verified the change in the DNA DSB repair gene expression by qPCR in irradiated K27M DIPG (SF8628, DIPG-007) and H3 wild-type GBM cells (SF9427) with and without GSK-J4 treatment (Fig. 1C). Radiation treatment (RT) increases the expression of DNA DSB repair genes including PCNA, XRCC1, FANCA, and POLD1 while GSK-J4 decreases the expression of these genes in K27M DIPG cells. Furthermore, radiation-induced DNA DSB repair gene expression was significantly reduced by combination treatment with GSK-J4 and RT. In contrast to K27M DIPG cell lines, the DNA DSB repair gene expression was not changed by GSK-J4 treatment in irradiated SF9427 H3 wild-type GBM cells (Fig. 1C).

To investigate the influence of GSK-J4 on genome-wide DNA accessibility, we performed the assay transposase accessible chromatin sequencing (ATAC-Seq) and found an overall concordance between ATAC-Seq and RNA-seq data. Based on ATAC-Seq, we sought to compare the overall accessibility between GSK-J4 treatment and DMSO treatment samples based on the identified binding peaks. We found a trend toward reduced DNA accessibility as indicated by the long-tail of marginally downregulated...
binding sites in SF8628 DIPG cells treated with GSK-J4 when compared with DMSO treated cells (Supplementary Fig. S1C). This is consistent with our previous study showing the chromatin-repressive role of GSK-J4 through increased H3K27 trimethylation and dimethylation in K27M DIPG cells (10). To investigate the enriched transcriptional factor among the decreased ATAC-Seq binding sites, we created a union of differential peaks between DMSO- and GSK-J4–treated samples ($P < 0.05$). We performed HOMER motif analysis in SF8628 DIPG cells and found a significant enrichment of DNA sequencing motifs involving neuron developmental transcriptional factors such as GLIS2, PITX1 (Supplementary Table S2). Interestingly, the promoters of two DNA-repair genes, PCNA and XRCC1, have diminished binding in the GSK-J4–treated SF8628 and DIPG-007 cells (Fig. 1D). Taken together, our results suggest that GSK-J4 promotes the transcriptionally silent state through an increase in H3K27 methylation and represses the genes involving DNA DSB repair in K27M DIPG cells.

GSK-J4 enhances radiation-induced DNA damage

To determine the effect of GSK-J4 on radiation-induced DNA damage, fluorescence immunocytochemistry of DSB markers
γH2AX and 53BP1 was examined to quantitate the extent of DNA damage in irradiated K27M DIPG cells (SF8628) and H3 wild-type GBM cells (SF9427) either in the presence or absence of GSK-J4. SF8628 K27M DIPG cells treated with GSK-J4 showed increase γH2AX and 53BP1 foci relative to untreated cells. However, GSK-J4 did not cause DNA damage in SF9427 wild-type GBM cells. In both SF8628 K27M DIPG and SF9427 wild-type GBM cells, γH2AX and 53BP1 foci increased 1 hour following radiotherapy, indicating increased DNA damage by irradiation. At 24 hours after irradiation, γH2AX and 53BP1 foci were largely reduced in those cells due to successful repair of the previously noted DNA damage. However, irradiated SF8628 K27M DIPG cells treated with GSK-J4 sustained high levels of γH2AX and 53BP1 at 24 hours compared with irradiated SF9427 wild-type GBM cells treated with GSK-J4 (Fig. 2A). This might be due to the lack of response to GSK-J4 inhibitory effect in SF9427 wild-type GBM cells, as previously reported (10). Furthermore, Western blotting revealed that radiation-induced γH2AX was increased and sustained until 48 hours in SF8628 K27M DIPG cells subjected to combination of GSK-J4 and radiation, unlike cells treated with radiation alone (Fig. 2B). These findings suggest that GSK-J4 uniquely inhibits DNA damage repair in irradiated K27M DIPG cells to sustain radiation-induced DNA damage.

