Dual Inhibition of PI3K/AKT and MEK/ERK Pathways Induces Synergistic Antitumor Effects in Diffuse Intrinsic Pontine Glioma Cells[1]

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Abstract

Diffuse intrinsic pontine glioma (DIPG) is a devastating disease with an extremely poor prognosis. Recent studies have shown that platelet-derived growth factor receptor (PDGFR) and its downstream effector pathway, PI3K/AKT/mTOR, are frequently amplified in DIPG, and potential therapies targeting this pathway have emerged. However, the addition of targeted single agents has not been found to improve clinical outcomes in DIPG, and targeting this pathway alone has produced insufficient clinical responses in multiple malignancies investigated, including lung, endometrial, and bladder cancers. Acquired resistance also seems inevitable. Activation of the Ras/Raf/MEK/ERK pathway, which shares many nodes of cross talk with the PI3K/AKT pathway, has been implicated in the development of resistance. In the present study, perifosine, a PI3K/AKT pathway inhibitor, and trametinib, a MEK inhibitor, were combined, and their therapeutic efficacy on DIPG cells was assessed. Growth delay assays were performed with each drug individually or in combination. Here, we show that dual inhibition of PI3K/AKT and MEK/ERK pathways synergistically reduced cell viability. We also reveal that trametinib induced AKT phosphorylation in DIPG cells that could not be effectively attenuated by the addition of perifosine, likely due to the activation of other compensatory mechanisms. The synergistic reduction in cell viability was through the pronounced induction of apoptosis, with some effect from cell cycle arrest. We conclude that the concurrent inhibition of the PI3K/AKT and MEK/ERK pathways may be a potential therapeutic strategy for DIPG.

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Introduction

Diffuse intrinsic pontine glioma (DIPG), diagnosed in children at a median age of 6 to 7 years, accounts for approximately 15% of all malignant pediatric central nervous system tumors and is the most common pediatric brainstem tumor [1,2]. Despite efforts over the past several decades, the prognosis for children with DIPG remains dismal, with a median survival of less than 1 year [1,2]. Due to the diffusely infiltrative nature of DIPGs, radiation therapy remains the standard of care, although its benefits are not durable [3]. In addition, repeated clinical trials investigating various adjuvant chemotherapies failed to improve patient outcomes long term when compared to radiotherapy alone [3,4].

A key barrier to the development of effective therapies has been a limited understanding of DIPG biology. Now, genomic and molecular data have become increasingly available due to a rise in diagnostic biopsies and autopsy programs. In particular, amplifications in the receptor tyrosine kinase (RTK)/PI3K/AKT/mTOR signaling pathway have been identified in approximately 50% of DIPGs, with platelet-derived growth factor receptor alpha (PDGFRA) as the most commonly amplified RTK [5]. Amplification of this pathway contributes to the aggressive phenotypic characteristics of this tumor [6].

Although the PDGFR/PI3K/AKT/mTOR signaling pathway presents potential druggable targets, inhibition of this pathway alone has thus far proven to produce insufficient clinical responses in trials investigating multiple malignancies including lung, gynecological, prostate, colorectal, and bladder cancers [7–11]. In DIPG, clinical trials using molecularly targeted therapies against RTKs, such as EGFR, or other signal transduction effectors also have not conferred any clinical advantage over other historical trials or radiation therapy alone [12,13]. In addition, activated PDGFR transduces signals through many downstream pathways other than PI3K/AKT that play important roles in tumorigenesis, including Src kinase, PLC/PKC, and Ras/Raf/MEK/ERK pathways [14]. The MEK/ERK pathway is concurrently activated with the PI3K/AKT pathway in multiple human cancers [15], including gliomas [16]. Both pathways are frequently mutated or amplified, which constitutively activates proliferation and survival signals that ultimately lead to tumorigenesis. Although there is currently no direct evidence that there is concurrent activation of both pathways in the same DIPG sample, extensive nodes exist that facilitate cross-talk between these two signaling pathways, and they act as barriers to molecularly targeted therapy using single agents [17]. Inhibition of one pathway induces compensatory signaling in the other, mediating treatment resistance [18,19]. As a result, combination therapy with PI3K/AKT and MEK/ERK pathway inhibitors may be an effective therapeutic strategy and has been studied in many cancer types, with particular success in *BRAF*-mutated melanoma and renal cell carcinoma [15]. However, this combination has not been explored in DIPG.

The aim of this study is to investigate the effectiveness of combinatorial therapy targeting these two signaling pathways with perifosine, a PI3K/AKT inhibitor, and trametinib, a MEK inhibitor, on DIPG. Perifosine, which had been promising in phase II clinical trials for multiple myeloma and colorectal cancer but not phase III trials [20], is currently being tested in combination with other chemotherapeutics in adult gliomas. Previously, perifosine was found to induce cell death but not increase survival in a mouse model of high-grade brainstem gliomas [21]. Trametinib, approved for melanoma, is currently undergoing clinical trials for multiple tumor types, including brain metastases. In this study, we show that the combination of perifosine and trametinib at their GI_{50} concentrations in patient-derived DIPG cell lines *in vitro* synergistically inhibits tumor cell proliferation and induces cell death. We also show that the inhibition of both pathways simultaneously may not be sufficient to suppress AKT phosphorylation, suggesting the activation of other compensatory pathways.

