Title
Targeting PI3K in personalized treatment of BRAF-mutated pediatric low-grade gliomas

Permalink
https://escholarship.org/uc/item/5kz3j3gs

Journal
MOLECULAR CANCER THERAPEUTICS, 14(7)

ISSN
1535-7163

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Publication Date
2015-07-01

DOI
10.1158/1538-8514.PI3K14-B42

Peer reviewed
BRAF Status in Personalizing Treatment Approaches for Pediatric Gliomas

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Abstract

Purpose: Alteration of the BRAF/MEK/MAPK pathway is the hallmark of pediatric low-grade gliomas (PLGGs), and mTOR activation has been documented in the majority of these tumors. We investigated combinations of MEKi/2, BRAFV600E and mTOR inhibitors in gliomas carrying specific genetic alterations of the MAPK pathway.

Experimental Design: We used human glioma lines containing BRAFV600E (adult high-grade: AM-38, DBTRG, PLGG: BT40), or wild-type BRAF (pediatric high-grade: SF188, SF9427, SF8628) and isogenic systems of KIAA1549:BRAF expressing NIH/3T3 cells and BRAFV600E expressing murine brain cells. Signaling inhibitors included everolimus (mTOR), PLX4720 (BRAFV600E), and AZD6244 (MEKi/2). Proliferation was determined using ATP-based assays. In vivo inhibitor activities were assessed in the BT40 PLGG xenograft model.

Results: In BRAFV600E cells, the three possible doublet combinations of AZD6244, everolimus, and PLX4720 exhibited significantly greater effects on cell viability. In BRAFWT cells, everolimus + AZD6244 was superior compared with respective monotherapies. Similar results were found using isogenic murine cells. In KIAA1549:BRAF cells, MEKi/2 inhibition reduced cell viability and S-phase content, effects that were modestly augmented by mTOR inhibition. In vivo experiments in the BRAFV600E pediatric xenograft model BT40 showed the greatest survival advantage in mice treated with AZD6244 + PLX4720 (P < 0.01).

Conclusions: In BRAFV600E tumors, combination of AZD6244 + PLX4720 is superior to monotherapy and to other combinatorial approaches. In BRAFWT pediatric gliomas, everolimus + AZD6244 is superior to either agent alone. KIAA1549:BRAF-expressing tumors display marked sensitivity to MEKi/2 inhibition. Application of these results to PLGG treatment must be exercised with caution because the dearth of PLGG models necessitated only a single patient-derived PLGG (BT40) in this study. Clin Cancer Res; 22(21); 5312–21. ©2016 AACR.

Introduction

Pediatric low-grade gliomas (PLGGs) constitute the most common group of central nervous system tumors in children (1). Although they exhibit rare malignant transformation and relatively slow growth, PLGGs pose great morbidity, including vision loss, endocrine dysfunction, and poor cognition. Viewed as a chronic disease, ideal therapy that would carry limited side effects can be best achieved through selective, targeted therapy. Despite the known heterogeneity of PLGGs and characterized driver mutations that together offer the ideal, timely platform for personalized approaches to therapy, most children are still treated with standard chemotherapy protocols in a “one-treatment-fits-all” approach.

Genomic discoveries in recent years have significantly enhanced our understanding of the molecular pathogenesis of PLGGs. The most frequent aberration identified in PLGG implicates BRAF, a key regulatory kinase of the mitogen-activated protein kinase (MAPK) pathway. The majority of pilocytic astrocytomas (PAs) carry the BRAF fusion protein KIAA1549:BRAF, leading to MAPK signaling activation (2, 3). The impact of this fusion protein on clinical outcome remains controversial (4, 5). Clinical studies are currently investigating effects of specific inhibitors of the MAPK pathway, such as the MEKi/2 inhibitor AZD6244, for the treatment of PLGGs (ClinicalTrials.gov; Identifier NCT01089101). Activating BRAFV600E point mutations are
BRAF Alterations in Pediatric Low-Grade Glioma

