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Targeting the mitogen-activated protein kinase kinase and protein kinase A pathways overcomes acquired resistance to Selumetinib in low-grade glioma cells

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Abstract. The Ras/Raf/MEK/MAPK signaling cascade is frequently activated in human cancer and serves a crucial role in the oncogenesis of pediatric low-grade gliomas (PLGGs). Therefore, drugs targeting kinases among the mitogen-activated protein kinase (MAPK) effectors of receptor tyrosine kinase signaling may represent promising candidates for the treatment of PLGGs. The aim of the present study was to elucidate the anticancer effects of the MEK inhibitor Selumetinib on two low-grade glioma cell lines and the possible underlying effects on intracellular signal transduction. The two cancer cell lines displayed different levels of sensitivity to Selumetinib, as Res186 cells were resistant (IC_{50}>1 μM), whereas Res259 cells were sensitive (IC_{50}≤1 μM) to MEK inhibition. Despite the different levels of sensitivity, Selumetinib mediated the phosphorylation of AKT and MEK in both cell lines and suppressed the phosphorylated MAPK cascades. In addition, Selumetinib induced cell cycle arrest at the G_0/G_1 phase by downregulating the expression levels of cyclin D1 and p21 and upregulating those of p27 compared with those in the control cells. A Res259 cell line with acquired resistance to Selumetinib (Res259/R) was next established and biologically and molecularly characterized, and it was demonstrated that addition of a selective cAMP-dependent protein kinase A inhibitor to Selumetinib overcame drug resistance in Res 259/R cells. In conclusion, the results of the present study provided three low-grade glioma cell line models characterized by sensitivity, intrinsic and acquired resistance to Selumetinib, which may be usuful tools to study new mechanisms of chemoresistance to MEK inhibitors and to explore alternative therapeutic strategies in low-grade gliomas for personalization of treatment.

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Introduction

Brain tumors are the leading cause of cancer-related mortality in children, despite their lower incidence (1.12-5.14 cases per 100,000 individuals) compared with that of hematological malignancies in this age group (1,2). Pediatric low-grade gliomas (PLGGs) comprise a histologically heterogeneous group of World Health Organization grade I and II tumors (3), and they are the most common type of central nervous system tumor in children (4).

The prognosis and treatment of PLGGs depend on patient age, tumor location, dimensions and histopathological characteristics (5). When complete resection is possible, patients usually do not require any adjuvant therapy, whereas partial resection is generally associated with tumor recurrence and the patients may need additional treatment, such as chemotherapy or radiotherapy (6,7). Unfortunately, the use of ionizing radiation and conventional chemotherapies in pediatric patients may damage the developing brain, often leading to significant permanent neurocognitive and systemic complications including loss of vision and/or hearing, endocrinopathies and mood or behavior disorders (7-9). In addition, while the clinical symptoms of PLGG may take months to years to progress, its management by cytotoxic chemotherapy has been demonstrated ineffective due to the slow growth rate of the tumor.

Tumors exploit various pathways that are indispensable for the regulation of cancer cell biology (10). The extracellular signal-regulated kinase (ERK) pathway has been the subject of extensive research in recent years due to its key role in the regulation of cancer cell proliferation, survival and metastasis (11). The mitogen-activated protein kinase (MAPK)-ERK signaling

pathway has been demonstrated to be fundamental for the tumorigenesis of PLGGs, and pharmacological targeting of this pathway with small molecule kinase inhibitors may represent a viable therapeutic approach (11-13). Mitogen-activated protein kinase kinase (MEK)1 and MEK2 are dual-specificity protein kinases that function in the MAPK cascade, controlling cell proliferation and differentiation; MEK1/2 activate ERK 1/2, which have wide substrate specificity, resulting in the activation of a multitude of cellular responses involved in the control of cell proliferation, differentiation and apoptosis (14-16).

Selumetinib (AZD6244; ARRY-142886) is a selective, ATP-uncompetitive inhibitor of MEK1/2 (17). Selumetinib specifically binds to MEK1/2 and induces several conformational changes in the unphosphorylated MEK1/2 enzymes by inhibiting their catalytic activity, which results in the inhibition of ERK activation and the blockade of the signal transduction pathways (17,18). This small molecule was previously investigated in clinical trials for the treatment of melanoma (NCT01974752), biliary (NCT00553332), colorectal (NCT01116271), pancreatic (NCT00372944) and advanced non-small cell lung (NCT01783197) cancer as well as a variety of other malignancies (18-21).

The antitumor effect of Selumetinib on PLGGs has yet to be fully characterized. However, as activation of the MEK-ERK pathway inhibits apoptosis and promotes cell cycle progression through increasing cyclin D expression (22), inhibition of the MAPK pathway through specific targeted therapies appears to be a promising strategy for the treatment of PLGGs.

The aim of the present study was to investigate the antitumor activity of Selumetinib *in vitro* in two PLGG cell lines and to determine the effects of Selumetinib treatment on the key intracellular downstream signaling pathways. In addition, a cellular model of acquired resistance to Selumetinib was established in order to investigate whether the addition of a selective protein kinase A (PKA) inhibitor cAMP analogue to Selumetinib may represent a possible synergistic therapeutic strategy for reversing acquired resistance to this MEK inhibitor.

Materials and methods

Reagents. Selumetinib (AZD6244) was purchased from AdooQ Bioscience, dissolved to 67.3 mM in DMSO and stored at -20˚C. 8-Chloroadenosine 3',5'-cyclic-monophosphate (8-Cl-cAMP) was obtained from Sigma-Aldrich; Merck KGaA, dissolved in a solution of 0.1 M NH_4OH to 27.49 mM and stored at -20˚C.

Cell lines. The pediatric glioma cell lines Res186 and Res259 were kindly provided by Professor Chris Jones (Glioma Team, Centre for Evolution and Cancer Divisions of Molecular Pathology and Cancer Therapeutics, The Institute of Cancer Research, London, UK). Res186 and Res259 cells were derived from a 3-year-old female patient with pilocytic astrocytoma and a 4-year-old female patient with diffuse astrocytoma, respectively (23). The PLGG cell lines were cultured at 37˚C with 5% CO_2 in DMEM F-12 medium (Corning, Inc.) supplemented with 10% fetal bovine serum (FBS; PAN-Biotech GmbH), 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin.