Materials and Methods

Cell Culture

The patient-derived DIPG cell lines, SU-DIPG-IV and SU-DIPG-XIII, were obtained from the laboratory of Dr. Michelle Monje (Stanford University School of Medicine, Stanford, CA). SU-DIPG-IV cells harbored an H3.1K27M mutation, *MDM4* amplification, and *ACVR1* G328V mutation, while SU-DIPG-XIII only exhibited an H3.3K27M mutation. Both cell lines were *P53* wild type (Supplemental Table) [22]. All cells were cultured as neurospheres in tumor stem media containing Neurobasal-A (Thermo Fisher, Waltham, MA), antibiotic-antimycotic (Thermo Fisher), B-27 supplement minus vitamin A (Thermo Fisher),

human-bFGF (20 ng/ml) (Shenandoah Biotechnologies, Warwick, PA), human-EGF (20 ng/ml) (Shenandoah), human PDGF-AB (20 ng/ml) (Shenandoah), and heparin (10 ng/ml). All cells were kept in a humidified atmosphere at 37°C with 5% CO_2 and were passaged weekly.

Drugs

Perifosine was purchased from LC Laboratories (Woburn, MA), and trametinib was provided by the Developmental Therapeutics Program at the National Cancer Institute (Bethesda, MD). Stock concentrations of perifosine were prepared in Dulbecco's modified Eagle's medium at 10 mM and diluted to working concentrations in tumor stem media. Stock trametinib was prepared in DMSO at 10 mM and diluted to working concentrations in tumor stem media. Stock concentrations were stored in aliquots at –20°C.

Cell Viability Assay, GI_{50}, and Combination Index Determination

Five thousand cells per well were seeded in 96-well plates overnight prior to drug treatment. Cell viability was assessed 72 hours after drug treatment in triplicates at concentrations indicated in each experiment. Drugs were reconstituted in DMSO, and cell viability was assessed through quantification of ATP levels (CellTiter-Glo Luminescent Cell Viability Assay, Promega, Madison, WI) according to the manufacturer's recommended protocol. Luminescence values were normalized to vehicle control, and GI_{50} values were determined as the dose at which cell viability was decreased by 50% compared to control. Combination Index (CI) was calculated by the Chou-Talalay method [23], and synergy assessment was performed using CalcuSyn software (Biosoft, Cambridge, UK). CI values were interpreted as follows: less than 1 is synergism, equal to 1 is additivity, and greater than 1 is antagonism.

Apoptosis Assay

Cells were treated with GI_{50} concentrations of the drugs alone or in combination. Caspase-3/7 activity within the cells was measured 4 hours after drug treatment using Caspase-Glo 3/7 Assay (Promega) according to the manufacturer's protocol. Luminescence values were normalized to untreated control and presented as fold increases of control.

Cell Cycle Analysis

Cells treated with indicated concentrations of perifosine, trametinib, or their combination for 24 hours were trypsinized and fixed in 70% ethanol at –20°C overnight. After centrifugation and washing with PBS, cells were resuspended in FxCycle PI/RNase Staining Solution (Thermo Fisher; #F10797) containing propidium iodide dye and ribonuclease A and incubated for 30 minutes at room temperature, protected from light. DNA content was measured by flow cytometry using FACSCanto (BD Biosciences, San Jose, CA), and at least 10,000 cells per sample were analyzed. Cell cycle population percentages were calculated using FlowJo software (Ashland, OR; version 9).

Western Blot Analysis

Cells were grown to ~70% confluence, and drug doses were added based on GI_{50} data determined from cell viability assays. Lysates from cells cultured for 24 and 48 hours were prepared by washing with cold PBS followed by lysis with modified RIPA buffer (150 mM NaCl, 50 mM Tris-HCl [pH 8], 1% NP-40, 0.5% Na deoxycholate, and 0.1% SDS) supplemented with protease and phosphatase inhibitor cocktail