GSK-J4 inhibits DNA damage repair through HR

DNA DSBs are repaired by two major pathways: via HR in late S and G₂ phase and via nonhomologous end-joining (NHEJ) throughout the cells cycle (20, 21). During HR, the missing information is copied into the DSB site from a homologous sequence, whereas during NHEJ joins, the broken ends are joined without homology (22). In order to quantify the DSB repair pathways, we used GFP-reconstitution reporter cassettes for detection of HR and NHEJ (16). Using reporter constructs for each DSB repair pathway, we studied the DNA-repair ability of SF8628 K27M DIPG cells and human astrocytes expressing K27M (Astro KM) and wild-type (Astro WT) H3F3A genes, in the presence and absence of GSK-J4. GSK-J4 exerted an inhibitory effect on the cells’ ability to repair DNA via the HR pathway while largely sparing the NHEJ pathway (Fig. 2C; Supplementary Fig. S2A). This inhibitory effect of GSK-J4 on DNA damage repair was observed in the cells expressing the K27M H3F3A gene (SF8628 DIPG and Astro KM) but not in the cells expressing the H3 wild-type H3F3A gene (Astro WT; Fig. 2C and Supplementary Fig. S2A).

To better understand the effect of GSK-J4 on DNA-repair dynamics, the cell-cycle distribution was evaluated in K27M DIPG (SF8628), Astro KM, and Astro WT cells treated with GSK-J4, radiation, and the combination of GSK-J4 and radiation (Supplementary Fig. S2B). In the SF8628 K27M DIPG and Astro KM cells, GSK-J4 treatment increased the S phase fraction (SF8628: 28.8%, Astro KM: 25.9%) relative to control (SF8628: 12.2%, Astro KM: 12.0%). Radiation increased G₂-M cell fraction (SF8628: 28.9%, Astro KM: 25.5%) relative to control (SF8628: 21.8%, Astro KM: 19.3%) or GSK-J4 alone (SF8628: 4.5%, Astro KM: 9.3%). Combination treatment of GSK-J4 and radiation also increased S phase fraction (SF8628: 21.2%, Astro KM: 22.4%) relative to radiation alone (SF8628: 11.2%, Astro KM: 17.4%). Thus, GSK-J4 maintained a high proportion of S phase fraction during response to irradiation in SF8628 K27M DIPG and Astro KM cells. In the Astro WT cells, GSK-J4 showed the least increase S phase fraction (13.4%) relative to control (11.2%).

Western blot analysis revealed that treatment with GSK-J4 prior to radiation causes a time-dependent reduction in the level of phosphorylated RAD50 and total RAD51 protein, which both play a major role in HR DNA DSB repair pathway, in SF8628 K27M DIPG cells (Fig. 2B). Fluorescence immunocytochemistry of RAD51 in SF8628 K27M DIPG cells showed that RAD51 foci formation increased in a time-dependent manner with peak at 24 hours following radiation (Supplementary Fig. S2C). GSK-J4 inhibited RAD51 foci formation at each time point, indicating inhibition of HR DNA damage repair by GSK-J4.

Effect of GSK-J4 on cell proliferation in combination with radiation

Next, we examined the effect of GSK-J4 on radiation response in K27M DIPG cells. Clonogenic survival assay was carried out using two K27M DIPG cell lines (SF8628 and DIPG-007) and H3 wild-type GBM cell line (SF9427). Cells were treated with GSK-J4 administered at either 24 or 72 hours before or after radiation. GSK-J4 treatment for 72 hours before radiation resulted in the largest enhancement of radiation effect compared with that of radiation-only treatment in K27M DIPG cells (SF8628: DEF of 1.34, DIPG-007: 1.25; Fig. 2D). Exposure of DIPG cells to GSK-J4 for either 24 or 72 hours after radiation showed the least radiation dose enhancement (SF8628: DEF of 1.08 for 24 hours and 1.08 for 72 hours; DIPG-007: DEF of 1.03 for 24 hours and 1.13 for 72 hours; Fig. 2D). GSK-J4 treatment for 24 hours before radiation was intermediate in its radiation-enhancing effect (SF8628: DEF of 1.19; DIPG-007: DEF of 1.19; Fig. 2D). There is a minor effect of GSK-J4 on enhancing the response to radiation in SF9427 (DEF between 0.93 and 1.06). GSK-J4 treatment for 72 hours in the absence of radiation inhibited the clonal growth of K27M DIPG cells, but had no effect on the clonal growth of H3 wild-type GBM cells (Supplementary Fig. S2D).