The Selumetinib-resistant cell line, referred to as Res259/R, was generated by continuous exposure (6 months) of the parental cell line Res259 to gradually increasing Selumetinib concentrations (1-25 μM) and maintained in a medium containing 25 μM Selumetinib. The half maximal inhibitory concentration (IC_{50}) values in Selumetinib-sensitive and resistant Res259 cell lines was 1 and 25 μM, respectively, with a resistance index (RI) of ~25-fold. Experiments were performed using cells cultured in a Selumetinib-free medium for ≥15 days.

Cell viability assay. Res259, Res186 and Res259/R cells were seeded in 6-well plates overnight in medium supplemented with 10% FBS at 37˚C. Each cell line was optimized for seeding cell number to ensure a similar degree of confluence at the end of the experiment in the untreated (control) wells (Res259, $4x10^4$; Res186, $5x10^4$; Res259/R, $3.5x10^4$ cells/well). After 24 h, the cells were starved in DMEM F-12 medium with 0.5% FBS. Vehicle (DMSO) or serial concentrations (0.01, 0.05, 0.25, 0.5, 1, 2.5, 10 and 25 μM) of Selumetinib were added to the medium, and the cells were cultured for 72 h at 37˚C. Cell number and viability, as well as the number of dead cells were assessed by an automated cell counter (NucleoCounter 100®; Chemometec), and expressed as a percentage of treated cells relative to the untreated control cells. All experiments were repeated at least five times in duplicate. The IC_{50} was calculated using GraphPad Prism version 6.0 (GraphPad Software, Inc.).

BRAF mutational analysis. DNA extraction from Res186 and Res259 cells was performed using the QIAamp DNA Mini kit (Qiagen GmbH). Exons 11 and 15 of the BRAF gene were amplified using the following primers: Exon 11 forward, 5'-TTATTGATGCGAACAGTGAATAT-3' and reverse, 5'-TTACAGTGGGACAAAGAATTG-3'; exon 15 forward, 5'-TCATAATGCTTGCTCTGATAGGA-3' and reverse, 5'-GGCCAAAAATTTAATCAGTGGA-3'. Briefly, DNA (100-200 ng) was amplified in a mixture containing 1X PCR buffer (20 mM Tris, pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$), dNTPs (200 mM each), primers (20 pM each) and 0.5 unit GoTaq polymerase (Promega Corporation) in a final volume of 25 μl. PCR conditions were as follows: Initial denaturation at 95˚C for 8 min, followed by 35 cycles of 95˚C for 40 sec, 55˚C for 40 sec and 72˚C for 40 sec. After visualization on agarose gels, the PCR products were treated with ExoSAP-IT (USB Corporation) according to the manufacturer's protocol, amplified for 25 cycles of 95˚C for 10 sec, 50˚C for 5 sec and 60˚C for 4 min with a BigDye Terminator cycle sequencing kit (version 3.1; Applied Biosystems; Thermo Fisher Scientific, Inc.) using forward and reverse primers, and sequenced with an ABI PRISM 3100-Avant Genetic Analyzer with Sequencing Analysis Software™ v.3.6.2 (Applied Biosystems; Thermo Fisher Scientific, Inc).

Cell cycle analysis by flow cytometry. Res186, Res 259 and Res 259/R cells were exposed to Selumetinib (1 and 2.5 μM for Res186; 0.5 and 1 μM for Res259; 5 and 25 μM for Res259/R) and collected after 24, 48 or 72 h. At the end of each incubation period at 37˚C, adherent cells were trypsinized, harvested and washed with cold PBS. The cells were then counted by an automated cell counter, gently fixed in 70% v/v cold ethanol

Table I. Antibodies used for western blotting.

Protein	Dilution	Cat. no.	Supplier
AKT	1:1,000	9272	Cell Signaling Technology, Inc.
p-AKT (Ser473)	1:1,000	9271	Cell Signaling Technology, Inc.
Bax (N-20)	1:200	sc-493	Santa Cruz Biotechnology, Inc.
Caspase-3 (H-277)	1:200	sc-7148	Santa Cruz Biotechnology, Inc.
Caspase-9 (H-83)	1:200	sc-7885	Santa Cruz Biotechnology, Inc.
Cyclin D1 (DCS-6)	1:200	sc-20044	Santa Cruz Biotechnology, Inc.
Cytochrome c (H-104)	1:200	sc-7159	Santa Cruz Biotechnology, Inc.
MEK1/2	1:1,000	9122	Cell Signaling Technology, Inc.
p-MEK1/2 (Ser217/221)	1:1,000	9121	Cell Signaling Technology, Inc.
NF1	1:1,000	A300-140-M	Bethyl Laboratories, Inc.
p44/42 MAPK	1:1,000	9102S	Cell Signaling Technology, Inc.
p-p44/42 MAPK (Thr202/Thr204)	1:1,000	9101S	Cell Signaling Technology, Inc.
p21 Waf1/Cip (12D1)	1:1,000	2947	Cell Signaling Technology, Inc.
p27 (F-8)	1:200	sc-1641	Santa Cruz Biotechnology, Inc.
PARP (46D11)	1:1,000	9532	Cell Signaling Technology, Inc.
PI3K p85	1:1,000	4292	Cell Signaling Technology, Inc.
β-actin (C-2)	1:200	sc-8432	Santa Cruz Biotechnology, Inc.
HRP-anti-rabbit	1:5,000	# 32460	Thermo Fisher Scientific
HRP-anti-mouse	1:5,000	# 32230	Thermo Fisher Scientific

p, phosphorylated.

and incubated at -20˚C for ≤7 days. Fixed cells were stained with a PBS solution containing RNase (5 μl RNase solution, 10 mg/ml per 2x10^5 cells) and 50 μg/ml propidium iodide (PI) and stored at 4˚C overnight. Flow cytometric DNA ploidy analysis was performed by acquiring a minimum of 3x10^4 cells at a low flow rate for each sample using a six-parameter (two scatter and four fluorescence signals) EPICS-XL flow cytometer (Beckman Coulter, Inc.). Analysis of cell cycle distribution was performed using FCS Express 7 Research Edition (De Novo Software). All experiments were repeated at least three times in duplicate.

