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Inhibition of Nucleotide Synthesis Targets Brain Tumor Stem Cells in a Subset of Glioblastoma

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Abstract

Inhibition of both the *de novo* (DNP) and salvage (NSP) pathways of nucleoside synthesis has been demonstrated to impair leukemia cells. We endeavored to determine whether this approach would be efficacious in glioblastoma. To diminish nucleoside biosynthesis, we utilized compound DI-39, which selectively targets NSP, in combination with thymidine (dT), which selectively targets DNP. We employed *in vitro* and *ex vivo* models to determine the effects of pretreatment with dT + DI-39 on brain tumor stem cells (BTSC). Here, we demonstrate that this combinatorial therapy elicits a differential response across a spectrum of human patient-derived glioblastoma cultures. As determined by apoptotic markers, most cultures were relatively resistant to treatment, although a subset was highly sensitive. Sensitivity was unrelated to S-phase delay and to DNA damage induced by treatment. Bioinformatics analysis

indicated that response across cultures was associated with the transcription factor PAX3 (associated with resistance) and with canonical pathways, including the nucleotide excision repair pathway, PTEN (associated with resistance), PI3K/AKT (associated with sensitivity), and ErbB2-ErbB3. Our *in vitro* assays demonstrated that, in sensitive cultures, clonal sphere formation was reduced upon removal from pretreatment. In contrast, in a resistant culture, clonal sphere formation was slightly increased upon removal from pretreatment. Moreover, in an intracranial xenograft model, pretreatment of a sensitive culture caused significantly smaller and fewer tumors. In a resistant culture, tumors were equivalent irrespective of pretreatment. These results indicate that, in the subset of sensitive glioblastoma, BTSCs are targeted by inhibition of pyrimidine synthesis. *Mol Cancer Ther*; 15(6); 1–8. ©2016 AACR.

Introduction

Glioblastoma is a devastating disease with no cure, a dire prognosis, and a median survival of approximately one year (1). Novel treatments are necessary to reduce the malignancy of these brain tumors. One therapeutic strategy is to target brain tumor stem cells (BTSC), as they are the cells believed to be responsible for tumor formation and recurrence (2–4). Evidence for the existence of BTSC came from several laboratories (5–7) and has been supported by several studies (8, 9). Indeed, further studies have substantiated that BTSCs do exist in models of

glioblastoma and are responsible for chemoresistance and tumor formation (10–13). Therefore, identifying agents that target BTSC is a promising strategy in the development of chemotherapeutics to treat glioblastoma.

Personalized medicine that tailors therapy to each individual patient based on tumor characteristics is a potential strategy for the treatment of glioblastoma (14, 15). Verhaak and colleagues have classified glioblastoma tumors into four distinct groups based on gene expression that correlates with specific mutations (16). Whether or not this classification system distinguishes cohorts that respond to targeted therapy is an area of current study. One way to test the hypothesis that expression profiling can assist in tailored therapy is to classify glioblastoma patient-derived gliomasphere cultures based on expression profiling and study their response to targeted therapy.

Rapidly dividing cells, such as cancer cells, require a balanced supply of dNTPs to replicate and repair DNA (17). Production of nucleotides is carried out by two main pathways, the *de novo* pathway (DNP), reliant on the enzyme ribonucleotide reductase (RNR), and the salvage pathway (NSP), reliant on the enzyme deoxycytidine (dC) kinase (dCK; Fig. 1). It has been demonstrated that NSP compensates for impaired DNP (18). In fact, this compensation of NSP for impaired DNP may explain why inhibition of the DNP alone, through the addition of thymidine (dT), has failed in clinical trials (19–21). The hypothesis that combinatorial targeting of both DNP and NSP may be an efficacious treatment of cancer has been validated in a study on acute lymphoblastic leukemia (ALL) cells (22). This study utilized a novel compound, DI-39, to inhibit dCK and target NSP, as well as dT to inhibit RNR and target DNP (Fig. 1). However, this approach has not been tested in solid tumors. In this study, we sought to test

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Figure 1.