(Cell Signaling Technology, Danvers, MA). Protein concentrations were determined using Bradford assay (Alfa Aesar, Haverhill, MA). Lysates were resolved by SDS-PAGE on 4% to 20% polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA), transferred on PVDF membranes (Thermo Fisher), and blocked for an hour with 5% blotting-grade blocker (Bio-Rad) in TBS/0.1% Tween-20. To probe the membrane, the following primary antibodies from Cell Signaling Technology were used: p-AKT^{Ser473} (1:1000; #4060), p-AKT^{Thr308} (1:1000; #13038), p-ERK1/2$^{Thr202/Tyr204}$ (1:1000; #4370), total ERK1/2 (1:1000; #4695), and GAPDH (1:1000; #5174). Total AKT1 (#60203-1-lg) diluted at 1:1000 was obtained from Proteintech (Rosemont, IL). Appropriate secondary antibodies conjugated with horseradish peroxidase (Abcam, Cambridge, UK) were incubated in blocking buffer at room temperature for 1 hour. Detection of protein-antibody complexes was performed with Amersham ECL kit according to the manufacturer's recommended protocol (GE Healthcare, Little Chalfont, UK). Quantification of immunoreactive signals was completed using ImageJ software (National Institutes of Health, Bethesda, MD), and the relative ratio of activated protein to loading control (GAPDH) was calculated for each experiment.

Statistical Analysis

All data represented here are mean ± SEM of at least three independent experiments, unless indicated otherwise. Analysis of variance followed by Tukey's *post hoc* test were used to compare effects of treatment between groups. Statistical analyses were performed using GraphPad Prism software (La Jolla, CA; Version 7). Differences with $P < .05$ were considered statistically significant.

Results

### Effects of Combinatorial Therapy on DIPG Cell Viability

We first evaluated the effects of perifosine and trametinib alone on DIPG cell viability. Patient-derived SU-DIPG-IV and SU-DIPG-XIII cells were exposed to perifosine (dose range 0.5 μM-1 mM) or trametinib (dose range 5 nM-10 μM), and cell viability was assessed 72 hours after drug treatment. We observed a concentration-dependent decrease in cell viability, with a GI$_{50}$ concentration of perifosine determined to be 10 μM in SU-DIPG-IV

and 45 μM in SU-DIPG-XIII (Figure 1A). The GI$_{50}$ concentration of trametinib was 0.25 μM in SU-DIPG-IV and 0.5 μM in SU-DIPG-XIII (Figure 1B). Overall, SU-DIPG-IV cells were more sensitive to both perifosine and trametinib than SU-DIPG-XIII cells, suggesting a cell line–specific effect.

Next, the effect of perifosine and trametinib together was investigated by combining the two drugs at their GI$_{50}$ concentrations. In SU-DIPG-IV cells (Figure 2A), combinatorial therapy with perifosine and trametinib significantly reduced cell viability (29.36%) compared to either perifosine (43.98%; $P = .02$) or trametinib alone (50.56%; $P = .003$). Similarly, combinatorial therapy in SU-DIPG-XIII cells significantly decreased cell viability (23.97%) compared to each drug individually (46.33% with perifosine, $P = .04$; 46.41% with trametinib, $P = .04$). The Chou-Talalay median-effect method [24] using CI was then used to define the nature of this drug interaction. In SU-DIPG-IV cells, the CI of perifosine and trametinib at their respective GI$_{50}$ concentrations was found to be 0.85 (Figure 2A, *inset*). The CI with perifosine and trametinib combined in SU-DIPG-XIII was 0.76 (Figure 2B, *inset*). These results indicate that the combination of perifosine and trametinib at GI$_{50}$ concentrations is synergistic in both DIPG cell lines.