Western blotting. Res186, Res259 and Res259/R cells treated with Selumetinib were lysed in lysis buffer (20 mM Tris pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin and 1 mM phenylmethylsulfonyl fluoride), and protein concentration was determined by a Dc protein assay (Bio-Rad Laboratories, Inc.). Total lysates (25 μg/lane) were subjected to electrophoresis on 8-12% polyacrylamide gels, transferred to a Hybond nitrocellulose membrane (Amersham; Cytiva) and probed with appropriate dilutions of the following primary antibodies: Anti-AKT, anti-phosphorylated (p)-AKT (Ser473), anti-MAPK, anti-p-MAPK (Thr202/Tyr204), anti-p-MEK1/2, anti-MEK1/2, anti-p27, anti-p21, anti-cyclin D1, anti-poly(ADP-ribose) polymerase (PARP), anti-caspase 9, anti-caspase 3, anti-cytochrome c, anti-p85, anti-neurofibromin (NF1), anti-Bax and anti-β-actin (Table I) overnight at 4˚C. The membranes were washed with 1X TBS and incubated with secondary anti-rabbit or anti-mouse antibodies conjugated with horseradish peroxidase for 2 h at room temperature. The membranes were developed using an ECL detection system (Amersham; Cytiva), and chemiluminescence signals were captured using the ChemiDoc XRS system (Bio-Rad Laboratories, Inc.). For phosphorylation analysis in Res186 and Res259 cells, the membranes were initially probed with antibodies for the p-epitopes, then stripped by incubation in 62.5 mM Tris-HCl pH 6.8, 2% SDS and 100 mM β-mercaptoethanol at 50˚C for 30 min, washed, blocked and reprobed with antibodies recognizing total AKT, MEK or MAPK.

Co-treatment experiments. Before testing the combination treatment in the *in vitro* glioma models, PLGG cell lines were treated with 0.5-30 μM 8-Cl-cAMP alone at 37˚C for 72 h, and cell viability was assessed by an automated cell counter. For the co-treatment experiment, Res259 (4x10^4 cells/well), Res186 (5x10^4 cells/well) and Res259/R (3.5x10^4 cells/well) were exposed to 0.01-5 μM Selumetinib and 0.5-40 μM 8-Cl-cAMP at a fixed drug ratio at 37˚C for 72 h. For each cell line, the drug ratio was selected based on the respective IC_{30} values of the two drugs as follows: Drug ratio=IC_{30} (Selumetinib)/IC_{30} (8-Cl-cAMP). Cell viability was assessed by an automated cell counter, and proliferation inhibition (%) was determined using the following equation: Proliferation inhibition (%)=1-(mean treated cell number)/(mean control cell number) x100. The results of the combination treatments were analyzed using the Chou-Talalay method (24), and the combination index (CI) was calculated using CalcuSyn software (Biosoft, version 1.0). CI values were interpreted as follows: <1, synergism; 1, additivity; >1, antagonism.

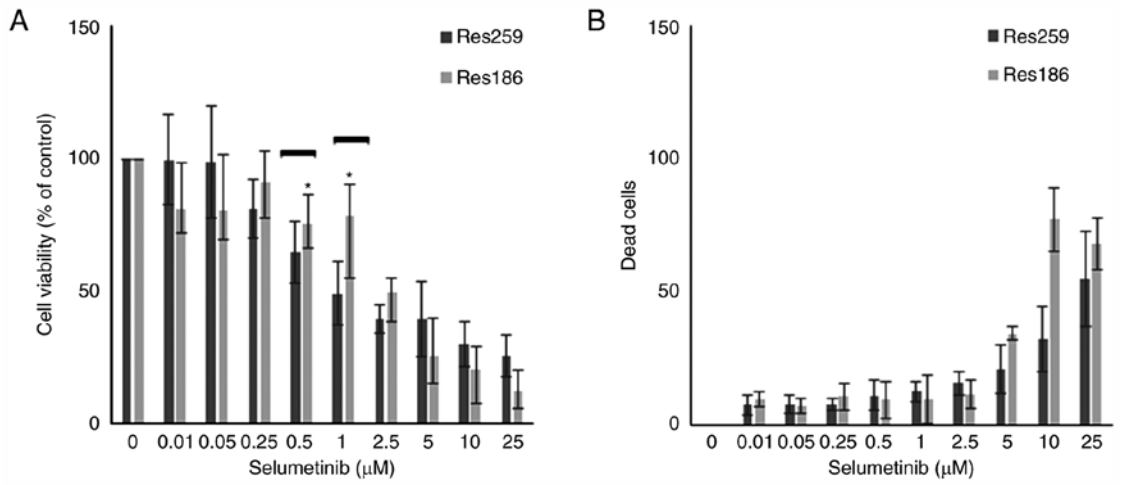


Figure 1. Effects of Selumetinib on the proliferation and viability of low-grade glioma cells. (A) The viability of Res186 and Res259 cells incubated with Selumetinib for 72 h was measured by an automated cell counter. Data are expressed as the percentage relative to the control cells (treated with DMSO for 72 h). Each point represents the mean value of five independent experiments, each performed in duplicate. *P<0.05. (B) Proportion of dead cells in the assays presented in A.

Statistical analysis. The data are presented as the mean ± standard deviation. GraphPad Prism software version 5.0 (GraphPad Software, Inc.) was used for statistical analyses. When comparing two groups, a two-tailed unpaired Student's t-test was used to determine statistical significance. When comparing more than two groups, one-way or two-way ANOVA was performed according to the design of the experiment. Dunnett's multiple comparison test was used to compare the treatment and control groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of Selumetinib on PLGG cell proliferation and viability. To determine the effects of Selumetinib on Res186 and Res259 cells, cell proliferation and viability following treatment with 0.01-25 μM Selumetinib were determined by an automated cell counter after 3-day treatment. As presented in Fig. 1A, Selumetinib reduced the proliferation of the Res186 and Res259 cells, with higher concentrations of Selumetinib achieving more effective inhibition. In Res259, the IC_{50} value was observed to be 1.0 μM , whereas in Res186, the IC_{50} value was 2.5 μM. Based on these results, Res259 cells were classified as sensitive (S), whereas Res186 cells were classed as resistant (R), with Selumetinib IC_{50} at 72 h of ≤1 and >1 μM, respectively. This cut-off value was selected on the basis of a previous phase I trial reported by Adjei *et al* (25), where 1 μM was the mean plasma drug concentration achieved in patients at the maximum tolerated dose.