Schematic of targeting the nucleotide synthesis pathways: DNP and NSP. DI-39 is a compound that inhibits the NSP by blocking the enzyme dCK. dT is converted to dTTP that inhibits *de novo* synthesis by blocking the enzyme RNR. Together, dT + DI-39 provide a combinatorial therapy that inhibits nucleotide synthesis from both compensatory pathways.

whether dual targeting of DNP and NSP would be an effective therapeutic strategy in the treatment of glioblastoma.

Materials and Methods

Tumor collection

Twenty-three surgical resections were collected under Institutional Review Board–approved protocols and graded by neuropathologists as described previously (23). One widely used (22, 24) tumor sample was obtained from Duke University (Durham, NC) after it had been resected and placed as a xenograft.

Neurosphere culturing

Neurospheres were cultured from glioblastoma tumor samples as described previously (6, 23).

In vitro removal assays

We studied the percentage of sphere formation for cultures at clonal density (plating 50 cells/100 μ L/well of a 96-well plate) after removal from pretreatment for three days. Glioblastoma cells were pretreated for three days with DMSO, dT (1 mmol/L) + dC (2.5 μ mol/L), DI-39 (500 nmol/L) + dC, or dT + dC + DI-39. To assess colony formation, we chose to use a cell density of 20 cells per well. In preliminary studies using retroviral-labeled cells, we found that even in very highly proliferative cultures, the chance of spheres containing contributions from more than one cell were under 10%, with some cultures showing 100% clonal spheres at this cell density. Limiting dilution assays also demonstrated that 20 cells per well were on the linear portion of the curve for the vast majority of cultures tested. We measured sphere diameter using MCID image analysis (<http://www.mcid.co.uk/>).

Statistical analysis

For comparison of small groups, we used a cut-off value of $P < 0.05$ to distinguish significant differences. Statistical analyses for comparing cell proliferation, sphere formation, and sphere diameter among groups of treated cells were done in GraphPad Prism software (<http://www.graphpad.com/scientific-software/prism/>), utilizing the paired Mann–Whitney test. Analysis of tumor formation utilized χ^2 tests using STATA 8.0 software (StataCorp, <http://www.stata.com/>). Linear regression of Annexin

versus doubling time for sphere cultures was assessed for significance by GraphPad Prism software.

In vivo intracranial xenotransplantation

Animal experimentation was done with institutional approval following NIH (Bethesda, MD) guidelines using NOD/SCID mice (Jackson ImmunoResearch Laboratories, <http://www.jacksonimmuno.com>). HK308 or HK296 FLUC-GFP GBM cells [1×10^5 ; constitutively expressing a construct of luciferase (FLUC) and GFP] were injected intracranially as orthotopic xenotransplants into female 2-month-old NOD/SCID, gamma null mice as described previously (23). Each of the two pretreatment groups [DMSO or dT (1 mmol/L) + dC (2.5 μ mol/L) + DI-39 (500 nmol/L)] consisted of 4 mice. After 12 to 13 weeks, mice underwent optical imaging to assess tumor size. Bioluminescent imaging of the tumors was carried out. Following perfusion with 4% paraformaldehyde, brains were removed, postfixed, and sunk in sucrose prior to sectioning on a cryostat. GFP signal for each tumor section was visualized and photographed to validate the bioluminescent imaging.

Optical imaging

Optical imaging was performed at the Crump Institute for Molecular Imaging at the University of California Los Angeles (Los Angeles, CA). Mice were anesthetized by inhalation of isoflurane. Intraperitoneal injection of 100 μ L of luciferin (30 mg/mL) was followed by 10 minutes of live uptake to interact with the luciferase-expressing FLUC–GFP cells and produce bioluminescence. The IVIS Lumina 2 Imaging System (Caliper Life Sciences) was utilized for *in vivo* bioluminescent imaging. A photograph of the mice is overlaid with a color scale of a region of interest representing total flux (photon/second) and quantified with the Living Image software package (Xenogen).

FACS analysis

For cell-cycle analyses, total DNA content was determined using 20 μ g/mL propidium iodide containing 5 μ g/mL RNase A. Annexin V staining was performed according to the manufacturer's instructions (Becton Dickinson). All flow cytometry data were acquired on a four-laser LSRII cytometer (Becton Dickinson) and analyzed using FlowJo v7.6.5 (Tree Star). Annexin V flow

cytometry data were normalized to the vehicle control for each respective cell line.

