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Therapeutic strategies for diffuse midline glioma from high-throughput combination drug screening

Grant L. Lin¹, Kelli M. Wilson², Michele Ceribelli², Benjamin Z. Stanton³, Pamelyn J. Woo¹, Sara Kreimer¹, Elizabeth Y. Qin¹, Xiaohu Zhang², James Lennon¹, Surya Nagaraja¹, Patrick J. Morris², Michael Quezada¹, Shawn M. Gillespie¹, Damien Y. Dubeau², Aleksandra M. Michalowski⁴, Paul Shinn², Rajarshi Guha², Marc Ferrer², Carleen Klumpp-Thomas², Sam Michael², Crystal McKnight², Paras Minhas¹, Zina Itkin², Eric H. Raabe⁵, Lu Chen², Reem Ghanem¹, Anna Geraghty¹, Lijun Ni¹, Katrin Andreasson¹, Nicholas A. Vitanza¹, Katherine E. Warren^{6,*}, Craig J. Thomas^{2,7,*}, Michelle Monje^{1,8,9,10,11,12,*}

¹. Department of Neurology, Stanford University School of Medicine, Stanford, CA 94305, USA.

². Division of Preclinical Innovation, National Center for Advancing Translational Sciences, National Institutes of Health, Rockville, MD 20850, USA.

³. Center for Childhood Cancer & Blood Diseases, Abigail Wexner Research Institute at Nationwide Children's Hospital, The Ohio State University College of Medicine, Columbus, OH 43205

⁴. Laboratory of Cancer Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA

⁵. Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, MD 21287, USA

⁶. Pediatric Oncology Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA

⁷. Lymphoid Malignancies Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA

⁸. Department of Neurosurgery, Stanford University School of Medicine, Stanford, CA 94305, USA.

⁹. Department of Pediatrics, Stanford University School of Medicine, Stanford, CA 94305, USA

¹⁰. Department of Pathology, Stanford University School of Medicine, Stanford, CA 94305, USA.

¹¹. Stanford Cancer Institute, Stanford University School of Medicine, Stanford, CA 94305, USA.

*To whom correspondence should be addressed: Katherine E. Warren (katherine_warren@dfci.harvard.edu), Craig J. Thomas (craigj@mail.nih.gov), Michelle Monje (mmonje@stanford.edu).

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12. Stanford Institute for Stem Cell and Regenerative Medicine, Stanford University School of Medicine, Stanford, CA 94305, USA.

Abstract

Diffuse midline gliomas (DMG) are universally lethal malignancies occurring chiefly during childhood and involve midline structures of the central nervous system, including thalamus, pons, and spinal cord. These molecularly related cancers are characterized by high prevalence of the histone-3K27M mutation. In search of effective therapeutic options, we examined multiple DMG cultures in sequential quantitative high-throughput screens (HTS) of 2,706 approved and investigational drugs. This effort generated 19,936 single-agent dose responses that inspired a series of HTS-enabled drug combination assessments encompassing 9,195 drug-drug examinations. Top combinations were validated across patient-derived cell cultures representing the major DMG genotypes. In vivo testing in patient-derived xenograft models validated the combination of the multi-histone deacetylase (HDAC) inhibitor panobinostat and the proteasome inhibitor marizomib as a promising therapeutic approach. Transcriptional and metabolomic surveys revealed substantial alterations to key metabolic processes and the cellular unfolded protein response following treatment with panobinostat and marizomib. Rescue of drug-induced cytotoxicity and basal mitochondrial respiration with exogenous application of nicotinamide mononucleotide (NMN) or exacerbation of these phenotypes when blocking nicotinamide adenine dinucleotide (NAD⁺) production via nicotinamide phosphoribosyltransferase (NAMPT) inhibition demonstrated that metabolic catastrophe drives the combination-induced cytotoxicity. This study provides a comprehensive single-agent and combinatorial drug screen for DMG and identifies concomitant HDAC and proteasome inhibition as a promising therapeutic strategy that underscores under-recognized metabolic vulnerabilities in DMG.

One Sentence Summary:

High-throughput screens in DMG identify promising treatments such as the combination of panobinostat and marizomib acting through metabolic collapse.

Introduction

Diffuse midline gliomas (DMGs) such as diffuse intrinsic pontine glioma (DIPG) are universally lethal central nervous system (CNS) tumors that occur chiefly during childhood(1). Despite decades of clinical trials, treatment is limited to radiotherapy. Even with radiotherapy, median overall survival for children with DIPG is only 9–11 months(2,3). Over the past decade, the molecular characterization of DIPG has meaningfully advanced our understanding of the genetic and epigenetic underpinnings of these tumors, including the identification of a recurrent H3K27M mutation in H3.3 (H3F3A) or H3.1 (HIST1H3B) histones(4,5). Mechanistically, the H3K27M mutation results in dysfunction of the Polycomb Repressive Complex 2 (PRC2) and consequent loss of H3K27 trimethylation, broad epigenetic dysregulation and oncogenic gene expression. DIPG has recently been reclassified into a broader category of midline gliomas that share the signature H3K27M mutation, including thalamic and spinal cord gliomas(6,7).

We previously reported a limited chemical screen against a panel of 83 agents in patient-derived DIPG cultures(8). That study identified the multi-HDAC inhibitor panobinostat as a promising clinical agent, exhibiting a disease-specific mechanism of restoring H3K27 methylation and normalizing oncogenic gene expression. Those results led to ongoing Phase I clinical trials of panobinostat in DIPG (NCT02717455, NCT03566199, NCT03632317)(9). Nevertheless, in preclinical DIPG models, resistance to panobinostat emerges, highlighting the need for combinatorial therapeutic strategies(8,10). Driven by the mechanistic implications of the H3K27M mutation, substantial effort has focused on epigenetic targets, including inhibitors of EZH2, CDK7, and BET family proteins(10–13). However, a full appreciation of the druggable landscape in DMG/DIPG remains lacking.

The application of combinatorial drug therapy has revolutionized prognoses for other cancers such as childhood leukemia(14). The development of chemogenomic compound libraries has enabled target-based drug discovery and, used in a combination-matrix approach, allows exploration of thousands of drug-drug pairs for potential synergy(15–17).

We sought to comprehensively characterize the druggable cell-intrinsic vulnerabilities in DMG/DIPG and identify promising therapeutic agents. High-throughput drug screening utilizing two mechanistically annotated collections of approved and investigational drugs in six well-characterized patient-derived DIPG cell cultures resulted in a dataset of 19,936 single-agent dose responses. Based on this dataset, we performed several combinatorial screens encompassing 9,195 discrete drug-drug combinations to identify potential synergies. These single-agent and combination drug responses were used to map the drug and drug-to-drug interaction landscape of the most promising agents based on potency, mechanistic class enrichment, and predicted blood-brain barrier penetrance (BBB). Promising drugs and drug combinations were evaluated in vitro across an expanded panel of patient-derived DMG cell cultures representative of the major genetic subtypes of DMG and in vivo within orthotopic xenograft models. These studies identified the combination of panobinostat and marizomib as a promising drug combination. Investigations of molecular mechanisms that drive the cytotoxic synergy of this drug combination revealed a metabolic vulnerability in DIPG/DMG.