BRAF mutation analysis. The sequencing analysis of BRAF mutations in the Res186 and Res259 cell lines demonstrated that both cell lines had a wild-type BRAF sequence (data not shown).

Effects of Selumetinib on the cell cycle in PLGG cell lines. Res186 and Res259 cells were exposed to the respective IC_{30} and IC_{50} of Selumetinib for 24, 48 and 72 h, and the effects on the cell distribution in different cell cycle phases was evaluated by flow cytometry. Res259 and Res186 cells were observed to accumulate at the G_0/G_1 phase of the cell cycle after 24- and 48-h treatment. In both cell lines, this effect was observed to be dose-dependent and was accompanied by a reduction of cells in the S phase. At 72 h, the percentages of cells in the G_0/G_1 phase were comparable between the treated and control cells (Fig. 2B), suggesting that the disruption of the cell cycle progression through the G_0/G_1 and S phases occurred within 48 h of drug exposure and was transient.

Short-term effects of Selumetinib on intracellular signaling in PLGG cell lines. To evaluate the effects of Selumetinib treatment on the key intracellular downstream signaling pathways, modulation of MEK1/2, MAPK and AKT was assessed by western blot experiments. Both cell lines were treated for various times with Selumetinib at their respective IC_{30} and IC_{50}. The results demonstrated that Selumetinib treatment promoted the phosphorylation of AKT and MEK1/2 after 30 min in the two cell lines at IC_{30} and IC_{50}, although a slightly lower pattern of activation was observed in the sensitive Res259 cell line. By contrast, Selumetinib completely suppressed MAPK phosphorylation in Res186 and Res259 cells. The levels of total AKT, MEK and MAPK did not change during the experiment (Fig. 3A).

The main mechanism underlying the effects of Selumetinib on the PLGG cell lines was further examined. Both PLGG cell lines exhibited an increase in the p27 protein expression levels after 24 h drug exposure at IC_{30} and IC_{50}, whereas the phosphorylation of p21 was observed only at 24 h in Res186 cells. In addition, p85 was activated after 15-min treatment in Res186 cells; by contrast, in Res259 cells, the phosphorylation of p85 was high at 24 h. Selumetinib did not induce the cleavage of PARP in either cell line within 24 h of treatment (Fig. 3B).

Establishment of a Selumetinib-resistant PLGG cell line and mechanisms associated with resistance development. To establish a PLGG cell line with an acquired resistance to Selumetinib, Res259 cells were cultured long-term (6 months) and gradually exposed to increasing concentrations of the drug. Continuous and prolonged treatment resulted in a loss of sensitivity to Selumetinib and the establishment of the

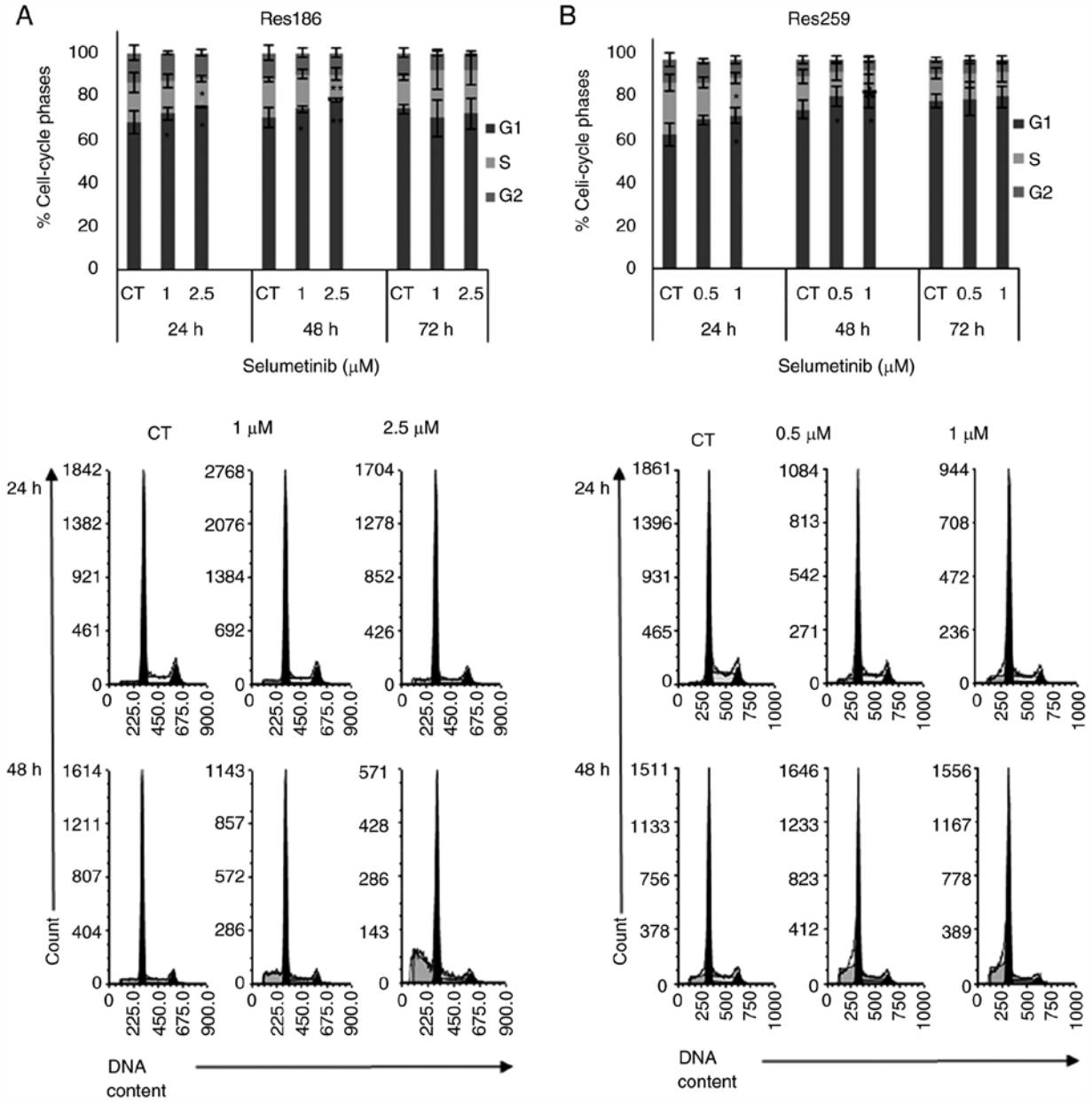


Figure 2. Selumetinib arrests the cell cycle progression of low-grade glioma cells at the G_0/G_1 phase. (A) Res186 and (B) Res259 cells were treated with the indicated concentrations of Selumetinib for 24, 48 and 72 h and subjected to cell cycle analysis by flow cytometry. Upper panels represent the stacked percentages of cells in the G_0/G_1, S and G_2/M phases of the cell cycle from at least three independent experiments. Lower panels, representative flow cytometry results in (A) Res186 and (B) Res 259 cell lines at 24 and 48 h. *P<0.05 vs. CT and **P<0.005 vs. CT. CT, control.