Effects of Combinatorial Therapy on PI3K/AKT and MEK/ERK Pathway Activation

One of the purposes of combinatorial therapy with perifosine and trametinib is to inhibit two cell proliferation and survival pathways, PI3K/AKT and MEK/ERK. To understand the molecular effects of combinatorial therapy, we measured the levels of AKT and ERK phosphorylation by immunoblotting to determine whether this drug combination effectively suppressed activation of these two signal transduction pathways. The phosphorylation status of AKT and ERK was used to assess the targeting efficacy of perifosine and trametinib, respectively. After 24 hours of drug treatment, perifosine alone effectively reduced AKT phosphorylation in both SU-DIPG-IV (Figure 3A) and SU-DIPG-XIII (Figure 3C) cells. Trametinib alone increased AKT phosphorylation in both cell lines (Figure 3). Interestingly, perifosine and trametinib in combination failed to suppress AKT phosphorylation to levels seen in either the perifosine alone or control cells at both 24-hour and 48-hour time points (Figure 3), and no difference in phosphorylation was observed at
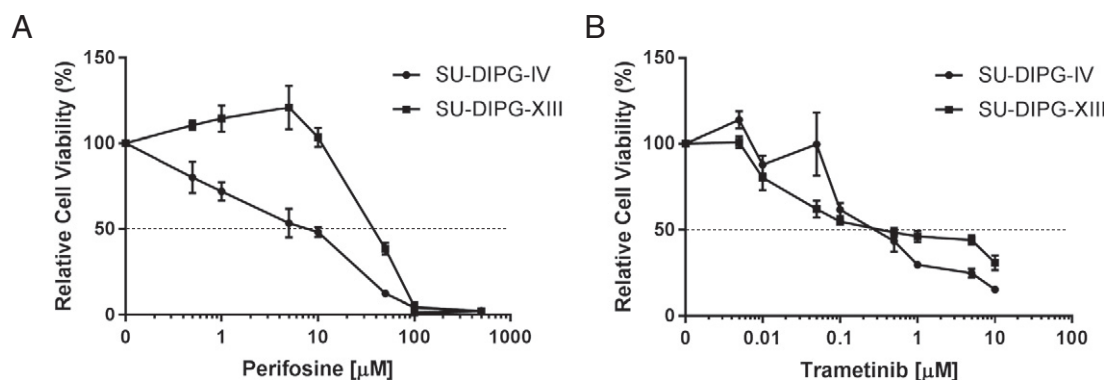
Figure 1. Treatment with perifosine and trametinib dose-dependently decreases DIPG cell viability. Cell viability with perifosine (A) and trametinib (B) was determined in two DIPG cell lines, SU-DIPG-IV and SU-DIPG-XIII, using CellTiter-Glo luminescent assay and relative to DMSO-treated controls. Cells were incubated in tumor stem cell medium for 72 hours with drugs at increasing concentrations. Data are expressed as mean ± SEM of at least three independent experiments done in triplicate. Dashed line, 50% growth inhibition (GI$_{50}$).
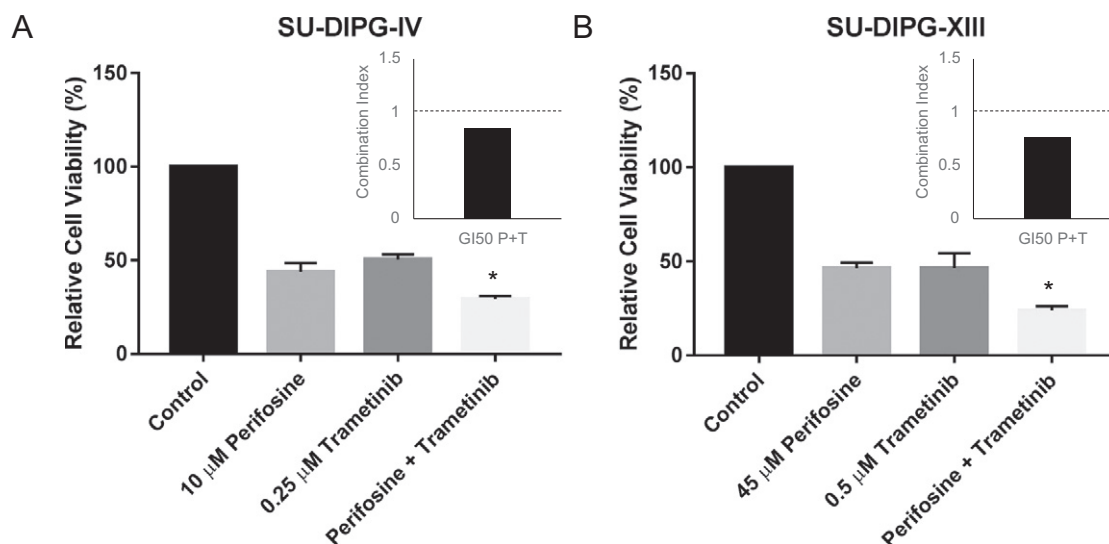
Figure 2. Combinatorial therapy with perifosine and trametinib synergistically decreases DIPG cell viability. GI_{50} concentrations of perifosine, trametinib, or both were used to treat SU-DIPG-IV (A) and SU-DIPG-XIII (B) cell lines for 72 hours. Cell viability was measured using CellTiter-Glo luminescent assay. Data expressed are mean ± SEM of three independent experiments done in triplicate. *$P < .05$ compared to drugs alone and to control. Combination indices calculated for GI_{50} concentrations of perifosine and trametinib in combination are shown in inset for each cell line, where CI < 1 indicates synergy.

the Thr308 site (Supplemental Figure 1). Previous studies showed that inhibition of AKT phosphorylation with a dual PI3K/mTOR inhibitor (NVP-BEZ235) can be overcome by 24 hours in some cell lines [25]. Therefore, an additional 4-hour time point was evaluated, and a 10-fold higher concentration of perifosine in combination with trametinib was assessed at this time point. We noticed no substantial inhibition in AKT phosphorylation (Supplemental Figure 2).