Results

Comprehensive high-throughput drug screening demonstrates classes of mechanistic vulnerabilities of DIPG

We performed multiple single-agent screens in a total of six DIPG cell culture models (JHH-DIPG-1, SU-DIPG-IV, SU-DIPG-VI, SU-DIPG-XIII, SU-DIPG-XVII, and SU-DIPG-XXV) using our internal mechanism interrogation plate (MIPE) 4.0 and 5.0 libraries. All data are publicly available and searchable via the Pubchem database (<https://pubchem.ncbi.nlm.nih.gov/>) (BioAssay Identifier AID: table S1 in data file S1) and at <https://matrix.ncats.nih.gov/>. These libraries contain a total of 2,706 unique agents (257 unique to MIPE 4.0, 764 unique to MIPE 5.0, and 1685 conserved in both libraries) (Fig. 1A, fig. S1A, table S2 in data file S1)(16). These libraries include multiple inhibitors for well-explored oncogenic targets (for instance PI3K, HSP90) while simultaneously encompassing mechanistic diversity, targeting over 860 distinct mechanisms-of-action

(MoAs; table S3 in data file S1). Altogether, these screens generated 19,936 dose response signatures. Analysis of the MIPE 5.0 outcomes highlighted similar drug-sensitivity profiles among the screened cell cultures, generating a coherent database of DIPG chemical vulnerabilities used to prioritize compounds for further analyses (fig. S1B, S1C). We utilized Z-transformed area under the curve (Z-AUC) to distinguish inactive and active drug responses. Agents with Z-AUC values less than -0.85 in at least three of the cell cultures in the MIPE 5.0 dataset were classified as ‘hits’, identifying 371 hits. Notably, several MoAs relevant to DIPG pathogenesis were enriched among these agents, including histone deacetylase (HDAC), proteasome, insulin-like growth factor receptor (IGFR), mitogen-activated protein kinase kinase (MEK), and Phosphoinositide-3-kinase (PI3K) inhibitors (Fig. 1B, table S4 in data file S1). Dose-response curves for representative agents of these MoAs are shown in Fig. 1C. Agents from these mechanistic classes demonstrated a relatively wide potency-range, with proteasome inhibitors being, on average, the most potent drug class (Fig. 1D, fig. S1D). Several hits outperformed agents in currently active or recently completed DIPG clinical trials (Fig. 1A, 1E, Table 1, table S5 in data file S1) and some agents currently in clinical studies for DIPG failed to elicit a meaningful cytotoxic response (see Table S1 for examples). Cautious interpretation of these data is advocated as several trials (examples detailed in Table S1) examine combinations of these drugs and/or the co-administration of radiation therapy. Our screens also examined drug classes of current preclinical interest in DIPG. Bromodomain inhibitors, for example, are of emerging interest in DMG and GBM(12). Of the fifteen bromodomain inhibitors included in our MIPE 5.0 screens, only two (ARV-771 and mivebresib) demonstrated activity beyond our Z-AUC cutoff.

Because the ability of these candidates to penetrate the blood-brain barrier is crucial for their development as systemically delivered DMG therapies, we incorporated CNS penetration as a metric to judge the translational potential of drugs from our screens. To that end, we applied the CNS Multi Parameter Optimization Desirability (MPO) scoring system to all agents in the MIPE 5.0 library(18). Reassessing the potential of ‘hits’ while considering their MPO scores (increasing MPO value indicates more likely CNS exposure) provided additional information (Fig. 1F). Akin to the cytotoxicity outcomes, there were a range of MPO scores for key drug classes and agents in current clinical evaluation in DIPG (Fig. 1G). Twenty-two agents possessed strong activity (average Z-AUC < -2.0) and predicted CNS exposure (MPO score > 4.4), including several with proven CNS exposure (Table 2, table S6 in data file S1). Among these was the next generation proteasome inhibitor marizomib (salinosporamide A), which possessed strong DIPG cell cytotoxicity ($AC_{50} < 40$ nM in all cell cultures) and validated CNS exposure in humans (Fig. 1H)(19,20).

Quantitative high-throughput drug-drug combination screening identifies potential combinatory approaches for DIPG

We next evaluated the actions of key drugs of interest from the single-agent screens in HTS-enabled combination assessments(16). Both the HDAC inhibitor panobinostat and the proteasome inhibitor marizomib were evaluated versus the entire MIPE 5.0 library, and a third combination experiment was conducted evaluating 45 selected agents in an all-versus-

all experiment exploring 990 drug-drug pairs. Additional combination experiments were performed amassing 9,195 discrete drug-drug combinations (<https://matrix.ncats.nih.gov/>).

The examination of panobinostat or marizomib versus the entire MIPE 5.0 library provided insight into the systems effect associated with HDAC and proteasome inhibitors (Fig. 2A). Panobinostat had broad synergistic interaction with several drug classes, including proteasome inhibitors and signaling modulators including PI3K, IGFR and MEK inhibitors (Fig. 2B, 2C, table S7 in data file S1). Marizomib displayed a more restricted combination profile, with synergistic cytotoxicity primarily when combined with HDAC inhibitors and, to a lesser extent, HSP90 inhibitors (Fig. 2B, table S8 in data file S1). The MIPE 5.0 library contains 35 different HDAC inhibitors with distinct selectivity profiles across individual HDAC isoforms. Examining each agent's combination profile with marizomib highlighted that pan-HDAC inhibitors (for instance panobinostat, romidepsin) targeting class I HDAC (HDAC1, HDAC2 and HDAC3) and class II HDAC yielded the highest degree of synergy (Fig. 2D, table S8). HDAC inhibitors that do not target class I HDACs did not synergize with nor potentiate marizomib (for instance TMP-195, PCI-34051). Selective inhibitors of HDAC6 (for instance ACY-775, tubastatin A), a cytoplasmic HDAC implicated in synergy with proteasome inhibitors in multiple myeloma, did not display synergy or potentiation with marizomib in DIPG(21,22).

To generate a multi-dimensional drug-interaction map, we examined all possible drug-pair interactions among 45 agents (including marizomib and panobinostat) chosen based upon single-agent screen outcomes, established synergies from the one-versus-all combination screens, and selected agents of mechanistic interest (Fig. 2E, fig. S2A, S2B, S2C, table S9 in data file S1). This experiment confirmed synergy between marizomib and panobinostat and established that the included proteasome inhibitors (marizomib, carfilzomib, ixazomib) were synergistic with the included class I HDAC inhibitors (panobinostat, vorinostat) (Fig. 2F). Additionally, panobinostat again synergized with MEK inhibitors (selumetinib, AZD-8330), IGFR inhibitors (BMS-754807) and PI3K inhibitors (buparlisib)(8). We also incorporated all drugs in current DIPG clinical evaluation into this screen. Combinations of these agents did not demonstrate notable synergy or potentiation (fig. S3). The correlation heat-map of these 45 agents based on their reciprocal drug-to-drug interaction landscapes (Fig. 2E, fig S2A, S2B, S2C) enabled us to identify groups of drugs (sub-clusters) with similar combination profiles. Panobinostat clustered alongside the HSP90 inhibitor alvespimycin (17-DMAG) (group 1) suggesting potential interplay between HDAC and HSP90. Proteasome inhibitors (ixazomib, carfilzomib, marizomib) clustered together and demonstrated a similar combination pattern with the vacuolar-type H⁺ ATPase inhibitor bafilomycin A (group 2). Several signaling inhibitors (group 3; for instance BMS-754807, buparlisib, selumetinib) clustered together alongside modulators of tubulin function (for instance vincristine, plinabulin, mebendazole). Notably, several drugs within this signaling group showed mutual synergy among each other (table S8), supporting the idea that IGFR, PI3K, and MEK signaling cooperate to sustain DIPG viability. As our screening data was generated using an ATP-dependent assay (Celltiter-Glo®) to measure cell viability, we also repeated key single-agent and combination screening experiments using alternative assays measuring live-cell protease activity (glycylphenylalanyl-aminofluorocoumarin substrate) and caspase activation (Caspase-Glo 3/7™). These data broadly confirm all CelltiterGlo® outcomes. These

collective screening experiments highlight several potentially exploitable mechanistic vulnerabilities in DIPG.