Translational Relevance

Pediatric low-grade gliomas (PLGGs) constitute the most common group of central nervous system tumors in children. Although the molecular underpinnings of PLGGs represent an ideal platform for personalized approaches, precision medicine has yet to affect PLGG therapy. Targeted therapeutics are available for many of the implicated pathways; however, follow-up biological studies defining the appropriate agents for each alteration are lacking. Here, we define the most effective combinations of discrete mTOR and BRAF/MEK/MAPK inhibitors for PLGGs harboring the most common BRAF genotypes. Significant limitations and challenges are posed by the dearth of PLGG models and, in fact, the only patient-derived PLGG in this study is BT40; therefore, these results and their application to PLGG treatment must be interpreted with caution and efforts should be renewed to generate faithful models that truly recapitulate PLGG cellular context. The work provides the preclinical rationale for targeted combinatorial approaches for the treatment of PLGGs and defines the framework for the next generation of clinical trials for PLGGs, in which molecular features of individual tumors guide rational therapies.

Materials and Methods

Cell lines, xenografts, and inhibitors

DBTRG, AM-38, and BT40 (BRAFV600E), as well as SF188, SF9427, and SF8628 (BRAFWT) were obtained from the Brain Tumor Research Center (BTRC) Tissue Bank at the University of California, San Francisco (UCSF) and authenticated by the UCSF Genomics Core using Promega Powerplex 1.2 System (Promega). SF9427 and SF8628 were established from pediatric high-grade gliomas, as described previously (ref. 16; Supplementary Table S1). BRAFV600E mutations were confirmed by the UCSF genomics core by DNA sequencing. The BRAFWT and KIAA1549-BRAF NIH/3T3 fibroblast isogenic cell model was generated as previously described (17). The isogenic system of murine cells differing only in BRAFV600E status was generated as described (18). The genotypes used in this study are BrafCA/WT Ink4a-arflox/lox (6390, 6392) and BrafWT/WT Ink4a-arflox/lox (6387, 6393). Mouse neurosphere cultures from the subventricular zone of P60 littermates were established and cultured as previously described (19). In brief, cells were maintained in neurobasal medium (Invitrogen) supplemented with human basic fibroblast growth factor, epidermal growth factor (Peprotech), and B27 (Invitrogen). Cultures were maintained at 37°C and 5% CO2. To induce BRAFV600E-expression and deletion of Ink4a-arf, SVZ neurospheres were transduced in vitro with adenovirus-Cre-GFP (Vector Biolabs) for 12 to 18 hours. A2Z6244 and everolimus were obtained from Selleckchem and PLX4720 from Plexxikon Inc.

Cell viability assay

Cell viability was measured by the CellTiter-Glo Luminescent Cell Viability Assay (Promega) per the manufacturer’s guidelines. Assays were performed after 72 hours of treatment with each inhibitor or combination. Each experiment was set up in six replicates and repeated at least three times. Measurements were normalized to DMSO control. Pairwise comparisons were determined by the Student t test using GraphPad Prism 6.0 Software (GraphPad Software). To evaluate drug synergy, combinatorial index (CI) values were calculated using the software CalcuSyn (Biosoft Inc; ref. 20). CI < 1 signifies synergistic effect, CI = 1 indicates additive effect, and CI > 1 defines drug antagonism.

Cell-cycle analysis

Cell-cycle distributions were determined using flow cytometry following staining with BrdUrd and 7-amino-actinomycin D (7-AAD). Cells were grown exponentially in appropriate media containing 10% FBS or as specifically indicated. Subsequently, cells were treated with different inhibitors, fixed, and stained 24 hours after treatment according to the manufacturer’s instructions (BD Pharmingen FITC BdtUrd Flow Kit). For combination treatments, both compounds were administered concurrently. Fluorescence was measured on a FAC-sort flow cytometer (FACScalibur, Becton Dickson), and data were analyzed using FlowJo (TreeStar, Inc). All measurements are represented as an average of at least four replicates. Error bars represent standard error. Statistical significance (P < 0.05) was determined with a one-way analysis of variance (ANOVA) and Bonferroni multiple comparisons correction using GraphPad Prism 6.0 Software (GraphPad Software).