Apoptotic response to dual treatment by dT + DI-39

After three days of treatment with dT (1 mmol/L) + dC (2.5 μ mol/L) + DI-39 (500 nmol/L), each culture tested was analyzed by FACS analysis for the percentage of cells with cell death as determined by Annexin V staining and propidium iodide. Each value was normalized to the nontreated, dC (2.5 μ mol/L) only response. Values were plotted using GraphPad Prism software.

Microarray

Concentration and quality of RNA samples were examined using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies) and the Agilent 2100 Bioanalyzer (Agilent Technologies). RNA samples were reverse transcribed and labeled according to the manufacturer's instructions and hybridized to Affymetrix high-density oligonucleotide HG-U133A Plus 2.0 Human Arrays. Microarray data analysis was performed as described previously (25). Briefly, array preprocessing was completed in the R computing environment (<http://www.r-project.org>) using Bioconductor (<http://www.bioconductor.org>). Raw data were normalized using the robust multiarray method (26). To eliminate batch effects, additional normalization was performed using the R package "ComBat" (<http://statistics.byu.edu/johnson/ComBat/>; ref. 27) with default parameters. Contrast analysis of differential expression was performed using the LIMMA package (28). After linear model fitting, a Bayesian estimate of differential expression was calculated using a modified *t* test. The threshold for statistical significance was set at $P < 0.005$ for differential expression analysis and $P < 0.01$ for explorative analyses (gene ontology and pathway analysis). Gene ontology and pathway analysis were carried out using the Database for Annotation, Visualization and Integrated Discovery and Ingenuity Pathway Analysis (IPA; www.ingenuity.com).

TFactS analysis

Sign sensitive analysis of transcription factor associations with our gene lists of interest was performed at <http://www.tfacts.org/TFactS-new/TFactS-v2/index1.html>. Transcription factors were considered to be significantly associated with gene lists if $E < 0.05$.

Gene trait correlations

Gene trait correlations and *P* values were obtained using the standard Pearson correlation coefficient *r* using the `cor()` function in R. A $P < 0.001$ threshold was used to select the most interesting candidates.

Comet assay

Cell cultures were treated with either Ctrl-H₂O or dT (1 mmol/L) + DI-39 (500 nmol/L) for three days. Comet assays were performed using OxiSelect Comet Assay Kit (Cell Biolabs, Inc) according to the manufacturer's instructions. Comet tails were counted, and a fraction of nuclei with comet tails was determined and depicted in the results. A minimum of 50 nuclei were counted per condition.

The Cancer Genome Atlas (TCGA) classification, *EGFR* amplification, *EGFRv3* status, and doubling time (proliferation rate) were determined as described previously (Laks and colleagues, 2016; ref. 29).

Results

We recently reported that the NSP, through dC metabolism, can compensate for DNP-induced lethality in T-ALL cells (30). To first test whether the NSP can compensate for DNP inhibition in glioblastoma cells, we treated the gliomasphere culture, HK157, with dT in the absence of dC. Upon the addition of dT, cell death rose to 28.8% (Fig. 2A, second panel). However, the addition of dC significantly abrogated apoptosis (8.2%; Fig. 2A, third panel). To confirm that the NSP is responsible for mitigating dT-induced apoptosis, we treated HK157 cells with the dCK inhibitor, DI-39, in the presence of dT and dC. The addition of DI-39 restored apoptosis (30.8%), suggesting that similar to T-ALL, glioblastoma can utilize the NSP to compensate for DNP inhibition. There was no effect of DI-39 monotherapy alone on apoptotic measures (Annexin V) or on cell-cycle arrest of either resistant or sensitive cell cultures (data not shown).