Panobinostat and marizomib emerge as a promising combination from in vitro evaluation of top combination candidates across representative patient-derived DIPG cultures

Given the ongoing clinical trials of the HDAC inhibitor panobinostat and its prominence as a top hit throughout our screens, we evaluated four drugs (marizomib, selumetinib, BMS754807, and buparlisib) in combination with panobinostat against six patient-derived DIPG cell cultures representing the three major genetic subtypes (H3F3A-K27M: SU-DIPG-VI, SUDIPG-XIII, SU-DIPG-XVII; HIST1H3B-K27M: SU-DIPG-IV, SU-DIPG-XXI; H3-wildtype: VUMC-DIPG-10, full culture data in table S10 in data file S1). We generated drug dose-response curves for these candidate agents alone and with 25 nM panobinostat, a sub-IC₅₀ dose, to examine the drug interaction across cultures (Fig. 3A). While all four candidates demonstrated a shift of their dose-response curves when combined with panobinostat, marizomib and BMS-754807 exhibited substantial decreases in cell viability in all cultures tested at concentrations achievable in the pons after systemic administration (IC₅₀ < 30 nM). Buparlisib in the presence of 25 nM panobinostat resulted in an IC₅₀ ranging from 430–1041 nM. Selumetinib exhibited divergent effects across the cultures tested, including a paradoxical increase in cell viability in SU-DIPG-IV such that a dose-response curve could not be calculated.

We next measured DIPG cell viability across different drug doses or combinations in each culture (Fig. 3B, fig. S4A). We used the CalcuSyn 2.0 software (Biosoft) to establish conventional metrics for each drug combination(23). This demonstrated consistent synergy (combination index [CI] < 1) between panobinostat and marizomib, panobinostat and BMS-754807, and to a lesser degree, panobinostat and buparlisib (Fig. 3C). The combination of panobinostat and selumetinib demonstrated synergy in 4 of 6 patient cultures, but no synergy in SU-DIPG-IV and antagonistic effects on SU-DIPG-VI. Due to the concentration required for cell killing and the lower degree of synergy, we removed buparlisib from subsequent analyses.

To assess cell proliferation and cell death in response to drug treatment, we performed flow cytometry analysis of 5-ethynyl-2'-deoxyuridine (EdU) incorporation, Annexin-V staining, and DAPI incorporation. Panobinostat and marizomib together compared to either drug alone had robust effects on cell proliferation and cell death (Fig. 3D, fig. S4B). Combining panobinostat and BMS-754807 also showed added but less pronounced effects (fig. S5A, S5B). Evaluation of panobinostat and selumetinib again demonstrated divergent effects across cultures, including an increase in EdU incorporation in SU-DIPG-IV and a decrease in Annexin-V staining in SU-DIPGVI (fig. S5C, S5D).

Panobinostat and marizomib demonstrate efficacy as combination treatment in patient-derived DIPG xenografts

We evaluated the remaining combinations in vivo (panobinostat with marizomib, BMS754807, or selumetinib). We first tested these candidates as single agents in an orthotopic patient-derived xenograft model engineered to express firefly luciferase (SU-

DIPG-VI). In vivo bioluminescence imaging was performed immediately before drug or vehicle control administration to assess baseline tumor burden. Intravenous marizomib treatment led to a significant decrease in tumor burden at each of two different doses, measured 4 weeks posttreatment initiation (Fig. 4A; top $p < 0.05$, bottom $p < 0.01$). Oral treatment with BMS-754807 and selumetinib (fig. S6A) did not show efficacy as single agents.

Using an aggressive patient-derived xenograft model (SU-DIPG-XIII-P*, a subclone of the patient-derived SU-DIPG-XIII pons culture that demonstrates more aggressive growth in vivo enabling survival analysis) to evaluate the effects of combination treatment, we found that the combination of panobinostat and marizomib, alternating weeks, led to a significant increase in median survival (Fig. 4B; top, $p < 0.01$). Single-agent treatment with panobinostat or marizomib led to smaller increases in overall survival. To control for the additional overall dosing of the combination arm, we tested a second cohort in which the single-agent arms received twice the dosing of their respective drugs (Fig. 4B). Combination treatment again significantly increased overall survival ($p < 0.01$). Panobinostat and marizomib again led to smaller increases in overall survival. To assess whether these agents cause acute neurotoxicity, we treated another cohort of mice and evaluated apoptosis in normal brain tissue by cleaved caspase-3 (CC3) immunostaining 24-hours after vehicle control, panobinostat, or marizomib administration. We found negligible cell death in normal brain tissue and no difference between treatment and control groups (fig. S6B).

We next tested BMS-754807 and selumetinib in combination with panobinostat using the SU-DIPG-XIII-P* xenograft model (fig. S6C). Survival was not extended in the BMS-754807 treatment cohort. In the selumetinib-treated cohort, only the single-agent panobinostat treated group exhibited modestly increased survival, whereas single-agent selumetinib treated mice demonstrated no change from vehicle-treated controls. Alarming, combination-treated mice demonstrated no change in survival despite receiving the same amount of panobinostat as the single-arm control, suggesting that selumetinib may abrogate the effect of panobinostat alone.

Panobinostat and marizomib demonstrate efficacy in other patient-derived DMG xenograft models

DIPG was recently reclassified with spinal cord and thalamic gliomas that bear the signature H3K27M mutation(6,7). Using patient-derived spinal cord glioma (SU-pSCG-1) and thalamic glioma (QCTB-R059) culture models, we evaluated whether single-agent panobinostat treatment also demonstrates efficacy across non-pontine DMGs. In vitro, treatment with panobinostat demonstrated efficacy at nanomolar concentrations (fig. S7A, S7B, SU-pSCG-1 $IC_{50} = 29.4nM$, QCTB-R059 $IC_{50} = 40.7nM$). To evaluate in vivo efficacy, we xenografted SU-pSCG1 into the medulla or QCTB-R059 into the thalamus. Subsequent bioluminescence imaging before and after one week of panobinostat treatment demonstrated significant reduction in orthotopic tumor progression in SU-pSCG-1 (fig. S7C; brain $p < 0.05$, spinal cord $p < 0.05$) and QCTB-R059 (fig. S7D; $p < 0.0005$) xenografts.