Annexin V cytometric analysis

Proportions of apoptotic cells were quantified by PI/FITC Annexin V flow cytometry (BD Bioscience), 72 hours after treatment. Samples were processed and stained as described in the manufacturer’s protocol. All measurements represent an average of at least four replicates. FITC fluorescence was measured on a FAC-sort flow cytometer (Becton Dickson), and data were analyzed using FlowJo (TreeStar, Inc).
In vitro response to coinhibition of the MAPK and mTOR pathways in BRAF<sup>V600E</sup>-mutated DBTRG and AM-38 glioma cells. Combination of AZD6244 + everolimus (A), PLX4720 + everolimus (B), and AZD6244 + PLX4720 (C) in DBTRG cells. For each combination, we display results of cell viability, apoptosis, and cell-cycle distribution (left to right). For cell viability, means of six replicates with standard errors are displayed. Measurements are normalized to DMSO control. Cell viability was assessed 72 hours after treatment. (Continued on the following page.)
Western blot analysis

To assess the modulation of pathway-specific signaling molecules, we performed Western blot analyses. Total protein extracts from cells were prepared using cell lysis buffer (50 mmol/L HEPES pH 7.0, 150 mmol/L NaCl, 10% glycerol, 1% Triton X-100, 0.5% sodium deoxycholate, 1% NP-40, 1.5 mmol/L MgCl₂, and 10 mmol/L EDTA) containing a complete protease and phosphatase inhibitor cocktail (Roche Diagnostics Corporation). Protein lysates were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes, then incubated with antibodies against p-Akt, total Akt, p-ERK, p-S6, total S6, β-actin (Cell Signaling Technology). Band intensities were visualized by ECL Western detection reagent (GE Healthcare).

Immunohistochemistry of tumor samples

For immunohistochemical analyses of in vivo drug responses, nude mice harboring subcutaneous BT40 (BRAFV600E) xenografts were treated for 10 consecutive days. Tumors were harvested 1 hour after the last treatment, fixed in 4% paraformaldehyde overnight, and processed through ethanol dehydration series prior to paraffin embedding. Five-micrometer sections were cut, rehydrated with decreasing ethanol concentrations, and hybridized using anti-Ki67 (Dako), p-S6, p-Erk (R&D Systems), detected with DAB substrate (Vector Labs), and counterstained with hematoxylin. Image-based quantification was performed for each stain. Five 20× pictures were taken per tumor (Leica, DMLS microscope), with positively stained cells scored both manually and by the ImageJ based ImmunoRatio plugin.

Animals and surgical procedures

The UCSF Institutional Animal Care and Use Committee approved all animal protocols. Four- to 6-week-old female NOD SCID mice were purchased from The Jackson Laboratory. Animals were housed under aseptic conditions. BT40 tumor cells were implanted in the flank of mice as described previously (21). For each condition, 10 mice were injected but not all animals developed tumors. For the final analysis, the following numbers of animals were included: control n = 6, AZD6244 n = 7, everolimus n = 7, PLX4720 n = 7, everolimus + PLX4720 = 9, AZD6244 + PLX4720 = 7, and AZD6244 + everolimus = 7.

Treatments were initiated when tumors reached 100 to 200 mm³ in size. Tumor-bearing mice were randomized to vehicle control (OraPlus: Paddock Laboratories), AZD6244, everolimus, PLX4720, or combinations thereof. Mice were treated for a total of 10 days (Mo-Fri) with the following dosing schedule: 10 mg/kg of AZD6244 and/or 10 mg/kg everolimus once daily by oral administration and/or 10 mg/kg PLX4720 once daily by intraperitoneal (i.p.) administration. For combination treatment, inhibitors were given concurrently. All mice were examined daily for the development of symptoms related to tumor growth. Mice were euthanized when they exhibited symptoms indicative of significant impairment. In addition to the mice used for survival analyses, mice from each cohort were sacrificed 1 hour after completion of the treatment, with tumors resected and placed in 4% paraformaldehyde (PFA), then processed for IHC analysis.

The Kaplan–Meier estimator was used to generate survival curves, and differences between survival curves were calculated using a log-rank test. Differences between bioluminescence growth curves were compared using a two-tailed unpaired t test using Prism software (GraphPad Software).

