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The Collaborative Research Center for Neurological and Psychiatric Disorders
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Bromodomain inhibitor as a novel radiosensitizer for diffuse midline glioma

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Abstract

Diffuse midline glioma (DMG), including diffuse intrinsic pontine glioma (DMG), is a rare form of pediatric tumor that arises in the brainstem, and is the leading cause of death among children with a brain tumor. Despite numerous clinical trials, no drug has significantly prolonged survival of children with DMG. The current standard of care for DMG is radiotherapy, which offers only temporary benefit and a 5-year survival rate below 1%. To enhance the efficacy of radiation, multiple clinical trials have tested radiotherapy in combination with chemotherapeutics, but none have significantly benefited DMG patients. Genomic analyses have identified K27M mutation in histones H3 as unique hallmarks of DMG. Based on preliminary findings initially identified in an unbiased drug screen in combination with radiation using K27M-mutant DMG cells from genetically engineered mouse models (GEMMs) and patient derived xenograft (PDX) models, we hypothesize that BRD inhibitors can radiosensitize DMG cells. In this project, we will use GEMMs, harboring H3K27M or H3WT, as well as PDX DMG models to evaluate whether BRD inhibitors can be radiosensitizers in treating DMG patients. Specifically, we will use both *in vitro* and *in vivo* models to characterize the mechanism by which BRD proteins regulate the DNA damage response and radiosensitivity. We will evaluate clinical-grade BRD inhibitors to determine whether they significantly improve the survival of DMG-bearing mice to radiation. Successful completion of this study will advance our understanding of the molecular mechanisms of radiosensitizing effects of BRD4 inhibitors, and pave the way for future clinical trials for DMG.

A. INTRODUCTION

DMG is one of the devastating childhood cancers. Radiation therapy (RT) remains the only effective treatment yet provides a 5-year survival rate of only 1%. Several clinical trials have attempted to enhance RT efficacy by combining it with radiosensitizing agents, though none have been successful in doing so. Given the reality, there is a critical need to identify effective radiosensitizers in DMG. Our long-term goal is to understand mechanisms governing radiosensitivity in DMG, and to utilize associated discoveries to identify radiosensitizers to improve the efficacy of RT in treating DMG. We will evaluate the mechanisms and potential of BRD inhibitors in radiosensitizing DMG. With an eye towards advancing a BRD inhibitor into the clinic, we aim to characterize the mechanism(s) of action for clinical grade BRD inhibitors. We will prioritize a single candidate drugzhaving optimal *in vivo* brain penetration, efficacy, and low standalone toxicity for a future clinical trial.

B. MATERIALS AND METHODS

In an unbiased high throughput screen, we identified BET bromodomain (BRD) protein inhibitors as potent radiosensitizers of DIPG cells. We will test the

hypothesis that BRD inhibitors disrupt DNA repair responses in DIPG cells, thereby increasing the beneficial effect of RT. Our preliminary results suggest that multiple DNA damage response (DDR) pathways are affected by BRD inhibition, and that combining JQ1 BRD4 inhibitor treatment with RT improves animal subject survival in H3K27M DIPG patient-derived xenograft (PDX) models. To address our hypothesis, we propose the following specific aims and experimental approaches.

Aim 1. Characterize the mechanism of DIPG radiosensitization by BRD inhibition *in vitro* - We have shown that BRD inhibitors sensitize DIPG tumor cells to ionizing radiation (IR). We hypothesize that radiosensitization by BRD blockade occurs through epigenetically-driven changes in the expression levels of DNA damage response genes. We will inhibit BRD signaling through genetic ablation of BRD genes, and by pharmacological suppression with clinical-stage BRD inhibitors, and examine the efficacy, kinetics, and mechanism of action of BRD inhibition in tumor cells from genetically engineered mouse models (GEMMs) of DIPG, and in patient-derived DIPG cell lines.

Aim 2. Identify a single BET BRD inhibitor to move into the clinic as a radiosensitizer for DIPG - We will evaluate the *in vivo* efficacy of clinical-stage BRD inhibitors in radiosensitizing DIPG tumors with the goal of advancing a candidate for clinical evaluation. We will first perform pharmacokinetic and toxicity evaluations of BRD inhibitors in GEMMs and patient-derived xenograft (PDX) models. We will then evaluate the *in vivo* efficacy of the top two candidates in both mouse models, with results expected to reveal the most effective and safe compound for testing in DIPG patients. This study will be conducted under the protocols 2015-2583 (renewed in 03/2020) and G2022-0012, approved by IRB of Niigata University.