We next assessed the combination of marizomib with panobinostat against non-pontine DMGs. In vitro, the addition of 25nM panobinostat lowered the IC_{50} of marizomib to low

nanomolar concentrations in SU-pSCG-1 (2.468nM) and QCTB-R059 (5.918nM) (fig. S7E, S7F). We also measured tumor cell viability at different drug doses or combination (fig. S7G, S7H), and calculated drug synergy using the CalcuSyn 2.0 software (fig. S7I). In both models, panobinostat and marizomib demonstrated synergy at all doses tested.

To assess combination efficacy in vivo, QCTB-R059 cells were orthotopically xenografted into the thalamus. In vivo bioluminescent imaging was performed directly before treatment and 4 weeks after treatment, demonstrating a significant response (panobinostat 2.8-fold decrease, $p < 0.05$; marizomib 2.6-fold decrease, $p < 0.05$; combination 4.6 fold decrease vs. control, $p < 0.01$; Fig. 4C). We also assessed for tumor and normal cell death by CC3 immunohistochemistry in these mice after 4 weeks of treatment. We did not observe a difference in CC3 staining in all treatment conditions (fig. S8A), but notably saw negligible CC3 in non-tumor cells (fig. S8B), supporting that long-term treatment is not associated with meaningful neurotoxicity. These data support panobinostat and marizomib as an effective combinatorial strategy across non-pontine DMGs.

Combination-treated DMG cells exhibit altered transcriptional identity

To explore the mechanism underlying synergy between panobinostat and marizomib in DMG cells, we performed RNA sequencing on SU-DIPG-XIII sampled after 16 hours of exposure to DMSO, panobinostat, marizomib, or two combination doses (Fig. 5A, 5B). Panobinostat and marizomib exposure affected an orthogonal, but overall concordant, set of genes (fig. S9A). Direct comparison of the two combination doses did not demonstrate a pronounced difference (fig. S9B). We next performed gene set enrichment analysis (GSEA) of each treatment with respect to DMSO(24). Enriched gene sets were annotated for each comparison and analyzed by unsupervised hierarchical clustering of their Normalized Enrichment Scores (NES), demonstrating substantial overlap across comparisons. For example, cell cycle and oxidative phosphorylation gene-sets were consistently downregulated and apoptosis-related genes were consistently upregulated by all treatment groups (Fig. 5C, fig. S9C, S9D, S10). An 'unfolded protein response' (UPR) gene-set was unaffected by treatment with panobinostat but upregulated by marizomib. Upregulation of the UPR response was further potentiated by combination treatment. The UPR pathway was also identified when genes affected only by combination drug treatment were functionally categorized with Cytoscape Enrichment maps (Fig. 5D)(25,26). Leading edge analysis from the GSEA UPR gene-set highlighted genes that were strongly upregulated only in combination-treated cells (Fig. 5E). We repeated RNA sequencing experiments on two additional patient-derived cell cultures (SU-DIPG-VI and QCTB-R059) (fig. S11A). These data demonstrated overlapping signatures with the SU-DIPG-XIII dataset (fig. S11B). Importantly, upregulation of the UPR gene-set in marizomib and combination treated cells was a consistent finding across patient-derived cultures (fig. S11C, S11D).

Western blot analysis of treated SU-DIPG-XIII cells showed increased amounts of key modulators of the UPR response such as the endoplasmic reticulum (ER) chaperone protein BiP and the ER stress associated transcription factor CHOP in combination treated cells (Fig 5F, fig. S12A). Western blot analyses also showed increased apoptotic markers, as assessed by CC3 and cleaved-PARP, in combination-treated cells (Fig. 5F, fig. S12A). Treatment with

marizomib, either alone or in combination with panobinostat, resulted in increased ubiquitinated proteins and accumulation of the proliferation inhibitor p21 and the heat shock factor HSP70 (fig. S12B). Treatment with panobinostat, either alone or in combination with marizomib, resulted in increased H3 acetylation and α -tubulin acetylation (fig. S12C). Based upon the UPR outcomes, we incorporated several ER modulators into follow-up experiments including bafilomycin A, the sarco/ER Ca^{2+} ATPase (SERCA) inhibitor thapsigargin, the ER protein transport inhibitor brefeldin A, and the ER associated protein degradation (ERAD) inhibitor eeyarestatin. These agents exacerbated the cytotoxic potential of panobinostat, marizomib, or the combination of these agents (Fig. 5G, fig. S12D).

Panobinostat and marizomib combination-treated DIPG cells undergo metabolic collapse

Both GSEA and Cytoscape Enrichment Map analyses identified transcriptional downregulation of cellular metabolism and respiration in combination-treated cells (Fig. 5C, Fig. 6A, 6B, fig. S11A, S13A, S13B). The oxidative-phosphorylation gene-set was consistently downregulated by combination treatment. Moreover, analysis of oxidative-phosphorylation leading edge genes demonstrated effects mostly on the Complex I gene family (NADH:ubiquinone oxidoreductase subunits, NDUFs) and electron transport chain (ETC) modulators (for example, ATP5J2) (Fig. 6B, fig. S13A, S13B, fig. S14A). Interestingly, in the 45-drug all-vs-all screen, combining ETC inhibitors (the complex 1 inhibitor rotenone and the H^+ ATP synthase inhibitor oligomycin) with glycolytic flux inhibitors (the GLUT1 inhibitor BAY-876 and the MCT2 inhibitor AZD-3965) demonstrated the strongest synergy (Fig. 6C, fig. S14B). These two classes formed well-resolved sub-clusters in the all-versus-all correlation heat map (Fig. 2E). The ETC inhibitors (group #4) were antagonistic among themselves but possessed similar synergistic profiles with most agents tested, suggesting that inhibition of mitochondrial respiration generally increases the susceptibility of DIPG cells to cytotoxic agents (Fig. 6D, left). Conversely, glycolytic flux inhibitors (group #5) had striking synergy with only ETC drugs, and no other meaningful drug interaction (Fig. 6D, right).

To better define the metabolic consequences induced by panobinostat and marizomib, we performed liquid chromatography/mass spectrometry (LC/MS) analysis of DMG cellular metabolites across representative patient-derived cell cultures. Unsupervised hierarchical clustering of the fold-change of profiled metabolites compared to DMSO control demonstrated a distinct profile in combination-treated cells (Fig. 6E, fig. S14C, S14D). Key divergent metabolites included a combination-specific increase in oxidized glutathione (GSSG) and decrease in reduced glutathione (GSH), suggesting oxidative stress as a mechanistic driver of the combination-specific cytotoxicity (fig. S14C, S14D). To test this possibility, we repeated key combination studies with the reactive oxygen species (ROS) scavenger N-acetyl-cysteine (NAC). However, this did not rescue cell viability, suggesting that ROS induction is not a causal element of drug combination-driven cytotoxicity (fig. S15A, S15B). Another key divergent metabolite was a combination-specific upregulation of 3-phosphoglyceric-acid (3-PG), which can indicate pentose phosphate pathway arrest and decreased glycolytic flux. Additionally, downregulation of nicotinamide adenine dinucleotide (NAD^+) and accumulation of citric acid cycle members, hallmarks of general mitochondrial dysfunction, were more pronounced in combination-treated cells (Fig. 6F, fig.

S14C, S14D, fig. S16A). These results prompted us to analyze mitochondrial respiration in DMG cells following drug exposure. Using Seahorse-based assays, we observed marked decrease in basal cellular respiration and spare respiratory capacity of combination treated cells (Fig. 6G, fig. S16B).