Results

Combination therapy in BRAFV600E-mutant human glioma cells enhances cell-cycle arrest and apoptosis and reduces proliferation compared with monotherapy

We assessed effects of MEK1/2- (AZD6244), mTOR- (everolimus), or BRAFV600E-inhibitors (PLX4720) as monotherapy as well as in combination in BRAFV600E-mutated glioma cells DBTRG and AM-38. As shown in Figure 1A–C (DBTRG) and D-F (AM-38), the three possible doublet combinations of AZD6244, everolimus and PLX4720, led to significant decreases in cell proliferation compared with the respective single agents. The combination index (CI) theorem of Chou–Talalay offers quantitative definitions for additive effect (CI = 1), synergism (CI < 1), and antagonism (CI > 1) in drug combinations and demonstrated CI < 1 for all tested BRAFV600E glioma lines when PLX4720 was combined with AZD6244 (Supplementary Fig. S1A and S1B). Similarly, the most pronounced induction of apoptosis was seen after combined MEK1/2 and BRAFV600E inhibition (Fig. 1C; Supplementary Fig. S2A–S2C). All three combinations performed equally well in reducing S and G₂ phase accumulations; of note, PLX4720 was extremely effective as a single agent in arresting cell-cycle progression and therefore cell-cycle effects were not significantly enhanced when everolimus or AZD6244 was added to PLX4720 (Fig. 1A–C; Supplementary Fig. S2A–S2C). Western blot analyses revealed that treatment with everolimus significantly reduced p-S6. Treatment with PLX4720 or AZD6244 reduced expression of p-ERK, although AZD6244 was more effective in downregulating p-ERK. PLX4720 reduced p-ERK at 1 hour with a rebound in expression at 6 hours, a rebound that was prevented by the addition of AZD6244 to PLX4720. Treatment of BRAFV600E-mutant DBTRG cells with all examined single agents and combinations led to upregulation of p-AKT (Fig. 1G). Similar Western blot results were found in BRAFV600E-mutant AM-38 cells (data not shown).

Combination therapy in BRAFWT human glioma cells enhances cell-cycle arrest and apoptosis and reduces proliferation compared with monotherapy

We next turned to examining responses in BRAFWT-PLGGs by assessing proliferation, apoptosis, and cell-cycle distribution in SF188. We did not assess PLX4720 in BRAFWT cells because this would not be clinically relevant due to the known paradoxical activation of the MAPK pathway in BRAFWT tumor cells (22–24). As shown in Fig. 2A, combined inhibition of MEK1/2 and mTOR suppresses cell-cycle progression and increases levels of p-AKT and p-GSK3β, while reducing levels of p-ERK, p-p44/42, and p-S6 (Fig. 2B). Together, these results suggest that combined inhibition of MEK1/2 and mTOR may be a promising therapeutic strategy for the treatment of BRAFWT gliomas.

(Continued.)
decreased cell proliferation significantly more than single pathway inhibition. Similar results were also seen in SF8628 (Fig. 2B) and SF9427 BRAFWT cells (Fig. 2C). The combinatorial index calculation indicates strong synergy (CI<1) in the examined BRAFWT cells SF188 and SF9427 (Supplementary Fig. S3A and S3B). In addition, SF188 cells treated with AZD6244 + everolimus exhibited significantly greater apoptosis compared with single-agent treatments (Fig. 2D), a finding documented in SF8628 cells as well (Supplementary Fig. S4A). In addition to combinatorial effects on apoptosis and proliferation, we observed decreased S and G2 phase accumulation in SF188 and SF8628 cells treated with combination of AZD6244 + everolimus compared with the respective single-agent–treated cells (Fig. 2E; Supplementary Fig. S4B). Western blot analyses (Fig. 2F) revealed expected pathway modulations, including downregulation of p-ERK by AZD6244, reduction in p-S6 by everolimus, and upregulation of p-Akt by AZD6244, everolimus, and their combination.