Next, we evaluated MEK/ERK pathway activation using levels of ERK phosphorylation, a downstream target of MEK that trametinib inhibits [26]. In both SU-DIPG-IV and SU-DIPG-XIII cells, trametinib alone effectively inhibited ERK phosphorylation (Figure 3). Perifosine alone had no substantial effect on ERK phosphorylation in SU-DIPG-IV cells (Figure 3, A and B) but induced ERK phosphorylation in SU-DIPG-XIII cells (Figure 3, C and D), as previously reported [19]. However, the combination of perifosine and trametinib resulted in the suppression of ERK phosphorylation to levels seen with trametinib alone in both cell lines (Figure 3). Results were similar between the two time points (24 and 48 hours), and densitometric analysis of AKT and ERK phosphorylation levels supported these findings (Figure 3, B and D). Combinatorial therapy with perifosine and trametinib effectively suppressed the MEK/ERK pathway but incompletely inhibited PI3K/AKT pathway activation in these DIPG cell lines. Thus, inhibition of components of these proliferative pathways likely cannot fully explain the synergistic decrease in cell viability observed.

Effects of Combinatorial Therapy on Caspase-Mediated Apoptosis and Cell Cycle Arrest

To investigate the mechanism underlying the synergistic reduction in cell viability with perifosine and trametinib, we examined this

combination's effect on apoptosis by measuring caspase-3/7 activity levels, as described in Materials and Methods. SU-DIPG-IV and SU-DIPG-XIII cells were treated with perifosine, trametinib, or both at their respective GI_{50} concentrations for 4 hours. In SU-DIPG-IV cells (Figure 4A), combinatorial therapy with perifosine and trametinib increased caspase-dependent apoptosis from control cells 1.67-fold, which is significantly higher than the increase in apoptosis seen with either perifosine (1.21-fold, $P < .001$) or trametinib (1.34-fold, $P = .002$) alone. Similarly, combinatorial therapy with perifosine and trametinib dramatically increased caspase-mediated apoptosis 2.12-fold compared to control in SU-DIPG-XIII cells ($P < .001$; Figure 4B). In addition, the drug combination significantly increased apoptosis compared to either perifosine (1.18-fold, $P < .001$) or trametinib (1.50-fold) alone ($P = .01$; Figure 4B). Therefore, the induction of apoptosis is a significant contributor to the synergistic reduction in DIPG cell viability.