We hypothesized that the combination-induced cytotoxicity could be due to metabolic catastrophe mirroring the acute toxicity observed when combining ETC inhibitors and glycolytic flux modulators. To test this, we manipulated cellular NAD⁺ concentrations through exogenous addition of nicotinamide mononucleotide (NMN), the metabolic precursor to NAD⁺, or daporinad, a nicotinamide phosphoribosyltransferase (NAMPT) inhibitor. First, we demonstrated in SUDIPG-XIII cells that NMN increases cellular NAD⁺, daporinad reduces cellular NAD⁺, and NMN and daporinad together results in normalized NAD⁺ concentrations (fig. S16C). Daporinad alone increased cell death and, as expected, this effect was reversed by NMN supplementation (fig. S16C). In combination-treated cells, NMN supplementation restored physiological NAD⁺ concentrations, whereas daporinad exacerbated the combination-induced effect on cellular NAD⁺ (Fig. 6H). Consistently, daporinad administration significantly increased the cytotoxicity of the combination treatment, and NMN supplementation reversed this effect. Critically, NAD⁺ concentration mirrored DIPG cell viability and NMN supplementation completely blocked the combination-induced cytotoxicity (Fig. 6I). Given the profound rescue by NMN supplementation, we repeated this experiment on five representative patient-derived DMG cell cultures: H3.3K27M DIPG (SU-DIPG-VI), H3.1K27M DIPG (SU-DIPG-XXI), H3WT DIPG (VU-DIPG-10), H3.3K27M spinal cord glioma (SU-pSCG-1), and H3.3K27M thalamic glioma (QCTB-R059). We found that NMN robustly blocks the combination-induced cytotoxicity in 5 of 6 tested cultures, and significantly reduces the combination-induced cytotoxicity in the last culture (QCTB-R059) (fig. S16D). Importantly, cytotoxicity induced by the tubulin polymerization inhibitor vincristine, which does not act through metabolic pathways, was not blocked by NMN supplementation (fig. S16E). Altogether, these findings demonstrate that both H3K27M and H3-wildtype DMG cells are highly sensitive to metabolic perturbations and that metabolic dysfunction is a causal component of the cytotoxic effects of the panobinostat and marizomib combination in DMG.

Discussion

The urgent need for effective therapeutic strategies for DMGs is highlighted by the lack of improvement in overall survival despite decades of clinical trials. The recent development of patient-derived DMG cell cultures along with the comprehensive genomic characterization of pediatric brain tumors has moved us beyond empiric testing to targeted drug discovery for this fatal disease. Here, we report a comprehensive single-agent and combination drug screen against DIPG, with data publicly available to the research community to guide preclinical exploration of promising therapies for DMG and provide rationale for clinical trial design. Furthermore, we demonstrate that the combination of HDAC inhibition and proteasome inhibition has potent synergy and represents a promising clinical strategy for treating DMGs. Importantly, H3.3K27M, H3.1K27M, and H3WT gliomas appear to respond similarly to this combination, suggesting broad utility across subgroups of DMGs. Finally,

we find that this synergy is driven by a metabolic crisis within DMG cells, uncovering a metabolic sensitivity.

Caveats about the patient-derived models used here include limited models in which assessing survival is feasible. Patient-derived models of DMG typically require many months for lethality, limiting survival studies to only unusually aggressive PDX models such as SU-DIPG-XIIP*. It should be noted that the in vivo efficacy demonstrated here is modest. This is consistent with the clinical intractability of the disease, for which any measurable prolongation of survival would be a step in the right direction. Ultimately, curative therapy for DMGs will likely require a multi-pronged approach, targeting cell-intrinsic vulnerabilities as described here, together with targeting key microenvironmental dependencies {Venkatesh 2017} and leveraging immunotherapeutic opportunities {Mount 2018}.

The combination of HDAC and proteasome inhibition has been pursued as a therapeutic strategy in other tumors, most notably multiple myeloma, with demonstrated safety and tolerability(27–29). In adult glioma cell lines, an in vitro study described synergy between the HDAC inhibitor vorinostat and the proteasome inhibitor bortezomib(30). Several mechanisms have been proposed for the synergy between HDAC and proteasome inhibitors, including the combination of HDAC6-mediated aggresome formation and proteasomal degradation resulting in defective protein catabolism, while other studies have implicated HDAC6-independent mechanisms(21,22,31,32). Our marizomib-based combination assessments revealed class I HDAC inhibitor specific synergy in DIPG. Similar to previous reports, we observe that combining HDAC and proteasome inhibitors (for example, panobinostat and marizomib) results in upregulation of the UPR at the gene expression and protein level.

We noted several other acute changes to gene expression following panobinostat and marizomib treatments. Among the most striking features was the combination-specific downregulation of metabolism-related genes. The altered metabolic state of cancer cells is well studied and targeting key metabolic vulnerabilities in cancer is emerging as an attractive therapeutic modality(33,34). Here, we found that selected modulators of mitochondrial respiration and glycolytic flux create a state of synthetic lethality in DMG. Furthermore, combining panobinostat and marizomib alters the metabolic profile of DMG cells in a manner distinct from either single-agent treatment, including a substantial reduction in NAD⁺. Critically, restoring NAD⁺ concentrations with NMN supplementation completely rescues the combination-induced cytotoxicity, while NAD⁺ depletion via the NAMPT inhibitor daporinad alone leads to cell death and exacerbates the combination-induced cytotoxicity. Together, these findings indicate that DMG is highly dependent on adequate NAD⁺, and that the synergistic effect of panobinostat and marizomib is driven in large part by their effect on cellular metabolism. Further studies will more definitively establish the mechanistic basis for the observed synergy.

The application of marizomib, a blood-brain-barrier penetrant proteasome inhibitor, in CNS tumors has been proposed as a potential therapeutic strategy in GBM(19,20,35,36). This strategy is further supported by the observation that marizomib has an effect on CNS

metastases of multiple myeloma, providing evidence of brain penetrance(37). In DIPG, interim analysis of an ongoing clinical trial for panobinostat (NCT02717455) has demonstrated early evidence of clinical benefit(9). Given the role combination therapy has played in treating other cancers, our data support exploring a clinical trial of panobinostat together with marizomib in DMGs. While the effect size of the combination-induced increase in overall survival in this study is admittedly modest (~20%), this therapeutic benefit represents a substantial step towards changing the prognosis of DMGs. Ultimately, the development of effective, multi-pronged clinical strategies targeting cell-intrinsic vulnerabilities together with microenvironmental dependencies and immunotherapeutic opportunities provides hope for overcoming this devastating disease.