**Combination therapy in BRAFV600E and BRAFWT isogenic mouse cells is superior to single-agent treatments**

To confirm our results that combination treatment is superior to single agents in BRAFV600E and BRAFWT cells, we used an isogenic system of murine brain cells differing only in BRAFV600E status. In this BRAFV600E-isogenic system, BRAFV600E cells were as sensitive to everolimus as BRAFWT cells, but exquisitely more sensitive to AZD6244 than BRAFWT cells (Fig. 3). Comparing inhibitor combinations, each of the three combinations was superior to single agents in reducing cell proliferation in BRAFV600E-mutated cells.
Similar to our findings in BRAFV600E-mutated DBTRG cells (Fig. 1), the combination of PLX4720 + AZD6244 was superior to the other two doublet combinations, although AZD6244 + everolimus also showed marked combinatorial activity. The combination index of PLX4720 + AZD6244 was indicative of strong synergism at all dose levels. As expected, Western blot analyses in BRafV600E-mutated cells revealed downregulation of p-ERK by AZD6244 or PLX4720 and downregulation of p-S6 at 6 hours by everolimus (Fig. 3B). In BRafWT murine glioma cells, combination of AZD6244 + everolimus enhanced antiproliferative effects compared with single agents (Fig. 3C), with CI values below one, indicating synergistic effects of this combinatorial approach (data not shown). Western blot analyses showed no difference in basal p-ERK and p-S6 levels between KIAA1549:BRAF and BRafWT cells (Fig. 4C and D), as growth factors present in full serum media conditions cause persistent activation of wild-type BRAF.

Low serum conditions (0.5% FBS) augmented disparate activity of AZD6244 on KIAA1549:BRAF and BRafWT cells, demonstrating exquisite antiproliferative effects of single-agent MEK1/2 inhibition on BRAF fusion cells (Fig. 4E). Growth in low serum led to expected accumulation of cells in G1 phase, which was further augmented by MEK1/2 inhibition in KIAA1549:BRAF fusion cells but not in BRafWT cells (Fig. 4F). Western blot analyses revealed that when grown in low serum, BRafWT cells had lower basal levels of p-ERK and p-S6 than KIAA1549:BRAF fusion cells (Fig. 4H). This difference was not seen when cells were grown in full serum conditions as growth factors cause persistent activation of wild-type BRAF. Low serum conditions (0.5% FBS) augmented disparate activity of AZD6244 on KIAA1549:BRAF and BRafWT cells, demonstrating exquisite antiproliferative effects of single-agent MEK1/2 inhibition on BRAF fusion cells (Fig. 4E).

NIH/3T3 cells carrying the KIAA1549:BRAF fusion protein are highly sensitive to MEK1/2 inhibition compared with isogenic BRafWT-expressing cells.

We next turned to examining responses in BRAF fusion cells. In contrast to BRafWT and BRafV600E, no patient-derived LGG lines expressing BRAF fusions have been successfully developed to permit preclinical testing. This is despite significant efforts to grow primary cells in culture by numerous laboratories, including our own. To address this, we recently generated a set of BRAF fusion heterologous expression systems consisting of NIH/3T3 expressing BRafV600E or the most common BRAF alteration in PLGGs, the KIAA1549:BRAF fusion protein (Fig. 4). Under 10% serum conditions, MEK1/2 and/or mTOR inhibition produced similar decreases in cell proliferation in cells carrying the KIAA1549:BRAF fusion protein compared with BRafWT (Fig. 4A). In contrast, BRafWT cells showed minimal change in cell-cycle distribution in response to MEK1/2 inhibition, mTOR inhibition, or the combination, whereas the KIAA1549:BRAF cells showed significant reduction in S and G2 phase after MEK1/2 inhibition but not after mTOR inhibition (Fig. 4B). Western blot analyses showed no difference in basal p-ERK and p-S6 levels between KIAA1549:BRAF and BRafWT cells (Fig. 4C and D), as growth factors present in full serum media conditions cause persistent activation of wild-type BRAF.
In vitro response to coinhibition of the MAPK and mTOR pathways in NIH/3T3 BRAF<sup>WT</sup> and KIAA1549:BRAF fusion cells. A and E, cell viability in BRAF<sup>WT</sup> and KIAA1549:BRAF-expressing NIH/3T3 cells in response to AZD6244, everolimus, or combination thereof under high (10% DBS) and low (0.5% DBS) serum (E) conditions. Means of six replicates with standard error are displayed. Measurements are normalized to DMSO control. Cell viability was assessed 72 hours after treatment. B and F, cell-cycle distribution of BRAF<sup>WT</sup> and KIAA1549:BRAF-expressing NIH/3T3 cells after treatment with AZD6244 and/or everolimus under high (10%) and low (0.5%) serum condition (F). Cell-cycle analyses were performed 24 hours after treatment and depicted are averages of four experiments. Bar graphs display distributions of cells within each cell-cycle phase. Error bars indicate standard error. Western blot analyses of p-Akt, p-ERK, and p-S6 in (C, G) NIH/3T3 BRAF<sup>WT</sup> cells and (D, H) KIAA1549:BRAF-expressing NIH/3T3 cells after treatment with AZD6244, everolimus, or combination thereof. β-Actin was used as a loading control. Levels of p-Akt, p-ERK, and p-S6 were determined 1 and 6 hours after treatment. For all of the above results, in samples receiving combination therapy, inhibitors were administered simultaneously.