Cell cycle analysis was then conducted to investigate the effects of combinatorial therapy on cytotoxic mechanisms other than caspase-mediated apoptosis. After 24 hours of treatment with GI_{50} concentrations of perifosine, trametinib, or both, cell cycle was analyzed by propidium iodide flow cytometry. Combinatorial therapy with perifosine and trametinib significantly induced cell cycle arrest at G0/G1 phase (40.18%) of the cell cycle compared to control (31.0%; $P < .001$) and reduced the number of cells undergoing S phase replication (2.16% vs 8.92% in control, $P = .007$) in SU-DIPG-IV cells (Figure 5A). The drug combination did not significantly change G2/M phase arrest in these cells (Figure 5A). Similarly, in SU-DIPG-XIII cells (Figure 5B), perifosine and trametinib together increased G0/G1 arrest (70.80% vs 56.83% in control, $P < .001$) while reducing S phase replication compared to control (18.81% vs 28.38%, $P = .04$). In addition, combination therapy in these cells also significantly increased G2/M cell cycle arrest (23.38% vs 11.90% in control, $P = .008$; Figure 5B). In both cell
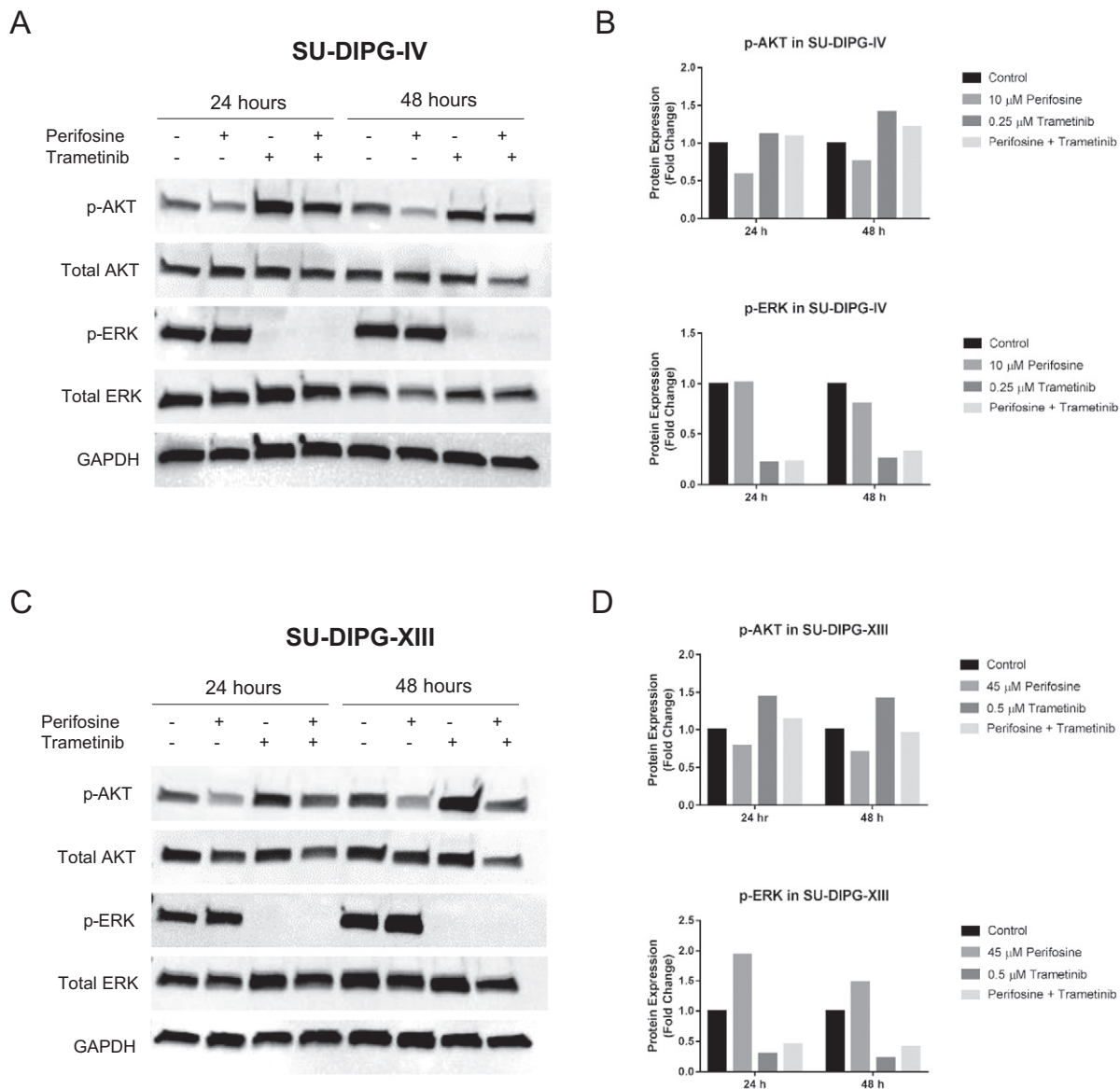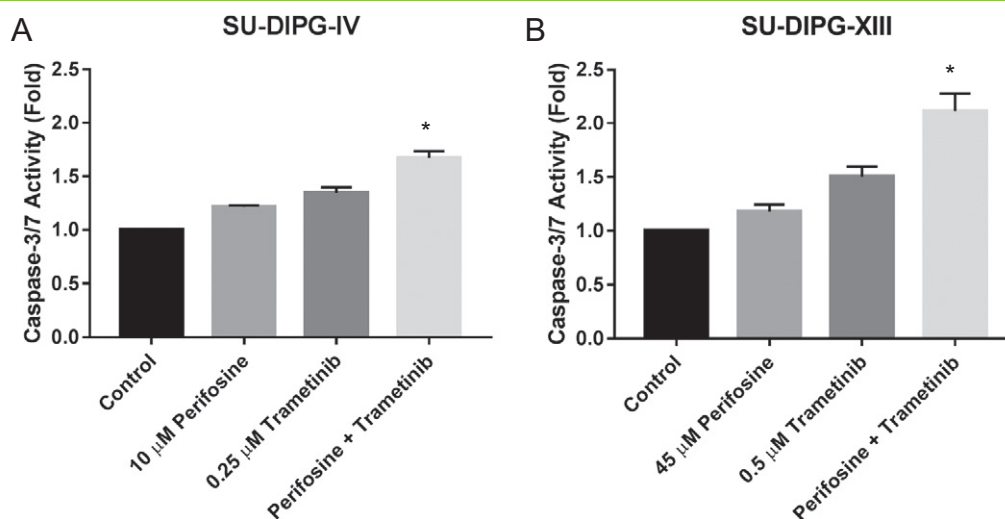
Figure 3. Perifosine and trametinib in combination decrease ERK but not AKT phosphorylation. Western blots show phosphorylation status of AKT and ERK from SU-DIPG-IV (A) and SU-DIPG-XIII (C) cells treated for 24 or 48 hours with GI_{50} concentrations of perifosine, trametinib, or both. A representative blot of at least three experiments with similar results is shown. Densitometric analysis was performed using ImageJ for each cell line (B and D), and protein expression levels of p-AKT and p-ERK were normalized to GAPDH loading control. Values shown are means of fold change from untreated control cells.

lines, we observed a significant increase in G0/G1 arrest and inhibition of proliferation with trametinib alone ($P < .0001$). No significant cell cycle arrest was noted with perifosine treatment alone, with the exception of SU-DIPG-XIII cells arresting at G2/M (Figure 5).