Materials and Methods

Experimental Design

This study was designed to identify and validate combination therapeutic candidates for diffuse midline gliomas. High-throughput single agent and combination drug screening was performed as described and as previously reported(16). In vitro and in vivo xenograft experiments were conducted for top combination candidates. Selection of compounds for combination testing was determined by considering individual agent efficacy, current clinical status, brain penetrance, and mechanistic interest/enrichment. *Randomization*: Mice xenografted with luciferase-expressing tumors were randomized into treatment conditions following baseline imaging to normalize for initial bioluminescence signal. Mice xenografted with SU-DIPG-XIII-P* tumors were randomized into treatment groups prior to treatment initiation. *Blinding*: Experimenters were blinding to condition for histological image acquisition and analysis. *Replication*: All in vitro validation experiments were performed at least in triplicate. Sample sizes for all in vivo xenograft experiments are reported in their respective figure legends. For single-agent bioluminescence experiments, a minimum group n of 3 was calculated to have 80% power to detect a 2-fold change in signal on two-tailed t-test, with estimated variance as calculated from previous experiments. For ANOVA of combination-agent bioluminescence experiments, a minimum group n of 3 was calculated to have 80% power given four treatment groups with estimated variance as calculated from previous bioluminescence experiments. For survival analyses, a total cohort size of 13 animals was calculated to have 80% power to detect a 20% increase in overall survival.

Statistical analysis

Plots were drawn using GraphPad Prism and R software. Original numerical data are provided in table S11 in data file S1. Error bars represent mean $\pm$ SEM. Statistical significance for *in vitro* and *in vivo* experiments was calculated using appropriate t-test, ANOVA analyses, or Tukey's multiple comparisons test using GraphPad Prism as indicated in figure legends. Survival curve analysis was analyzed using log-rank statistical testing using GraphPad Prism. Differentially expressed genes was estimated with DESeq2 Wald test with adjusted p value = 0.05. An alpha level of 0.05 with two-sided testing was used unless otherwise specified. All data were normally distributed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Drug candidates identified through high-throughput drug screening in DIPG.

(A) Heat-map representation of drug activities for four DIPG cell cultures (JHH-DIPG-1, SU-DIPG-XIII, SU-DIPG-XVII, SU-DIPG-XXV) screened versus the MIPE 5.0 library. Activity scores are based upon Z-score transformed area-under-the-curve values (Z-AUC). Tick-marks on the right side of the plots highlight the relative ranking of proteasome and HDAC inhibitors, as well as the relative rankings of agents in active clinical evaluation in DIPG. (B) Mechanistic drug classes enriched among the 371 'hits' selected based on consistent potency across DIPG cell cultures. Enrichment was defined as Hits# 2 and

target coverage 35%. (C) Dose response curves for selected agents from key enriched mechanistic classes (Fig. 1B) including panobinostat (HDAC), selumetinib (MEK), BMS-754807 (IGFR), buparlisib (PI3K). (D) Potency (AC_{50}) distribution for proteasome (red) and HDAC (blue) inhibitors compared with the 371 potency-selected hits (gray). (E) Potency (AC_{50}) distribution for proteasome (red) and HDAC (blue) inhibitors compared with agents in active clinical evaluation in DIPG (mustard). (F) For the 371 potency-selected hits, Z-AUC values were compared to predicted CNS penetration using Multi Parameter Optimization Desirability (MPO) scores. The color scheme is the same of Fig. 1E. (G) Distribution of MPO scores for proteasome inhibitors (red), HDAC inhibitors (blue), and agents in active clinical evaluation in DIPG. (H) Dose-response curves for the proteasome inhibitor marizomib.

Fig. 2. Synergistic drug-drug interactions in DIPG identified via HTS-enabled combination drug screening.

(A) Schematic layout of the ‘drug-versus-all’ screen for panobinostat and marizomib versus the entire MIPE 5.0 library. (Top) Each drug pair was tested in a 6×6 matrix block, reflecting 5 doses plus DMSO control for each individual matrix block. (Middle) An exemplar 1536 plate containing 6×6 blocks. (Bottom) Examples of % Response and Bliss heat-maps for additivity, synergy or antagonism outcomes. (B) The entire panobinostat (left panels) and marizomib (right panels) ‘drug-versus-all’ screen results were ranked by synergy, as

assessed by the ExcessHSA metric (gray). Each panel highlights drugs from key mechanistic classes including HDAC, proteasome, MEK, IGFR and PI3K inhibitors. **(C)** (Left) ExcessHSA values for the combination of panobinostat with the proteasome inhibitor marizomib, the IGFR inhibitor BMS754807, the MEK inhibitor selumetinib and the PI3K inhibitor buparlisib. (Right) % Response and Bliss heat-maps for BMS-754807, selumetinib, and buparlisib. **(D)** (Left) Thirty HDAC inhibitors from the MIPE 5.0 library were ranked based on the ExcessHSA values versus the marizomib. Class I (*) and Class II/III (**) HDAC inhibitors are highlighted. (Right) %Response and Bliss heat-maps for example class I pan-HDAC inhibitors (romidepsin and dacinostat) or Class II HDAC6 inhibitors (ACY-775 and tubastatin A). **(E)** (Left) The correlation heat-map of the 45 drug 'all-versus-all' combination screen. Sub-groups of drugs with similar combination profiles are highlighted (group 1: blue; group 2: brown; group 3: orange; group 4: light green; group 5: dark green). (Right) The original ExcessHSA values for the groups 1, 2 and 3. Ranking is based on the average ExcessHSA within each group. These figures are expanded in fig. S2. **(F)** 10×10 %Response heat-maps and Bliss heat-maps for the combinations of panobinostat with either marizomib or BMS-754807.

Fig. 3. In vitro validation of top drug combinations across representative DIPG cell cultures. (A) Dose-response curves of cell viability as measured by CellTiterGlo compared to DMSO control of patient-derived DIPG cell cultures after 72 h exposure to top candidate therapeutic agents marizomib, BMS-754807, buparlisib, and selumetinib alone (top) or with 25 nM panobinostat (bottom). Vertical dashed line is at 1 μ M, representing an approximate range for achievable concentrations in vivo. (B) Cell viability compared to DMSO control after 72 hours exposure of six patient-derived DIPG cell cultures to varying doses of panobinostat (blue), marizomib (red), or both (purple). Similar measurements for the other top drug

combination candidates can be found in fig. S3A. **(C)** Calculated median-effect drug synergy combination index (CI) scores (Biosoft Calcsyn 2.0) across dose levels for each of the four candidate drug combinations. Horizontal dashed line indicates a CI = 1, where points below the line indicate synergy and points above the line indicate antagonism. **(D)** Cell proliferation as measured by flow cytometric analysis of EdU incorporation (left) and cell death as measured by surface labeling of Annexin V (middle) and permeability to DAPI (right) of six patient-derived DIPG cell cultures. For EdU analysis, cells were incubated with DMSO vehicle (Control, grey), panobinostat (blue), marizomib (red) or combination panobinostat and marizomib (Combo, purple) for 16 hours, and then exposed to 10 μ M EdU for 24 hours before analysis. For Annexin V and DAPI analysis, cells were incubated for 48 hours before analysis. Individual flow cytometry histograms can be found in fig. S3B.

Fig. 4. Panobinostat and marizomib in xenograft models of DIPG and other diffuse midline gliomas.