Res259/R cell line. As demonstrated in Fig. 4A, the IC_{50} value at 72 h was 25 μM, which was 25-fold higher compared with the sensitive Res259 cell line (RI, ~25).

To determine the potential molecular biomarkers for Selumetinib resistance in the newly established cell line, the expression levels of the principal intracellular proteins involved in the response to Selumetinib were first determined. In Res259/R cells, higher levels of p-MEK and p-AKT were observed compared with those in the sensitive parental glioma cell line. Furthermore, the resistant cells appeared to express a slightly higher level of p-MAPK compared with their sensitive counterparts, as illustrated in Fig. 4B. Since the resistant and sensitive cell lines appeared to exhibit different degrees of AKT, MEK and MAPK phosphorylation, the basal expression of NF1, the upstream negative regulator of MEK and AKT signaling, was next analyzed (26,27). The results demonstrated that Res259/R cells appeared to exhibit lower basal NF1 levels compared with those in the sensitive cell line Res259, suggesting that in Res259/R cells, low NF1 expression may lead to uncontrolled activation of its downstream effectors.

The effects of Selumetinib on the cell cycle progression in resistant cells were determined. Notably, it was observed that the distribution of Res259/R cells in the various phases of the cell cycle differed slightly from that in the parental Res259 cell line, as higher numbers of Res259/R cells were observed in the proliferative phases of the cell cycle ($S\text{-}G_2/M$) compared with those of Res259 cells (≥50 and <30%, respectively) at 24 h of culture. In Res259/R cells, Selumetinib triggered cell cycle arrest at the G_0/G_1 phase of the cell cycle after 24 and 48 h (Fig. 4C). Following 72-h drug exposure, no

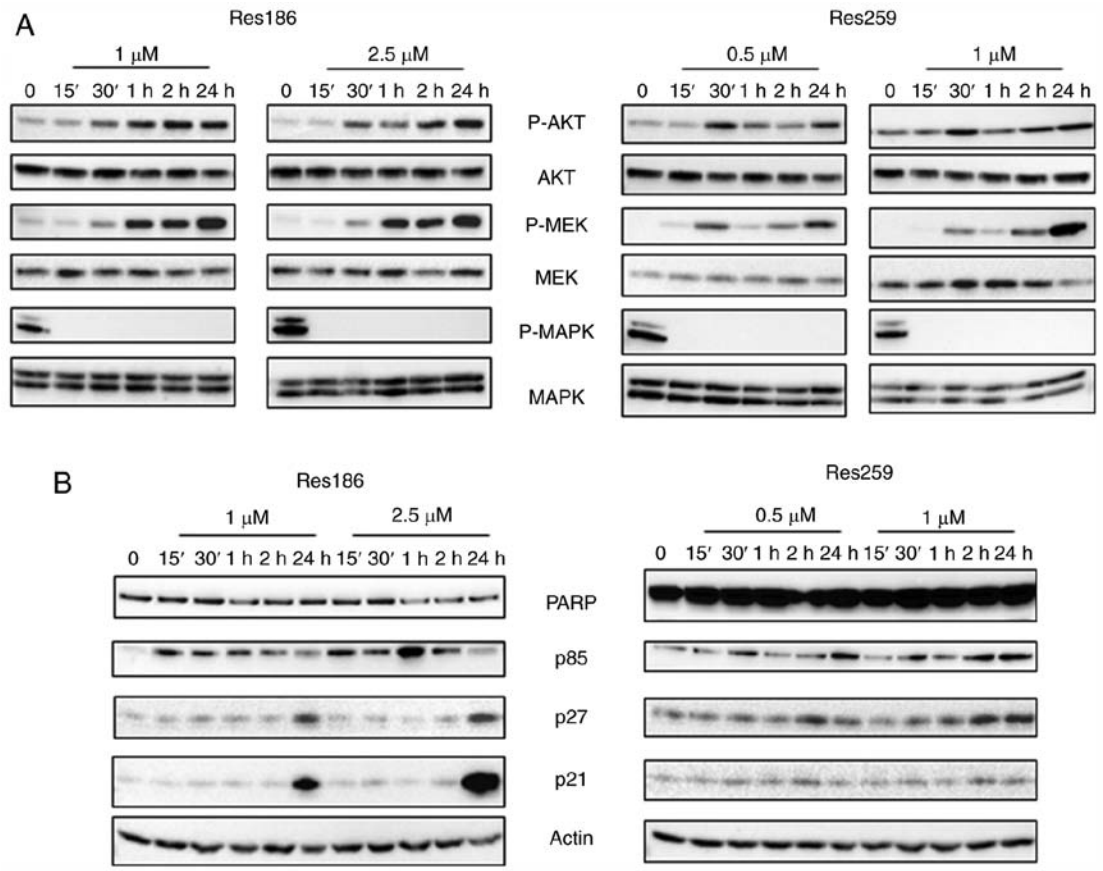


Figure 3. Western blot analysis of intracellular signaling proteins in low-grade glioma cells following treatment with Selumetinib. (A) Western blotting was used to assess the levels of total and phosphorylated extracellular-signal regulated kinase signaling proteins AKT, MEK1/2 and MAPK in Res186 and Res259 cells following Selumetinib treatment. (B) Western blotting was used to assess the levels of p85, p27, p21 and PARP in pediatric low-grade glioma cells after Selumetinib treatment for 24 h. The images are representative of at least two independent experiments with similar results. p, phosphorylated; PARP, poly(ADP-ribose) polymerase.

marked accumulation of cells was observed in the G_0/G_1 phase, similar to the parental Res259 cells (Fig. 2B).