Discussion

DIPGs are devastating pediatric brainstem tumors with few effective therapeutic options, but recent advances have identified amplifications in the signal transduction pathway RTK/PI3K/AKT/mTOR as driving mutations for a large proportion of DIPGs. Furthermore, the PI3K/AKT/mTOR and RAS/RAF/MEK/ERK pathways are often concurrently dysregulated in human tumors, as growth factors can initiate both signaling pathways through RTKs. These two important pathways also converge downstream to regulate cell survival, growth,

and proliferation, among other key cellular functions [26]. The current study investigated the effects in human DIPG cells of perifosine, a PI3K/AKT inhibitor, and trametinib, a MEK inhibitor, in combination to demonstrate that targeting both pathways has greater therapeutic efficacy than each alone. Results indicate that this drug combination synergistically reduces cell viability in two DIPG cell lines, SU-DIPG-IV and SU-DIPG-XIII. Synergy of PI3K/AKT and MEK/ERK pathway inhibitors is well documented both *in vitro* and *in vivo* and in multiple cancer types [18,27–34]. In these DIPG cells, the induction of caspase-mediated apoptosis was a significant contributor to the synergistic inhibition of cell proliferation. Our results are consistent with prior studies in glioblastoma, lung cancer, thyroid cancer, colorectal cancer, and other tumors, which show that concomitantly inhibiting the PI3K/AKT and MEK/ERK pathways significantly induces apoptosis compared to the inhibition of either pathway alone [27–29,32,34].
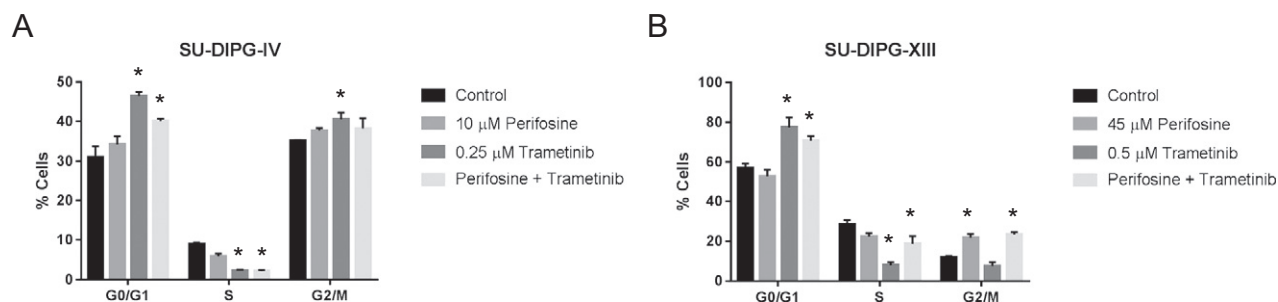
Figure 4. Perifosine and trametinib increase apoptosis. Caspase-3/7 activity was used as a measure of apoptosis in SU-DIPG-IV (A) and SU-DIPG-XIII (B) cells after 4 hours of treatment with GI_{50} concentrations of perifosine, trametinib, or both. Values are fold increases to untreated control and are shown as mean $\pm$ SEM of three independent experiments in triplicate. *$P < .05$ when compared to all other treatment groups.

Cell cycle arrest at G0/G1 and G2/M phases was also observed in a cell line–dependent manner, where the combination of perifosine and trametinib induced arrest at the G0/G1 phase in SU-DIPG-IV cells and at both the G0/G1 and G2/M phases in SU-DIPG-XIII cells. In both these cell lines, the proportion of cells undergoing S phase dropped significantly. Similar results, including the induction of G_1 arrest and a reduction in S phase fraction, have been observed with concurrent inhibition of the PI3K/AKT and MEK/ERK pathways in other cancer cell lines [29]. When induction of cell cycle arrest for each drug individually is examined, the effects of perifosine appear to be cell line–dependent, which could at least partially explain the variation in cell cycle populations between SU-DIPG-IV and SU-DIPG-XIII. Studies have also reported that perifosine induces arrest at both G1 and G2 phases of the cell cycle in different cancers, including squamous cell carcinoma and glioma [35,36]. In medulloblastoma cells, the effects of perifosine on cell cycle were different for the two cell lines investigated and were attributed to different activation pattern of various cell cycle regulatory proteins [37]. In addition, while perifosine induced G2/M arrest in hepatocellular carcinoma [38], it induced G0/G1 arrest in T-cell acute lymphoblastic leukemia [39]. In the present study, perifosine alone did not have a

significant effect on SU-DIPG-IV cells but induced G2/M arrest in SU-DIPG-XIII cells. Trametinib alone induced G0/G1 arrest in DIPG cells, which is consistent with results from prior studies [26].