Figure 4.

In vitro response to coinhibition of the MAPK and mTOR pathways was evident only in the combined treatment arm (Fig. 4D and H).

In vivo efficacy of combination therapy in BRAF<sup>V600E</sup> model of PLGG

To address the in vivo efficacy of combinations of PLX4720, everolimus, and AZD6244, we used the BRAF<sup>V600E</sup>-PLGG model BT40. Mice treated with a combination of AZD6244 plus everolimus or PLX4720 showed significantly prolonged survival and tumor volume reduction compared to single-agent treatment, with AZD6244 + PLX4720 appearing superior to AZD6244 + everolimus (Fig. 5A–C).

As observed in our in vitro studies, we found reduced expression of Ki67, a marker of proliferation, in xenografts from mice...
treated with everolimus, AZD6244, PLX4720, or combinations thereof, with the greatest reduction of Ki67 documented in mice treated with the combination of PLX4720 and AZD6244. Xenografts treated with everolimus showed reduced expression of p-S6, as expected. Treatment with AZD6244 and PLX4720 reduced p-ERK expression in xenografts, as anticipated, an effect that was significantly more pronounced after AZD6244 than after PLX4720 treatment.

**Discussion**

In this article, we provide the preclinical rationale for targeted therapy options based on the most commonly found genetic alterations in PLGG. Our studies indicate that combination therapies with PLX4720, everolimus, or AZD6244 for PLGGS carrying the **BRAF**V600E mutation or **BRAF**WT are superior to single-agent therapy. We further demonstrate that the KIAA1549:BRAF fusion protein renders cells highly sensitive to MEK1/2 inhibition, and combination therapy...
therapy is marginally superior compared to single-agent therapy.

Although BRAF\textsuperscript{V600E}-specific inhibitors such as vemurafenib and dabrafenib have only recently entered clinical practice for children whose tumors carry this mutation, initial reports are encouraging (25–27). Case reports indicate that vemurafenib is highly effective and have led to complete regression in BRAF\textsuperscript{V600E}-expressing pediatric high-grade gliomas (26). However, based on our experience and that of others, we also know that some patients progress on single-agent therapy with BRAF\textsuperscript{V600E} inhibitors. Feedback activation of EGFR and downstream effectors has been proposed as a potential escape mechanism, further supporting combination strategies that address the MAPK as well as the PI3K pathways (28, 29). Furthermore, combination of MEK and BRAF\textsuperscript{V600E} inhibition improves tolerability with decreased frequency of squamous cell carcinomas, a rare but serious side effect of vemurafenib (30).

In all our model systems of BRAF\textsuperscript{V600E}-mutated gliomas [isogenic murine, BRAF\textsuperscript{V600E} PLGG (BT40) and BRAF\textsuperscript{V600E} adult high-grade glioma (DBTRG, AM-38)], whether in vitro or in vivo, treatments with the three possible doublet combinations of AZD6244, everolimus, and PLX4720 were superior to their respective monotherapies, with PLX4720 + AZD6244 appearing the superior combination overall, showing consistently reduced cell viability, increased apoptosis, and cell-cycle arrest in vitro and, most prominently, prolonged survival and reduced tumor growth in vivo. In the in vitro models, rebound in p-ERK expression after 6 hours of PLX4720 treatment was completely obliterated by the addition of AZD6244 to PLX4720. These results, in addition to already published data, provide a strong clinical rationale for combination therapies in BRAF\textsuperscript{V600E}, positive gliomas. One limitation of our in vitro studies is the use of a flank model rather than an orthotopic model. However, BT40, the only available pediatric model that carries the BRAF\textsuperscript{V600E} mutation, grows only as a flank tumor, and there are no intracranial models currently available to study PLGG. Planned clinical trials will test the combination of BRAF\textsuperscript{V600E}-specific inhibition in combination with MEK inhibition in pediatric patients (NCT02124772).