There is a differential response to combinatorial treatment of dT + DI-39 across a panel of glioblastoma cultures (Fig. 2B). The normalized Annexin response (apoptotic index) is plotted with the sensitive cell cultures situated on the right side of the graph, and the resistant cell cultures situated on the left side of the graph (Fig. 2B). Apoptotic response (Annexin V) to DI-39 was not related to *EGFRv3* in culture (χ^2 , $P = 0.143$), *EGFR* amplification in tumor sample (χ^2 , $P = 0.247$), *PTEN* status in culture (χ^2 , $P = 0.776$), or TCGA classification of culture (χ^2 , $P = 0.080$). In addition, linear regression indicated that apoptotic response (Annexin V) to DI-39 was not significantly related to the baseline proliferation rate of the cultures in the absence of drug ($P = 0.0963$).

Each cell culture was processed for RNA content and assessed by microarray analysis to generate gene expression levels prior to treatment with DI-39. We performed gene by gene correlation analysis with the Annexin response (sensitivity) to treatment with dT + DI-39 and generated a list of 1,536 genes that were associated with response ($P < 0.001$). Next, we used Qiagen's IPA (www.qiagen.com/ingenuity) to distinguish canonical pathways associated ($P < 0.05$) with this list of genes. IPA determined 66 canonical pathways associated with response, including ErbB2-ErbB3 signaling (undetermined direction of association), *PTEN* signaling (associated with resistance, Z-score = -1.291), PI3K/AKT signaling (associated with sensitivity, Z-score = 0.832), and the nucleotide excision repair pathway (undetermined direction of association, Supplementary Table S1). We analyzed the list of genes associated with DI-39 response in TFactS (www.tfacts.org), in a sign sensitive manner, and the only transcription factor predicted to be associated with this list was *PAX3* ($E = 0.01734$). *PAX3* was predicted to be inhibited in relation to the list of genes, meaning that the activation of *PAX3* is associated with resistance to treatment by dT + DI-39.

Although dual treatment of DI-39 and dT effectively induced S-phase delay in all cultures treated, only certain sensitive cultures responded with an increase in cell death (Fig. 3A and B). For example, combined targeting of DNP (with dT) and NSP (with DI-39) in HK296 glioblastoma cells promoted S-phase delay (Fig. 3A, top right), but no lethality (Fig. 3A, bottom right). In contrast, the sensitive culture, HK308 cells, responded to dual treatment with delay in S-phase (Fig. 3B, top right) and with a dramatic increase in cell death by apoptosis (Fig. 3B, bottom right). Furthermore, cell death response was not related to the amount of DNA damage induced by treatment (Fig. 3C and D). Both the sensitive culture,

A HK157 **B** **Figure 2.**

Differential response to DI-39 treatment. A, FACS analysis plots of apoptosis (Annexin V) demonstrate an important role for the NSP in glioblastoma (GBM). The y-axis displays propidium iodide that labels DNA, and the x-axis displays Annexin V that labels apoptotic markers on cells. The first panel (far left) is vehicle (DMSO) treatment of HK157 glioblastoma cells and displays 4.91% basal levels of cell death (top right quadrant). The next panel is dT (1 mmol/L)-treated cells and displays 28.8% cell death upon inhibition of *de novo* nucleotide synthesis (by dT) in the absence of dC, which, if present, would activate the NSP. The next panel displays a rescue of the effect (only 8.18% cell death) of dT (inhibition of DNP) by the addition of dC (2.5 μ mol/L) that activates the NSP. The panel on the far right displays the effect of combined inhibition of DNP and NSP for nucleotide synthesis with 30% cell death, as the addition of DI-39 (500 nmol/L) inhibits the NSP that would otherwise be activated by the addition of dC. B, differential response across a panel of 23 glioblastoma cultures to combinatorial targeting of DNP and NSP for nucleotide synthesis. The x-axis displays the identification numbers for each patient-derived human glioblastoma culture. The y-axis displays the percentage of Annexin V from FACS analysis normalized to vehicle treated controls. Each measurement represents the mean $\pm$ SEM from two separate experiments. Each culture was treated for three days with 1 mmol/L dT, 2.5 μ mol/L dC, and 500 nmol/L DI-39. This plot displays cultures that are sensitive to this treatment (right) and those that are resistant to this treatment (left).