Further, immunoblot analyses indicated that the synergistic action of perifosine and trametinib in DIPG cells was likely not due to molecularly targeted inhibition of their respective cell proliferation pathways. In fact, although the drug combination successfully inhibited MEK/ERK pathway activation, even when perifosine alone appeared to increase ERK phosphorylation in SU-DIPG-XIII, it failed to effectively suppress PI3K/AKT pathway activation in either cell line. Prior studies have also reported an increase in the activation of the PI3K/AKT signaling pathway with MEK inhibition in many other cancer cells [25,40]. Increasing the perifosine dose 10-fold and shortening the duration of drug exposure also failed to make a notable difference. While some studies using perifosine and a MEK inhibitor in combination found a pronounced decrease in AKT phosphorylation levels, such as those in multiple myeloma and lung cancer [33,41], other studies in colorectal and thyroid cancer cell lines did not [31,42]. These findings provide further evidence that the effects of perifosine are cell type-dependent. A study in colorectal cancer found that PI3K/AKT and MEK/ERK pathway inhibitors in

Figure 5. Perifosine and trametinib induce cell cycle arrest. Propidium iodide flow cytometry was used to show cell cycle arrest in SU-DIPG-IV (A) and SU-DIPG-XIII (B) cells after 24 hours of treatment with indicated GI_{50} concentrations of perifosine and trametinib individually and in combination. Plotted are mean $\pm$ SEM of triplicate experiments. *$P < .05$ when compared to control.

combination did not prominently suppress AKT phosphorylation, and only the dual inhibitor NVP-BEZ235 (PI3K/mTOR inhibitor) at 10 times its GI_{50} concentration effectively inhibited AKT phosphorylation when in combination with a MEK inhibitor, but the PI3K-specific inhibitor failed to produce this result [31]. It is thus likely that mTOR plays an important role in modulating cellular response to PI3K/AKT and MEK/ERK signaling. It appears that other compensatory pathways exist that may also account for the lack of inhibition of AKT phosphorylation in the presence of both perifosine and trametinib. Moreover, PI3K-independent AKT activation may be directly achieved through various other tyrosine kinases, serine/threonine kinases, and even DNA repair machinery that can phosphorylate AKT at its canonical Ser473 site [43]. Furthermore, activating mutations of *ACVR1*, a component of the BMP signaling pathway, were found in 21% of DIPGs [44] and in SU-DIPG-IV [22]. These mutations were recently demonstrated to play a role in conferring resistance to RTK inhibitors in lung cancer [45]. Future experiments to identify compensatory mechanisms by which the PI3K/AKT pathway can continue to remain active after treatment with perifosine and trametinib in combination may reveal new therapeutic targets and open avenues for further investigation.

Taken together, these results suggest that perifosine and trametinib in combination synergistically reduce cell viability in SU-DIPG-IV and SU-DIPG-XIII cells primarily via apoptosis, with some effect from cell cycle arrest. However, an important limitation of this study is the use of only two heterogeneous patient-derived cell lines, which inherently have different mutations and may not reflect all DIPGs. Investigations using other cell lines may be necessary to increase generalizability of the presented data. The choice of perifosine, which was found to have heterogeneous cell line-dependent effects, was also a limitation, and future studies investigating these pathways will be conducted using more potent PI3K/AKT inhibitors. Because this is an *in vitro* study, further investigation is required to translate these findings to the clinical setting. For example, there is concern that targeting these two common pathways can lead to significant systemic toxicity, and more studies will be performed to optimize dosing and to identify molecular markers that could predict treatment response to these inhibitors. Importantly, one strategy to circumvent systemic toxicity is to deliver agents locally. In the past, the blood-brain barrier has presented a significant challenge for effective drug delivery. Both perifosine and trametinib have been documented to poorly penetrate the central nervous system [46,47]. A study in rhesus monkeys found that the ratio of CSF to plasma exposure via gavage administration of perifosine was only 0.16% [46]. In another study, trametinib only achieved approximately 20% of plasma concentrations after multiple doses, but it did not significantly inhibit extracellular signal–regulated kinase phosphorylation, suggesting that the concentration achieved may be insufficient for antitumor activity [26,48]. However, convection-enhanced delivery has been found to be an effective strategy in bypassing the blood-brain barrier and delivering chemotherapeutic agents directly to the injection site, thereby reducing systemic toxicity [49]. Finally, future studies are required to elucidate the molecular landscape of DIPG, to investigate the effects of PI3K/AKT and MEK/ERK pathway inhibition *in vivo*, and to optimize treatment response by testing various agents that can inhibit these pathways, with particular emphasis on more potent inhibitors or different classes of inhibitors. Nevertheless, our data demonstrate that the simultaneous inhibition of the PI3K/AKT and MEK/ERK pathways is a viable approach to the treatment of DIPG.

Conflicts of Interest

None.

Author Contributions

Y. L. W. designed the experiments with assistance from R. S., M. W., U. B. M., and M. M. S. Y. L. W., M. S., and R. C. carried out the experiments. Y. L. W. analyzed the data. Y. L. W., U. B. M., and M. M. S. interpreted data. Y. L. W. wrote the manuscript. U. B. M. and M. M. S. reviewed and revised the manuscript. All authors read, provided feedback on, and approved the manuscript.

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