Finally, after 24-h treatment, Selumetinib promoted AKT activation in sensitive Res259 cells, whereas the high levels of p-AKT observed in the Res259/R cell line were suppressed by Selumetinib treatment. Notably, in both glioma models of resistance and sensitivity to Selumetinib, MAPK phosphorylation was inhibited following drug exposure (Fig. 4D).

Long-term effects of Selumetinib treatment on PLGG cell lines. In order to explore the long-term effects of Selumetinib treatment on the *in vitro* PLGG models, Res259, Res259/R and Res186 cells were treated with Selumetinib for 3 and 7 days at their respective IC_{30}, and the expression levels of a range of proteins involved in the regulation of the cell cycle and apoptosis were assessed by western blot analysis. The modulation of the main molecular key mediators of the cell cycle following Selumetinib treatment were first examined in the resistant and sensitive glioma cell models. As presented in Fig. 5, MEK inhibition by Selumetinib induced an apparent reduction in cyclin D1 expression in Res186 and Res259 cells at different treatment times, whereas a slight decrease of this cell cycle regulator was only observed in Res259/R cells after 7 days of drug exposure. In the intrinsically resistant glioma cell line Res186, the decrease in cyclin D1 expression levels appeared to be accompanied by an increase in the levels of p21 after 3 days of drug exposure. By contrast, in Res259/R cells, a moderate reduction in cyclin D1 expression was observed after 7-day drug exposure, which was associated with upregulation of p27 rather than p21. Similarly, in the Res259 cells, a decrease in the protein expression levels of cyclin D1 was observed to be associated with a slight enhancement of p27 levels after 7 days of drug exposure. Notably, in Res259/R cells, the 6-month treatment with Selumetinib appeared to induce a reduction in the basal levels of the checkpoint proteins of the G_1-S transition (cyclin D1 and p21) compared with those in the parental cell line Res259. These results may indicate a more aggressive pattern of proliferation in the cellular model with acquired resistance Selumetinib compared with that in the sensitive cell line. It was then investigated whether the apoptosis machinery was activated by Selumetinib treatment. The results demonstrated that in Res259/R cells, Selumetinib appeared to slightly enhance the expression of cleaved caspase-9 following 7-day drug exposure, which was in accordance with caspase-3 activation at the same time. Furthermore, after 7 days of treatment, the 89-kDa cleaved PARP fragment was detected, and the levels of the pro-apoptotic factor Bax appeared to be increased. The resistant cell line Res186 exhibited different modulation of apoptosis regulators compared with those in Res259/R, suggesting that the underlying mechanisms of apoptosis and cell cycle arrest may vary between the two models of intrinsic and acquired Selumetinib resistance; in Res186 cells,

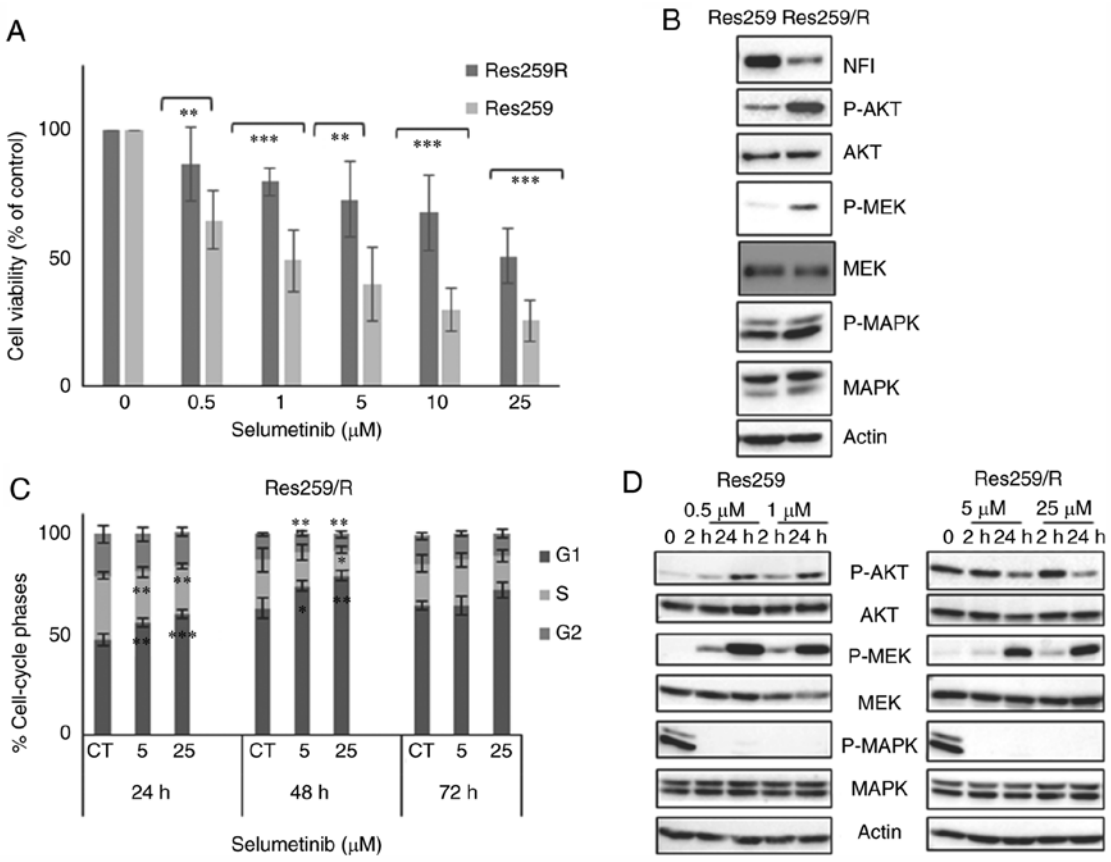


Figure 4. Establishment of a pediatric low-grade glioma cell line with acquired resistance to Selumetinib. (A) Res259 and Res259/R cells were treated with various concentrations of Selumetinib for 72 h, and cell viability was assessed by an automated cell counter. Each point represents the mean value of five independent experiments performed in triplicate. **P<0.005 and ***P<0.0005. (B) Western blotting demonstrated the protein levels of total and phosphorylated extracellular-signal regulated kinases AKT, MEK1/2 and MAPK, and NF1 in Res259 and Res259/R cells. (C) Res259/R cells were treated with Selumetinib, and the cell cycle distribution was determined by flow cytometry. The percentage of cells in each phase of the cell cycle is presented as the mean of at least three separate experiments. *P<0.05, **P<0.005 and ***P<0.0005 vs. CT. (D) Western blotting demonstrated the levels of total and phosphorylated extracellular-signal regulated kinase signaling proteins AKT, MEK1/2 and MAPK in Res259 and Res259/R cells following Selumetinib treatment for 2 or 24 h at IC_{30} and IC_{50}. The images are representative of at least two independent experiments with similar results. CT, control; p, phosphorylated.