GSK-J4 enhances radiation antitumor effect in human DIPG xenografts

Based on the effects of GSK-J4 on radiation-induced DNA DSB repair and clonogenic survival in irradiated DIPG cells, we hypothesized that GSK-J4 treatment would enhance radiation antitumor activity to suppress tumor growth and increase survival benefit in mice with orthotopic human DIPG xenografts. To address this, mice with SF8628 K27M DIPG xenografts were treated with a daily dose of 100 mg/kg of GSK-J4 for 10 days in the presence or absence of radiation treatment (RT) at total dose of 3 Gy (0.5 Gy per day for 3 days a week for 2 weeks). GSK-J4 monotherapy resulted in reduced tumor growth rate and conferred substantial survival benefit against human DIPG xenograft model, as indicated by bioluminescence monitoring (Fig. 3A and B) and survival of treated mice (**, P = 0.0021; Fig. 3C), consistent with a previous study (10). Similarly, radiation monotherapy provided a significant benefit (**, P = 0.0416 in Fig. 3A; ***, P < 0.0001 in Fig. 3C). We found that combination treatment of DIPG xenografts with GSK-J4 and RT significantly increased therapeutic benefit, outperforming either monotherapy (GSK-J4 vs. GSK-J4 + RT: ***, P = 0.0429 in Fig. 3A; ***, P = 0.0014 in Fig. 3C; RT vs. GSK-J4 + RT: *, P = 0.0348 in Fig. 3C). This DIPG xenograft experiment included mice that were euthanized at the end of treatment to obtain intracranial tumor samples to analyze the expression level of K27m3, JMJD3, DNA damage repair (PCNA, XRCC1, and POLD1), tumor cell proliferation (Ki-67), and apoptosis (TUNEL). IHC analysis of tumors obtained from mice treated...
Figure 2.