(A) In vivo bioluminescence imaging of SU-DIPG-VI GFP-luc xenografts after four weeks of treatment with marizomib at 150 μ g/kg once every two weeks (top, n = 3 vehicle controls, n = 3 treated mice) or 150 μ g/kg twice every two weeks (bottom, n = 5 vehicle controls, n = 3 treated mice). Two-tailed t-test. (B) Overall survival of SU-DIPG-XIII-P* xenografted mice treated with vehicle; panobinostat alone (top: 5 mg/kg, bottom: 10 mg/kg, 3 times per week, every other week); marizomib alone (150 μ g/kg, top: 1 time per week, bottom: 2 times per week, every other week); or combination (panobinostat 5 mg/kg, 3 times per week, every other week, and marizomib 150 μ g/kg, 1 time per week, every other week in both cohorts). Log rank test. (C) In vivo bioluminescence imaging of QCTB-R059 GFP-luc xenografts after four weeks of treatment with vehicle control, panobinostat (5 mg/kg, 3 times weekly, every other week), marizomib (150 μ g/kg, once weekly, every other week), or combination panobinostat and marizomib, alternating every week. One-way ANOVA with Tukey's multiple comparisons test. * p < 0.05, ** p < 0.01.

Fig. 5. RNA-seq analysis of the combination of panobinostat and marizomib in SU-DIPGXIII cells.

(A) Heat-map representations of the Log2 gene expression changes (Log2FC) following treatment of SU-DIPG-XIII with 50 nM panobinostat, 20 nM marizomib, or two combination doses (50 nM + 20 nM or 100 nM + 50 nM, panobinostat and marizomib, respectively). Differentially expressed genes were selected based on either panobinostat vs. DMSO (left) or marizomib vs. DMSO (right) comparisons, and heat-maps depicting all the four treatments set were then generated accordingly by unsupervised hierarchical clustering.

(B) Volcano plot of each individual treatment set with respect to the DMSO control. Statistically significantly downregulated or upregulated genes are highlighted in blue or red, respectively. **(C)** A fold-change pre-ranked list of each treatment versus DMSO was used to run GSEA analysis against the Hallmark (shown here) and Reactome gene sets (fig. S9C). Unsupervised hierarchical clustering of Normalized Enrichment Scores (NES) was used to generate a comprehensive heat-map visualization of the functional transcriptional outputs of the four treatment sets. **(D)** Cytoscape Enrichment Map visualization of top gene programs represented by significantly upregulated genes present in combination-low treated cells but not either single-agent treated condition. Node size represents number of genes, node color represents significance (FDR), and edge thickness represents number of shared genes. Clustered gene programs are labeled. **(E)** The gene expression changes for the ‘leading-edge’ genes from the Reactome-UPR gene-set are shown. Leading selection and ranking were based on the combo high (h) treatment set. **(F)** Western blot analysis of the indicated endoplasmic reticulum (ER) stress/UPR or apoptosis biomarkers is shown for all the four treatment sets. Black triangles denote cleaved PARP and cleaved caspase-3. β -actin was used as a loading control. **(G)** %Response and Bliss heat-maps are shown for the combination of either panobinostat or marizomib with the ER modulators eeyarestatin and bafilomycin A1.

Fig. 6. Targeted metabolic profiling of the combination of panobinostat and marizomib in SU-DIPG-XIII cells.

(A) Cytoscape Enrichment Map visualization of top gene programs represented by significantly downregulated genes present in combination low treated cells but not either single-agent treated condition. Node size represents number of genes, node color represents significance (FDR), and edge thickness represents number of shared genes. Clustered gene programs are labeled. (B) Gene expression changes for the ‘leading-edge’ genes from the Hallmark-Oxidative Phosphorylation gene-set. Leading selection and ranking were based on

the combo high (h) treatment set. Complex I gene family members (NDUFs) are bolded. **(C)** %Response and Bliss heat-maps highlighting the ‘synthetic lethality’ for the combination of electron transport chain inhibitors (the complex 1 inhibitor rotenone or the H⁺ATP synthase inhibitor oligomycin A) with glycolytic flux inhibitors (the GLUT1 inhibitor BAY-876 or the MCT2 inhibitor AZD-3965). **(D)** ExcessHSA values for well resolved sub-clusters of electron transport chain (group 4) or glycolytic flux (group 5) inhibitors originally identified in the correlation heat-map for the 45 drugs ‘all vs all’ screen (Fig. 2E). Ranking is based on the average ExcessHSA within each group. This plot is expanded in fig. S2. **(E)** Heat-map displaying unsupervised hierarchical clustering of fold-change of metabolites with respect to DMSO control, quantified by liquid chromatography/mass spectrometry (LC/MS). **(F)** Relative abundance of NAD⁺ and 3-phospho-glycerate in each treatment set with respect to the DMSO control. **(G)** Basal respiration and spare respiratory capacity, as assessed by Seahorse experiments, are reported for each treatment set. **(H)** Relative NAD⁺ concentration in combination treated (24h) cells with respect to the DMSO control, in the presence or absence of the NAD⁺ precursor nicotinamide mononucleotide (NMN), the NAMPT inhibitor daporinad, or both. **(I)** Cell death in combination-treated (24 hours) cells with respect to DMSO control, in the presence or absence of NMN, daporinad or both. **p < 0.005, ***p < 0.0005, ****p < 0.0001.

Table 1.Cellular cytotoxic response to agents in active clinical evaluation in DIPG (excluding biologics)¹.

Drug Name	Mech. Of Action	Clinical Trial Identifier	MPO Score ²	CNS Pent. ³	JHH-DIPG-1 ⁴	SU-DIPG-XIII ⁴	SU-DIPG-XVII ⁴	SU-DIPG-XXV ⁴
Doxorubicin	Topo II Inh.	NCT02758366 ⁵	2.79	Limited ¹⁶	1.048 μ M	0.934 μ M	0.235 μ M	1.176 μ M
Irinotecan	Topo I Inh.	NCT03086616 ⁶	2.94	Yes	I.C.	I.C.	0.263 μ M	I.C.
Lenalidomide	Cereblon Inh.	NCT01222754 ⁷	5.42	Yes	I.C.	I.C.	I.C.	I.C.
Mebendazole	Tub. Depol. Inh.	NCT01837862 ⁸	5.09	Yes	0.468 μ M	0.417 μ M	0.093 μ M	0.331 μ M
Vincristine	Tub. Pol. Inh.	NCT01837862 ⁸	2.88	Limited	0.029 μ M	0.331 μ M	0.030 μ M	0.030 μ M
Abemaciclib	CDK 4/6 Inh.	NCT02644460	3.11	Yes	I.C.	I.C.	I.C.	I.C.
Ribociclib	CDK 4/6 Inh.	NCT03355794 ⁹	4.30	Yes	I.C.	I.C.	I.C.	I.C.
Erlotinib	EGFR Inh.	NCT02233049 ¹⁰	4.25	Limited	I.C.	I.C.	I.C.	I.C.
Dasatinib	Multikinase Inh.	NCT01644773 ¹¹	3.54	Yes	I.C.	I.C.	I.C.	I.C.
Crizotinib	Multikinase Inh.	NCT01644773 ¹¹	3.02	Limited	0.590 μ M	0.332 μ M	0.468 μ M	0.468 μ M
Everolimus	mTOR Inh.	NCT03355794 ⁹	1.94	Limited	I.C.	I.C.	I.C.	I.C.
Temsirolimus	mTOR Inh.	NCT02420613 ¹²	2.06	Yes	I.C.	I.C.	I.C.	I.C.
Adavosertib	Wee1 Inh.	NCT01922076 ⁷	3.67	Yes	I.C.	I.C.	I.C.	I.C.
ONC-201	Multiple reports	NCT03416530	5.66	Unknown	I.C.	I.C.	I.C.	I.C.
Valproic acid	HDAC Inh.	NCT00879373 ¹³	5.62	Yes	I.C.	I.C.	I.C.	I.C.
Vorinostat	HDAC Inh.	NCT01189266 ^{7, 14}	4.82	Unknown	1.481 μ M	1.176 μ M	1.864 μ M	0.833 μ M
Panobinostat	HDAC Inh.	NCT02717455 ¹⁵	5.25	Unknown	0.166 μ M	0.166 μ M	1.176 μ M	0.148 μ M