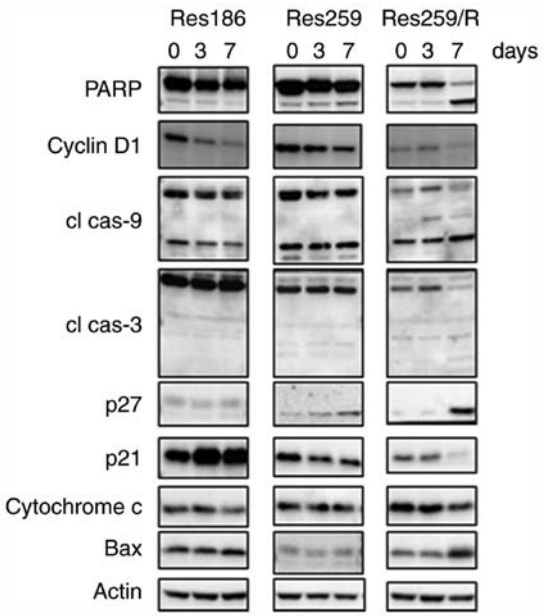


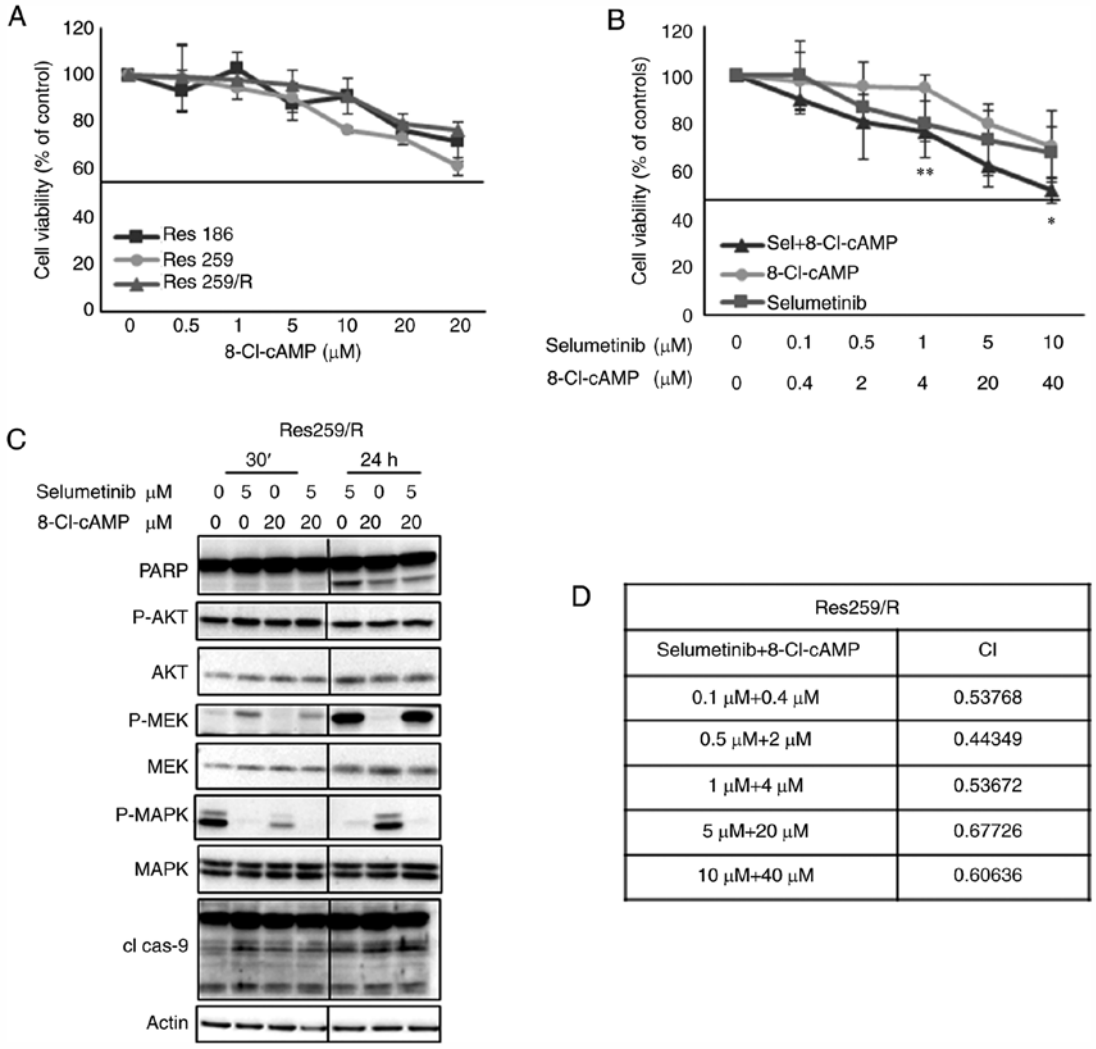
Figure 5. Effects of Selumetinib on intracellular signaling in Selumetinib-sensitive and resistant pediatric low-grade glioma cells. Cells were treated with DMSO for 3 days or Selumetinib for 3 or 7 days at their respective IC_{30} and were analyzed by western blotting. The images are representative of at least two independent experiments. cl cas, cleaved caspase.

Selumetinib induced a reduction of total PARP levels, but no caspase activation was noted even after 7 days of treatment.