GSK-J4 enhances radiation-induced DNA damage. A, Effect of GSK-J4 (6 μmol/L) on γH2AX and 53BP1 foci formation in irradiated SF8628 K27M DIPG and SF9427 H3 wild-type GBM cells. Left, representative images of nuclei from each treatment, showing γH2AX (top) and 53BP1 (bottom) foci. Right, mean and SD values for γH2AX and 53BP1 foci/nucleus are shown and are based on foci counts from three independent experiments. Unpaired t test values for γH2AX comparisons between treatments: ***, P < 0.0001; *, P = 0.0126 between untreated vs. GSK-J4 only for SF8628. Unpaired t test values for 53BP1 comparisons between treatments: ***, P < 0.0001; **, P = 0.0047 between untreated vs. GSK-J4 only for SF8628. B, Western blot results showing effects of GSK-J4 (6 μmol/L) on expression of γH2AX and HR repair pathway proteins, phospho-Rad50 and total Rad51 in SF8628 K27M DIPG cells. C, DNA-repair assay showing effect of GSK-J4 (6 μmol/L) on HR and nonhomologous end-joining (NHEJ) pathways in SF8628 K27M DIPG cells. Repair efficiency represents the ratio of GFP+ to DsRed+ cells normalized to 100% of vehicle control (DMSO). Values shown are based on averages from duplicate or triplicate samples, and error bars represent SD (mean ± SD). Unpaired t test values for comparisons between samples: ***, P = 0.0002; ***, P = 0.0047 between untreated vs. GSK-J4 only for SF8628. D, Clonogenic survival results for K27M DIPG cells (SF8628 and DIPG-007) and H3 wild-type glioma (SF9427) cells treated with GSK-J4 (2.5 μmol/L) for 24 or 72 hours before or after radiation (RT alone; black line; RT and GSK-J4; red line). Survival fractions, shown as mean ± SD based on averages from triplicate samples, were normalized to plating efficiency for each cell line. DEF for each cell line was calculated at the 10% survival level. n.s., not significant.
Figure 3.
GSK-J4 enhances radiation antitumor activity in vivo. Mice with SF8628 intracranial tumor were randomized to four treatment groups: control (DMSO, n = 9), GSK-J4 alone (100mg/kg/day for 10 consecutive days, n = 9), radiation treatment (RT) alone (0.5 Gy, 3 times a week for 2 consecutive weeks for a total dose of 3 Gy, n = 9), and GSK-J4 + RT (n = 7). A, Left, dot plot representation of tumor bioluminescence values in mice at day 64 after tumor cell injection. Horizontal bars indicate the mean value for each treatment group. Unpaired t test values for comparisons between treatments: control vs. RT, \(^{\ast}\), \(P = 0.0416\); control vs. GSK-J4 + RT, \(^{\ast\ast}\), \(P = 0.0389\); GSK-J4 vs. GSK-J4 + RT, \(^{\ast\ast}\), \(P = 0.0429\). Right, tumor bioluminescence overlay images showing relative bioluminescence intensities. B, Growth plots for intracranial tumors. Tumor bioluminescence values show mean and standard deviation (SD) for normalized against bioluminescence values obtained at day 36 after tumor cell injection. C, Corresponding survival plots for each experiment. Statistical analysis was performed using a log-rank test with Holm adjustment: control vs. GSK-J4, \(^{\ast}\), \(P = 0.0369\); GSK-J4 vs. GSK-J4 + RT, \(^{\ast\ast}\), \(P = 0.0429\); control vs. RT, \(^{\ast\ast\ast}\), \(P < 0.0001\); control vs. GSK-J4 + RT, \(^{\ast\ast\ast}\), \(P = 0.0001\); RT vs. GSK-J4 + RT, \(^{\ast}\), \(P = 0.0348\). D, Left, images of representative Ki-67 and TUNEL staining for intracranial tumor from mice euthanized at the end of treatment. Right, mean and SD values representing the average number of positive cells in 4 high-powered fields in each tumor. Unpaired t test values for comparisons between treatments. Ki-67: control vs. GSK-J4 + RT, \(^{\ast\ast\ast}\), \(P < 0.0001\); GSK-J4 vs. GSK-J4 + RT, \(^{\ast\ast}\), \(P = 0.0005\). TUNEL: control vs. GSK-J4 + RT, \(^{\ast}\), \(P = 0.0348\); GSK-J4 vs. GSK-J4 + RT, \(^{\ast\ast}\), \(P = 0.0092\); RT vs. GSK-J4 + RT, \(^{\ast}\), \(P = 0.0433\).
with GSK-J4 showed that GSK-J4 treatment significantly increased tumor cell K27me3 positivity (***, P = 0.0002) and decreased tumor cells JMJD3 positivity (**, P = 0.0022; Supplementary Fig. S3A). Immunohistochemical (IHC) analysis of DNA damage repair revealed that RT significantly increased the expression of DNA damage repair proteins including PCNA (***, P < 0.0001), XRCC1 (**, P = 0.0046), and POLD1 (**, P = 0.0102) relative to control, while GSK-J4 decreased the expression level of these proteins. In addition, the expression level of DNA damage repair proteins was significantly reduced by combination treatment with GSK-J4 and RT compared with RT alone (PCNA: ***, P = 0.0024; XRCC1: ****, P < 0.0001; POLD1: **, P = 0.0291). Analysis of intratumor Ki-67 staining showed all therapies significantly reduced DIPG xenograft cell proliferation relative to the control group (GSK-J4: ***, P = 0.0174; RT: **, P = 0.0107; GSK-J4 + RT: ****, P < 0.0001; Fig. 3D). There were significantly fewer Ki-67–positive cells in the samples treated with combination therapy compared with either monotherapy (***, P = 0.0005 in relation to GSK-J4 alone; **, P = 0.0031 in relation to RT alone). TUNEL staining results showed the highest proportion of positive cells in tumors from mice receiving combination therapy of GSK-J4 and RT relative to either monotherapy (***, P = 0.0092 relative to GSK-J4 alone; **, P = 0.0433 relative to RT alone). No TUNEL positivity was evident in normal brain surrounding tumor in mice receiving any of the combination therapy treatments (Fig. 3D). In contrast to K27M tumors, xenografts established from GBM43 cells expressing wild-type H3 showed no response to GSK-J4 treatment and no therapeutic benefit from combination therapy of GSK-J4 and radiation relative to radiation monotherapy.