HK308, and the resistant culture, HK296, showed equivalent increased levels of DNA damage as demonstrated by comet assay after three days of treatment with DI-39 + dT ($P < 0.0001$ for both increases, Mann-Whitney test; Fig. 3C and D). For HK296, the resistant culture, there was an increase in comet tails of 51.9% upon treatment as compared with control, and for HK308, the sensitive culture, there was an increase in comet tails of 62.1% upon treatment as compared with control. The fraction of comet tails was not significantly different between HK296 and HK308-treated cultures ($P = 0.2724$, Mann-Whitney test).

In glioblastoma cultures sensitive to treatment by dT + DI-39, clonal sphere formation was impaired after pretreatment, whereas in resistant glioblastoma cultures, clonal sphere formation was slightly increased after pretreatment (Fig. 4). We performed removal assays, where cells were pretreated with control dC (2.5 μ mol/L), dT (1 mmol/L) + dC, DI-39 (500 nmol/L) + dC, or dT + DI-39 + dC. After pretreatment, cells were seeded at clonal density (20 cells/well of a 96-well plate) and allowed to form sphere colonies in the absence of treatment. Clonal sphere diameter was not changed by pretreatment in any glioblastoma cell culture tested (Fig. 4A, C, E, and G). However, in cultures that were sensitive to dT + DI-39 treatment, sphere formation was significantly reduced after pretreatment with dT + DI-39 (Fig. 4B, D,

and F). In the glioblastoma culture tested that was resistant to treatment by dT + DI-39, sphere formation increased after pretreatment with dT + DI-39 (Fig. 4H). These data indicate that dT + DI-39 depletes the cells responsible for sphere formation in the subset of sensitive cultures but slightly enriches for sphere-forming cells in at least one culture that was resistant to treatment.

To determine whether *in vivo* tumor initiation is affected with treatment, we performed intracranial tumor-initiating assays after pretreatment with dT and DI-39. Tumor formation and tumor size were reduced after pretreatment with dT + DI-39 in a sensitive glioblastoma cell culture, but not in a resistant glioblastoma culture (Fig. 5). In HK308 cells, a culture that is sensitive to DI-39 + dT treatment *in vitro*, orthotopic xenotransplanted cells that were pretreated with DI-39 + dT formed tumors that were significantly smaller than cells pretreated with control-dC ($P = 0.0286$, Mann-Whitney test; Fig. 5A). Only one fourth of the mice transplanted with DI-39-pretreated HK308 cells formed detectable tumors, whereas all of the mice transplanted with control-dC-treated HK308 cells formed tumors, and this represents a significant reduction in tumor formation due to DI-39 pretreatment ($P = 0.028$, χ^2 test; Fig. 5B). Figure 5C illustrates the GFP-labeled tumor cells at the site of the tumor bulk/injection site. In these representative images of the treatment groups, the

Figure 3.

Dual targeting of DNP and NSP for nucleotide synthesis results in S-phase delay and, in certain sensitive glioblastoma cultures, apoptosis and cell death. All treatments are three days. A, in a resistant glioblastoma culture, HK296, combinatorial targeting of DNP and NSP results in S-phase delay, but not in a substantial increase in cell death. In the top two panels, the x-axis displays propidium iodide that indicates DNA levels in each cell. The y-axis displays the cell count. The first panel on the left hand side displays a normal distribution for the cell cycle, with most cells at 2N (85 K) and a minority at 4N (170 K), with few cells in between (S-phase). However, in the top panel to the right, upon the inhibition of DNP and NSP with the addition of dT and DI-39, the cell cycle is disrupted and there is an S-phase delay with a buildup of cells in between 2N and 4N. Despite this S-phase delay, the FACS analysis plots below display no substantial increase in cell death. B, in contrast, in a sensitive glioblastoma culture, HK308, the S-phase delay is accompanied by a substantial increase in cell death upon combinatorial inhibition by dT and DI-39. C, representative images of comet assay tails after three-day treatment conditions of Ctrl-H₂O-treated cells or DI-39 (500 nmol/L) + dT (1 mmol/L). D, equivalent increase in DNA damage between sensitive and resistant cell cultures after treatment (both changes were $P < 0.0001$, Mann-Whitney test). Quantification of the fractions of comet tails under each condition (same conditions as in Fig. 2D) after three days treatment is shown. Results are mean $\pm$ SEM. Ctrl, control.