C. RESULTS/OUTCOMES

Aim 1. Characterize the mechanism of DIPG radiosensitization by BRD inhibition *in vitro*.

Previously, we have shown that genetic and pharmacological inhibition of BRD4 activity suppressed DMG growth, BrdU incorporation, and increased apoptosis *in vitro*. We confirmed radiaosensitizing effects of BRD4 inhibition by clonogenic survival assay. We also found that BRD4 inhibition increased radiation-induced DNA damage by inhibiting HR and/or NHEJ DNA repair pathway in K27M-mutated DMG cells. These results were described previous progress report.

In the recent study, we evaluated the effects of BRD4c inhibition on senescence (beta-galactosidase assay), and sphere formation assays. The beta-galactosidase assay revealed increasing senescence-associated beta-galactosidase staining in the DMG cells treated with either AZD5153 or IR monotherapy. 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. 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 and neurosphere formation in compared to either monotherapy. 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.

We also analyze RNA-sequencing in the K27M DIPG cells treated with AZD5153. In our **recent 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. 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, including **3301 up-regulated** and **3591down-regulated genes** in response to the BET bromodomain inhibitor. Gene Set Enrichment Analysis (GSEA) and Gene Ontology (GO) pathway analysis 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*, *MAP1LC3B*) and catabolism pathways including **glycolysis and protein/macromolecule catabolic pathways** (e.g., *SIRT1*, *MTOR*). 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).

For **next round of grant periods**, we will analyze epigenetic profile in the K27M DIPG cells treated with AZD5153 CUT-RUN, and ATAC-sequence. We also analyze the effects of BRD4 inhibition on transcriptional machinery including transcriptional elongation by RNA polymerase II and R-loop (DNA-RNA hybrid) formation.

Aim 2. Identify a single BET BRD inhibitor to move into the clinic as a radiosensitizer for DIPG.

Previously, we have shown that the combination of AZD5153 and radiation significantly inhibited tumor growth and increase survival benefit relative to each monotherapy in PDX models. We have **recently** tested the anti-tumor activity of the combination therapy in DMG genetically engineered mouse models (**GEMM-DMG**: Ntv-a; p53fl/fl; PDGFA; H3K27M; Cr). The combination treatment of AZD5153 and radiation treatment (RT) prolonged the survival of GEMMs in compared to each monotherapy (** $P = 0.002$; AZD vs. AZD + RT, *$P = 0.011$; RT vs. AZD + RT, *$P = 0.012$).

D. DISCUSSION

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. The survival improvement of the combination therapy yet provides modest. One limitation of in vivo efficacy of BRD4 inhibitors is a poor brain penetration. To increase the drug concentration in the brain, we would further investigate new drug delivery systems such as disrupting the BBB using focused ultrasound or bypassing the BBB using convection-enhanced delivery and intranasal delivery. Nevertheless, our findings support the possible use of BET bromodomain inhibitor to increase radiation anti-tumor effect for the treatment of DMG.

BRD4 inhibition may sensitize H3K27me3-deficient tumors to radiation by reducing radioresistance phenotype and enhancing apoptotic response. We will further investigate the role of BRD4 inhibition in the transcription machinery associated with histone modification of H3K27me3 and H3K27ac for understanding radiation-induced DNA damage response in DMG.

E. CONCLUSION

Current our findings support the possibility that BRD4 inhibitor as a novel radiosensitizer and provide a rational for developing combination therapy with radiation for the treatment of DMG.

F. PUBLICATIONS

1. PAPERS

A part of the results from this project has published in the *Journal of Clinical Investigation* (Watanabe *et al,* 2024 May 21;134(13):e174794. PMID: 38771655).

2. PRESENTATIONS

Our results from this project are presented as poster at The SNO 7th Biennial Pediatric Neuro-Oncology Research meeting 2023 at Washington DC, and oral presentation in the Neuroscience Seminar 2024 titled “Epigenetic Dependence of Pediatric Brain Tumor” at Niigata, Niigata University, Japan.

G. APPLICATION AND REGISTRATION STATUS ON INTELLECTUAL PROPERTY RIGHTS

1. **PATENT**
   Not Applicable

2. **UTILITY MODEL REGISTRATION**
   Not Applicable

3. **OTHERS**
   Not Applicable