Discussion

DNA damage is thought to be the most deleterious effect caused by radiotherapy. Treatment of DIPG with fractionated focal radiotherapy often provides transient relief from tumor symptoms; however, tumor recurrence and patient demise is inevitable. We and others indicated that the majority of the DNA DSB caused by radiation is repaired within 24 hour of treatment (Fig. 2A; refs. 23–25). This DNA damage repair is possibly responsible for the tumor progression observed in DIPG after completion of radiotherapy, thereby ultimately conveying no survival benefits to the patients. It is essential to identify therapeutic agents that inhibit DNA DSB repair to enhance the antitumor effects of radiation, based on the understanding of the molecular mechanisms associated with chromatin modification in K27M DIPG.

We, as well as others, have identified the gain-of-function mechanism for the K27M mutation. The mutant protein sequences and functionally inactivates EZH2, a catalytic subunit of the K27 histone methyltransferase, polycomb repressive complex 2 (PRC2), leading to a dramatic reduction in global methylation at K27 residues in the K27M DIPG (7, 8). This reduction in H3K27 methylation leads to opening of the chromatin providing access for active transcription (26, 27). We have previously shown that inhibition of the H3K27 demethylase JMJD3 by GSK-J4 acts to restore K27 methylation in DIPG cells (10). Increased H3K27 methylation promotes heterochromatin, which is transcriptionally silent and decreases gene expression (27). In our current study, GSK-J4–mediated H3K27 methylation significantly decreases DNA accessibility at both promoter and distal binding sites (Supplementary Fig. S1C) and decreases expression of the genes involved in DNA damage repair pathways in K27M DIPG cells (Fig. 1A–C; Supplementary Table S1). GSK-J4 indirectly modifies chromatin structure to reduce chromatin accessibility, thereby providing a plausible mechanism to prevent access of DNA-repair proteins to the sites of DNA damage induced by radiation (Fig. 1D). Interestingly, GSK-J4 decreased enrichment of DNA sequencing motifs involving neuron developmental genes, while this observation has no direct relevance to the effect of GSK-J4 on DNA damage repair. The significant enrichment of genes involved in neuronal development has been identified by another group (9). Further investigations would be suitably addressed in future studies.

We observed that GSK-J4 increased radiation-induced DNA damage (Fig. 2A and B) by inhibiting HR DNA DSB repair pathway (Fig. 2C; Supplementary Fig. S2A) and enhanced radiosensitivity of K27M DIPG cell lines (Fig. 2D). These GSK-J4–mediated radiosensitizing effects were observed in two K27M DIPG cell lines (SF8628 and DIPG-007) but not in the H3 wild-type GBM cell line (SF9427). This might be due to the global H3K27 hypomethylation in K27M DIPG cells and GSK-J4’s inhibitory effect on histone demethylation increasing H3K27 methylation, as previously reported (10). Given that HR occurs in late S–G2 phase while NHEJ repairs DSB throughout the cell cycle (20, 21), our results suggest that GSK-J4 maintains a high proportion of S phase fraction to inhibit the HR DNA-repair pathway. These GSK-J4 effects on DNA damage repair were observed specifically in tumor cells expressing K27M-mutant H3F3A (SF8628 and Astro KM; Fig. 2C; Supplementary Fig. S2A). GSK-J4–mediated H3K27 methylation may function as an important mechanism to coordinate chromatin accessibility and transcription both at damage sites and in genes, and possibly to regulate repair of DNA lesions, while also orchestrating transcriptional responses to DNA damage (Fig. 4). The role of H3K27 methylation in radiation-induced DNA damage and repair has been investigated but remains unclear. Previous studies have shown that radiation-induced DNA damage recruits EZH2 to DNA DSB sites, leading to the accumulation of H3K27me3 at the damage sites (28–31). Genetic and pharmacologic inhibitions of EZH2 increase radiosensitivity in human bone osteosarcoma epithelial cells (U2OS; ref. 28) and atypical teratoid rhabdoid tumor cells (32), whereas it had no effect on the glioma cells (33). On the other hand, the expression of JMJD3 has been reported to increase in response to radiation-induced DNA damage (34). Consistent with in vitro results, our animal studies, using intracranial SF8628 K27M DIPG xenografts, demonstrated that GSK-J4 increased the genotoxic activity of RT, and that GSK-J4 and RT combination treatment had substantial growth inhibition and survival benefit that surpassed either agent (GSK-J4 or RT) administered as a monotherapy (Fig. 3A–C). This antitumor activity of GSK-J4 + RT combination therapy inhibits radiation-induced DNA damage repair, by showing significant decrease in PCNA, XRCC1, POLD1–positive cells compared with radiation alone (Supplementary Fig. S3B). GSK-J4 + RT combination therapy also promotes apoptosis and cell death as evidenced by significantly increasing in TUNEL–positive cells and decreasing Ki-67–positive cells in the combination therapy group compared with the monotherapy groups (Fig. 3D). The radiation dose used in the combination treatment with GSK-J4 was determined by a dose escalation study. We determined to use the fractionated radiation dose of 0.5 Gy/day for 3 days a week in 2 weeks, which showed intermediate survival benefit (**, P = 0.0016 relative to control,
median survivals extended by 64 days from 56.5 days of control group median survivals; Supplementary Fig. S3D).