pretreatment group formed no discernible tumor. These sections validated the optical imaging in Fig. 5B. In HK296 culture, a resistant culture *in vitro*, orthotopic xenotransplanted cells that were pretreated with DI-39 + dT formed tumors that were equivalent in size to cells pretreated with control-dC (Fig. 5D). In addition, all mice formed tumors regardless of pretreatment (Fig. 5E). Sectioning of the tumors validated the measurements generated by optical imaging (Fig. 5F). These findings confirm

that *in vitro* treatment with DI-39 + dT selectively depletes tumor-initiating cells in a sensitive culture, but not in a resistant culture.

Discussion

Effective inhibition of nucleotide synthesis requires combinatorial dT + DI-39 therapy for targeting both the DNP and the compensatory NSP, respectively. In this study, we demonstrate

A Sensitive lines (high Annexin response) **B** **C** **D** **E** **F** **G** Resistant lines (low Annexin response) **H** **Figure 4.**

Combinatorial targeting of DNP and NSP for nucleotide synthesis targets brain tumor stem cells in sensitive glioblastoma cultures, but not in resistant glioblastoma cultures. Results from removal assays, where cells are treated for 3 days and then removed into untreated conditions under clonal conditions (20 cells/well) until spheres form (14–21 days), are shown. Each plot represents two separate experiments. Each condition included 2.5 $\mu\text{mol/L}$ of dC to activate the NSP. A, sphere diameter does not change upon treatment in HK157 cells. Sphere diameter ($\mu\text{mol/L}$) for each measurement is depicted along with the mean and SEM. B, sphere formation is reduced in HK157 cells upon combinatorial treatment of dT (1 mmol/L) + DI-39 (500 nmol/L) in the presence of dC (2.5 $\mu\text{mol/L}$; $P = 0.0005$, t test). C, sphere diameter in HK374 cells cannot be measured as no spheres formed. D, sphere formation is reduced in HK374 cells upon combinatorial treatment of dT (1 mmol/L) + DI-39 (500 nmol/L) in the presence of dC (2.5 $\mu\text{mol/L}$; $P = 0.0176$, t test). E, sphere diameter does not change in HK308 cells upon treatment. F, sphere formation is reduced in HK308 cells upon combinatorial treatment of dT (1 mmol/L) + DI-39 (500 nmol/L) in the presence of dC (2.5 $\mu\text{mol/L}$; $P = 0.001$, t test). G, sphere diameter is not changed upon treatment in HK296 cells. H, sphere formation is increased upon treatment with dT + DI-39 in HK296 cells ($P = 0.003$, t test). Results are mean $\pm$ SEM.

that this therapeutic strategy targets sphere and tumor-initiating cells and successfully abrogates tumor formation in a subset of glioblastoma that are sensitive to treatment. This indicates that treatment with dT + DI-39 is effectively targeting BTSC in certain sensitive glioblastoma. Importantly, the difference between sensitive and resistant glioblastoma cultures is not dependent upon the degree of S-phase delay induced by treatment. Indeed, both

sensitive and resistant glioblastoma cell cultures respond to a similar degree with dramatic S-phase delay. Despite the similarity in S-phase delay in response to treatment, a differential response to DI-39 + dT treatment exists across our tested panel of glioblastoma in terms of apoptotic cell death.

Our findings highlight the importance of testing potential therapeutics across a spectrum of glioblastoma. One means that

Figure 5.