Notes:

¹. At the time of publication.². The Central Nervous System Multiparameter Optimization Desirability (MPO) Scores are based upon the design algorithm defined in Wager et al (ref. 18).³. Outcome definitions: 'Yes' means that published results confirmed CNS exposure in animal or human studies. 'Limited' means that published results suggest no or limited CNS exposure in animal or human studies. 'Unknown' means that published results were not available to the best of our knowledge.⁴. AC50 values are provided for agents with Z-transformed AUC values < -0.85 and are based upon the NCATS curve generator and are only provided for agents with a curve class designation of -1.1, -1.2 or -2.1 (otherwise noted as I.C. or 'incomplete curve') (see ref. 40 for curve class definitions). IC indicates no response at the concentrations tested.⁵. [NCT02758366](#) doses Doxorubicin utilizing a prolonged infusion alone or in combination with TMZ or radiation.⁶. [NCT03086616](#) doses Irinotecan utilizing a liposomal formulation.⁷. [NCT01189266](#), [NCT01222754](#), [NCT01922076](#) are trials combining the named drug with radiation therapy.

8. [NCT01837862](#) examines Mebendazole alone or in combination with Vincristine, Carboplatin, Irinotecan, Bevacizumab and/or TMZ.
9. [NCT03355794](#) examines a combination therapy involving Ribociclib and Everolimus following radiation therapy.
10. [NCT02233049](#) examines combinations of Erlotinib, Dastatinib and Everolimus.
11. [NCT01644773](#) examines a combination therapy involving Dasatinib and Crizotinib.
12. [NCT02420613](#) examines a combination therapy involving Temsirolimus and Vorinostat.
13. [NCT00879373](#) examines a combination therapy involving Valproic acid and Bevacizumab and radiation.
14. Vorinostat is also being explored in [NCT02420613](#). 15. [NCT03566199](#) doses Panobinostat utilizing a nanoparticle formulation.
15. Panobinostat is also being explored in NCG03566199. 16. In [NCT02758366](#), Doxorubicin is being dosed using a prolonged, slow-infusion which may increase the CNS exposure.

Table 2.Cellular cytotoxic response to agents with likely CNS exposure and cellular activity in DIPG¹.

Drug Name	Mech. Of Action	Development Phase ²	MPO Score ³	CNS Pent. ⁴	JHH-DIPG-I ⁵	SU-DIPG-XIII ⁴	SU-DIPG-XVII ⁴	SU-DIPG-XXV ⁴
Colchicine	Tub. Pol. Inh.	Approved	5.47	Unknown	0.029 μ M	0.052 μ M	0.030 μ M	0.041 μ M
Plinabulin	Tub. Pol. Inh.	Phase III	5.03	Yes	0.052 μ M	0.166 μ M	0.037 μ M	0.047 μ M
Combretastatin A-4	Tub. Pol. Inh..	Phase II	5.36	Yes	0.010 μ M	0.018 μ M	0.007 μ M	0.010 μ M
Azixa	Tub. Pol. Inh.	Phase II	5.08	Yes	0.011 μ M	0.009 μ M	0.008 μ M	0.006 μ M
Sepantronium	Survivin Inh.	Preclinical	5.63	Unknown	0.104 μ M	0.002 μ M	0.009 μ M	0.026 μ M
Marizomib	Proteasome Inh.	Phase I	5.50	Yes	0.029 μ M	0.014 μ M	0.033 μ M	0.018 μ M
PI-103	PI3K Inh.	Preclinical	5.40	Unknown	5.254 μ M	0.332 μ M	0.525 μ M	0.186 μ M
Daporinad	Nampt Inh.	Phase I	5.09	Yes	0.004 μ M	0.002 μ M	0.012 μ M	0.005 μ M
NAMPT-IN-1	Nampt Inh.	Preclinical	4.45	Unknown	0.047 μ M	0.037 μ M	0.263 μ M	0.083 μ M
GMX-1778	Nampt Inh.	Preclinical	5.16	Unknown	0.015 μ M	0.008 μ M	0.030 μ M	0.013 μ M
GDC-0623	MEK Inh.	Phase I	4.49	Yes	0.105 μ M	0.148 μ M	0.148 μ M	0.331 μ M
AZD-8330	MEK Inh.	Phase II	4.44	Unknown	0.059 μ M	0.132 μ M	0.166 μ M	0.296 μ M
Triptolide	XBP Inh.	Phase II	5.75	Yes	0.083 μ M	0.105 μ M	0.186 μ M	0.209 μ M
Triptonide	XBP Inh.	Preclinical	6.00	Yes	0.132 μ M	0.263 μ M	0.296 μ M	0.372 μ M
Ganetespib	HSP90 Inh.	Phase II	5.16	Yes	0.066 μ M	0.042 μ M	0.037 μ M	0.037 μ M
HSP-990	HSP90 Inh.	Preclinical	4.92	Unknown	0.037 μ M	0.042 μ M	0.030 μ M	0.030 μ M
NSC-319726	Ox. Stress	Preclinical	5.75	Unknown	0.026 μ M	0.024 μ M	0.009 μ M	0.013 μ M
Elesclomol	Ox. Stress	Phase I	5.21	Unknown	0.005 μ M	0.009 μ M	0.019 μ M	0.019 μ M
Dinaciclib	CDK Inh.	Phase I	5.20	Yes	0.042 μ M	0.037 μ M	0.037 μ M	0.030 μ M
CGP-60474	CDK Inh.	Preclinical	5.16	Unknown	0.118 μ M	0.083 μ M	0.132 μ M	0.132 μ M
SB-1317	CDK Inh.	Phase II	4.54	Unknown	0.166 μ M	0.132 μ M	0.083 μ M	0.093 μ M
Podofilox	Topo II Inh.	Approved	5.27	Unknown	0.030 μ M	0.030 μ M	0.042 μ M	0.047 μ M

Notes:

- ¹. Agents with an MPO score above 4.0 and an average Z-AUC below -1.9.
- ². Highest achieved clinical phase in any indication at the time of publication.
- ³. The Central Nervous System Multiparameter Optimization Desirability (MPO) Scores are based upon the design algorithm defined in Wager et al (ref. 18).
- ⁴. Outcome definitions: 'Yes' means that published results confirmed CNS exposure in animal or human studies. 'Unknown' means that published results were not available to the best of our knowledge.
- ⁵. AC50 values are provided for agents with Z-transformed AUC values < -0.85 and are based upon the NCATS curve generator and are only provided for agents with a curve class designation of -1.1, -1.2 or -2.1 (see ref. 40 for curve class definitions).
- ⁶. These agents are no longer in active clinical trials. 7. This agent is in active clinical evaluation as a prodrug named Minnelide.