GSK-J4 has been used to treat many kinds of tumors such as T-cell acute lymphoblastic leukemia (T-ALL), acute myeloid leukemia, diffuse large B-cell lymphoma, DIPG, neuroblastoma, prostate cancer, and gastric cancer (10, 35–41). However, GSK-J4 is not yet in clinical development. The major challenge of GSK-J4 in clinical development is that GSK-J4 is a prodrug and is rapidly converted to active drug GSK-J1 in vivo (10, 42). The highly polar GSK-J1 compound has restricted cellular permeability (41). Further development for stabilizing GSK-J4 in vivo would be required for clinical application for this compound. In addition to stabilizing GSK-J4, local drug delivery systems such as convection-enhanced delivery (43) would provide an alternative strategy for reducing drug metabolism and potentially increasing the efficacy of GSK-J4 in vivo.

As mentioned above, children with DIPG inevitably die of their disease. The only therapeutic modality that is used routinely for the treatment of DIPG is radiation. Yet, even with radiotherapy the median overall survival is approximately 10 months (3–5). Increased morbidity from RT (44–46), and the poor long-term outcomes of this cancer have necessitated the frequent inclusion...
of focal radiation in young patients. Given this reality, there is a critical need to identify new therapeutics that enhance RT anti-tumor activity and have the potential to enable the use of lower doses and/or volumes of radiation to limit long-term side effects in young patients. Present findings here support the possibility of using GSK-J4 with RT for increased antitumor effect, which could potentially lessen the amount of RT while still providing substantial relief from disease for patients.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Acknowledgments
We thank Dr. Yoshihiro Tanaka from the Department of Preventive Medicine, Northwestern University (Chicago, IL), for statistical comment. This work was supported by grants from the Bear Necessities Pediatric Cancer Foundation and Rally Foundation for Childhood Cancer Research (R. Hashizume), the Matthew Larson Foundation (R. Hashizume), St. Baldrick’s Foundation (R. Hashizume), Alex’s Lemonade Stand Foundation for Childhood Cancer (R. Hashizume), John McNicholas Pediatric Brain Tumor Foundation (R. Luulla, A. Shilatifard, and R. Hashizume), and the NIH grants R01NS093079 (R. Hashizume), F32CA216996 [D. Unruh], R01CA214035–15 (A. Shilatifard), and R35CA197569 (A. Shilatifard). Histology and fluorescent microscopy services were provided by the Mouse Histology and Phenotyping Laboratory and the Center for Advanced Microscopy/Nikon Imaging Center at Northwestern University, respectively.

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Received December 17, 2018; revised April 8, 2019; accepted June 18, 2019; published first June 21, 2019.

References


Clinical Cancer Research

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Clin Cancer Res  Published OnlineFirst June 21, 2019.

Updated version  Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-18-3890

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