In sensitive glioblastoma cultures HK308, *ex vivo* treatment with dT + DI-39 depletes cultures of BTSCs and drastically reduces tumor formation upon intracranial xenotransplantation. In contrast, in resistant culture HK296, there is no difference in tumor formation or tumor size after treatment. A, DI-39 + dT *ex vivo* pretreatment results in significantly less tumor burden in the sensitive glioblastoma culture HK308 ($P = 0.0286$, Mann-Whitney test). This plot shows quantification of individual measurements of tumor bioluminescent imaging after 12 weeks, with the mean and SEM depicted as bars. B, images of mice and their bioluminescent signals from transplanted cells expressing luciferase via lentiviral infection. Only one mouse out of four in the treated group has detectable levels of tumor cells, while the control group has uniform detection of large tumors. C, representative sections of GFP-expressing tumor cells from control (Ctrl) and dT + DI-39-pretreated cells display a large tumor in the striatum of the control and no tumor in the brain of the pretreated cells. D, quantification of tumor bioluminescence indicates that DI-39 + dT *ex vivo* pretreatment results in no change in tumor burden in the resistant glioblastoma culture HK296 ($P = 0.686$, Mann-Whitney test). This plot shows individual measurements of tumor bioluminescent imaging after 13 weeks, with the mean and SEM depicted as bars. E, images of mice and their bioluminescent signals from transplanted cells expressing luciferase via lentiviral infection. Both the control group and pretreated group have uniform detection of large tumors. F, representative sections of GFP-expressing tumor cells from control and dT + DI-39-pretreated cells display large tumors in the striatum of both the control and pretreated cells. Results are mean $\pm$ SEM.

might be used to achieve this goal would be to divide samples according to their classification groups as set out by Verhaak and colleagues (16) using the TCGA dataset or according to their key oncogenic mutations. However, our findings did not reveal group differences in response according to these schemes, suggesting that other means will need to be used to prospectively identify sensitive or resistant cohorts. A goal of future studies is to distinguish biomarkers that define the subset of glioblastoma, which are sensitive to this treatment strategy. Our gene by gene correlation with the percentage Annexin response to treatment indicated that response is related to the nucleotide excision repair pathway. This may provide a clue to the mechanisms of differential response by glioblastoma cultures to this treatment, as sensitivity may be dependent upon the cell's response to DNA damage rather than to the DNA damage itself. In support of this hypothesis, our comet assay data demonstrate an equivalent amount of DNA damage in both sensitive and resistant glioblastoma cultures. Although both sensitive and resistant cultures demonstrate equivalent DNA damage and S-phase delay, the responses to such stress and the ultimate fates of the cells differ considerably.

Our bioinformatics analysis indicated that resistance to treatment was related to activation of the transcription factor, *PAX3*. *PAX3* has been shown to regulate tumorigenicity of glioblastoma and, moreover, inhibit apoptosis (31). Perhaps, this role of *PAX3* in inhibiting apoptosis is associated to response to DNA damage induced by impaired nucleotide synthesis. Further work is necessary to determine whether *PAX3* serves a functional role in determining response to inhibition of nucleotide synthesis in glioblastoma. A previous study has demonstrated that *PAX3* depletion increases cytotoxicity of the DNA-damaging agent cisplatin (32). Our data are consistent with this role for *PAX3* in mediating apoptotic cell death in response to impaired DNA.

In our study, we were unable to test these hypotheses that *in vivo* treatment may reduce malignancy in sensitive glioblastoma but increase malignancy in resistant glioblastoma, as DI-39 has poor blood brain barrier penetration, and dT does not sufficiently penetrate to achieve therapeutic concentrations in experimental brain tumors (not shown). To develop inhibition of NSP as a therapeutic approach, we will need to design replacements for DI-39 and dT that can target brain tumors *in vivo*. However, the findings presented here represent a compelling foundation for the

role of nucleotide synthesis inhibition in targeting BTSC, upon which to build further investigations, with the objective of reducing tumor recurrence and malignancy in human patients.

Disclosure of Potential Conflicts of Interest

C.G. Radu has ownership interest (including patents) in Sofie Biosciences and Trethera Therapeutics. D.A. Nathanson has ownership interest (including patents) in Trethera Corporation. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: D.R. Laks, L. Ta, D.A. Nathanson, H.I. Kornblum
Development of methodology: D.R. Laks, L. Ta, C.G. Radu, D.A. Nathanson
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D.R. Laks, L. Ta, D.A. Nathanson
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Writing, review, and/or revision of the manuscript: D.R. Laks, L. Ta, D.A. Nathanson, H.I. Kornblum

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D.R. Laks, L. Ta, T.J. Crisman, C.G. Radu
Study supervision: L. Ta, T.J. Crisman, H.I. Kornblum

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