In examining responses in BRAF\textsuperscript{WT} pediatric high-grade gliomas, we show that the combination of AZD6244 + everolimus was superior to either single-agent therapy, as assessed by cell proliferation, cell-cycle arrest, and induction of apoptosis. In comparison with BRAF\textsuperscript{V600E} cells, BRAF\textsuperscript{WT} lines had reduced sensitivity to MEK inhibitor monotherapy consistent with previous reports (31, 32). We have shown that treatment with everolimus leads to activation of the MAPK pathway, most likely through a PI3K-dependent feedback loop. This has been shown in previously published studies and provides a strong rationale for combination therapy using MEK and mTOR inhibitors for BRAF\textsuperscript{WT} gliomas (33, 34). Ongoing studies are assessing the tolerability of the combination of such dual inhibition in adult patients with advanced cancers (NCT01347866), and studies in pediatric patients are under way.

In contrast to existing and available BRAF\textsuperscript{WT} and BRAF\textsuperscript{V600E} preclinical models, no patient derived glioma cell lines expressing BRAF fusions have been successfully established for preclinical studies. To circumvent this limitation, we used a BRAF fusion heterologous expression system in NIH/3T3 cells that we described previously (17). We have shown in vivo and in vitro that BRAF fusion expressing lines display robust resistance to and enhanced paradoxical activation by PLX4720 (17). Importantly, these models predict the clinical responses to BRAF targeting, as a recent phase II study found acceleration of PLGGs when treated with sorafenib, leading to early closure of the trial (35). Herein we found that BRAF fusion cells are highly sensitive to single-agent MEK1/2 inhibition with a small benefit derived from combining MEK1/2 and mTOR inhibition. Given the observed large sensitivity to MEK1/2 inhibition alone, single-agent activity may be sufficient to successfully inhibit growth of such tumors. Targeted therapy recommendations such as the one proposed herein will increase the need for tissue acquisition even for tumors such as optic pathway gliomas, a tumor type that classically has not been biopsied. The additional risk of biopsy might be justified if therapy recommendations would change based on the individual tumor characteristics. Several studies have shown that tissue acquisition is feasible and relatively safe, even for optic pathway gliomas (35, 36).

**Conclusion**

Using model systems of the most common PLGG BRAF alterations, we provide the preclinical rationale for therapeutic approaches for each genotype. Our results support treatment of BRAF\textsuperscript{V600E}-positive PLGGs with a combination of PLX4720 + AZD6244, although AZD6244 + everolimus also shows marked combinatorial activity. BRAF\textsuperscript{VT} gliomas appear to benefit from the combination of mTOR and MEK inhibition. Our studies are consistent with clinical reports of sensitivity of KIAA1549:BRAF fusion gliomas to MEK1/2 inhibition as a single agent.

**Disclosure of Potential Conflicts of Interest**

S. Mueller and D.A. Haas-Kogan report receiving commercial research grants from Novartis. No potential conflicts of interest were disclosed by the other authors.

**Authors’ Contributions**


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Olow, J. Meyerowitz, N. Gupta, D.A. Haas-Kogan

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Olow, S. Mueller, X. Yang, J. Meyerowitz, A.J. Waanders, D.A. Haas-Kogan

Writing, review, and/or revision of the manuscript: A. Olow, S. Mueller, X. Yang, W. Weiss, A.C. Resnick, A.J. Waanders, L.J.A. Stalpers, M.S. Berger, N. Gupta, C. Petritsch, D.A. Haas-Kogan

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**Grant Support**

This research was supported in part by the National Center for Advancing Translational Sciences, NIH, through UL1TR000143 (S. Mueller). NIH Brain Tumor SPORE grant P50 CA097257 (S. Mueller, D.A. Haas-Kogan, W. Weiss, M.S. Berger); NINDS R01NS080619 (C.D. James, C.K. Petritsch); NINDS R01NS096120 (D.A. Haas-Kogan, W. Weiss, A.C. Resnick), the Matthew Larson Foundation (S. Mueller), Grand Philanthropic Fund (D.A. Haas-Kogan), and the University of California Cancer Research Coordinating Committee (S. Mueller and A. Haas-Kogan).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked

Received May 8, 2015; revised May 2, 2016; accepted May 7, 2016, published OnlineFirst May 23, 2016.
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