Effects of the co-treatment with 8-Cl-cAMP and Selumetinib in PLGG cell lines. A number of studies have reported the importance of cAMP signaling in glioma (28-31) and the anti-proliferative effects of cAMP analogs on tumor cells (32-34). Thus, 8-Cl-cAMP was tested alone and in combination with Selumetinib to determine whether this cAMP analogue may overcome resistance to MEK inhibitors or interact with Selumetinib.

Before assessing the effects of the combination therapy, the antiproliferative effect of 8-Cl-cAMP on the PLGG cell lines was determined. Res186, Res 259 and Res259/R cells were treated with increasing concentrations of 8-Cl-cAMP for 72 h. As presented in Fig. 6A, a modest inhibition of cell viability was observed in the glioma cell lines only at high 8-Cl-cAMP concentrations (30 μM). The Selumetinib-resistant cell lines Res186 and Res259/R appeared to exhibit the same degree of sensitivity to 8-Cl-cAMP with an IC_{30} value of 20 μM, whereas the IC_{30} of Res259 was 10 μM.

For the combination experiments, PLGG cells were exposed to a range of concentrations of Selumetinib and 8-Cl-cAMP for 72 h at a fixed drug ratio for each cell line.



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Res259/R	
Selumetinib+8-Cl-cAMP	CI
0.1 μM+0.4 μM	0.53768
0.5 μM+2 μM	0.44349
1 μM+4 μM	0.53672
5 μM+20 μM	0.67726
10 μM+40 μM	0.60636

Figure 6. Effects of Selumetinib and 8-Cl-cAMP on Selumetinib-resistant cancer cells. (A) Cancer cells were treated with increasing concentrations of 8-Cl-cAMP for 72 h, and viable cells were counted by an automated cell counter. Data are expressed the percentage of the number of viable cells relative to that of the untreated controls. Each point represents the mean value of three independent experiments performed in duplicate. The horizontal line indicates 50% of cell proliferation inhibition. (B) Res259/R cells was treated with Selumetinib plus 8-Cl-cAMP at a fixed drug ratio for 72 h. Viable cell numbers were assessed by an automated cell counter. The values represent the mean of at least three independent experiments. $^{*}P<0.05$ and $^{**}P<0.005$ vs. 8-Cl-cAMP. (C) The Selumetinib resistant cell line Res259/R was treated with DMSO, Selumetinib, 8-Cl-cAMP or Selumetinib in combination with 8-Cl-cAMP at the indicated concentrations for 30 min or 24 h. Cell extracts were analyzed by western blotting. The images are representative of at least two independent experiments with similar results. (D) Combination index values were calculated according to the Chou and Talalay model for drug interactions. Sel, Selumetinib; 8-Cl-cAMP, 8-chloroadenosine 3',5'-cyclic-monophosphate; p, phosphorylated; cl cas, cleaved caspase.

Notably, the two resistant cell lines responded differently to the combination treatment; in the Res259/R cells, which exhibited acquired resistance to Selumetinib, co-treatment with Selumetinib and 8-Cl-cAMP induced a synergistic effect on cell viability, with the CI ranging between 0.44 and 0.68 (Fig. 6B and D). The addition of 8-Cl-cAMP did not sensitize the intrinsically resistant cell line Res186 to Selumetinib (data not shown). Similarly, in sensitive Res259 cells, combination treatment did not result in a synergistic effect (data not shown). Taken together, these results suggested that the addition of 8-Cl-cAMP to the single agent Selumetinib may overcome acquired, but not intrinsic resistance to this MEK inhibitor.

Western blot analysis was next performed on Res259/R cells to assess the activation of the apoptosis machinery and the principal effectors of the MAPK and AKT cascade following co-treatment with Selumetinib and 8-Cl-cAMP. Notably, the addition of 8-Cl-cAMP to Selumetinib appeared to reduce MEK phosphorylation induced by the Selumetinib alone within a shorter time period, but this effect was not maintained with longer exposure to the two drugs (Fig. 6C). In addition, the drug combination did not promote the expression of the main pro-apoptotic factors and did not affect p-AKT levels (Fig. 6C), thus suggesting that the synergistic effect on cell viability inhibition may involve other biochemical mechanisms. In addition, the protein expression levels of total AKT, MEK and MAPK did not change during the experiment.

Discussion

Over the last decade, gene expression profiling has provided new insights into the oncogenesis of PLGGs and has enabled the development of targeted therapies for these tumors (5). Uncontrolled activation of the MEK-MAPK signaling pathway is a well-known mechanism involved in the survival

and proliferation of cancer cells in several types of malignancy, including glioma (12,13). The highly selective small molecule MEK inhibitor Selumetinib has been demonstrated to be effective in clinical trials for adult cancer as a single agent (16,17,25) or as part of combination treatments (18-21), and is currently under evaluation for patients with refractory or recurrent PLGGs (clinical trial no. NCT01089101) (35,36). Despite MEK inhibition representing a promising chemotherapeutic approach, resistance to MEK inhibitors has been reported in the context of several types of cancer, such as melanoma and acute myelod leukemia (37-42). In the present study, the different sensitivity of two *in vitro* models of PLGG to single-agent Selumetinib treatment was demonstrated; Res259 was classified as a sensitive cell line, and Res186 as an intrinsically resistant cellular model. Notably, despite the different responses to the treatment, neither cell line harbored BRAF mutations (data not shown). BRAF mutations, particularly BRAFV600E, have been extensively reported to be associated with sensitivity to MEK inhibitors in various types of cancer (43-47), including melanoma, colorectal carcinoma and gliomas. Consistently with the results of the present study, previous studies by Ranzani *et al* (48) and Ming *et al* (49) have also demonstrated MEK inhibition sensitivity in a subset of BRAF wild-type cancer cell lines, which indicates that BRAF mutations *per se* may not predict sensitivity to Selumetinib. However, since pediatric patients with BRAF-mutated PLGGs may represent the best candidates for receiving Selumetinib as an alternative treatment to standard chemotherapy (50), a potential limitation of the present study was the lack of experiments using BRAF-mutated PLGG cells, which may have been more representative of the aforementioned group of patients.

The present study next evaluated whether inhibition of MAPK may be a potential biomarker for distinguishing between sensitive and intrinsically resistant cells. Previous studies on leukemia and colorectal cancer cells have revealed a substantial reduction of phosphorylated MAPK, regardless of sensitivity to the MEK inhibitor (51-53). Similar to these studies, Selumetinib suppressed MAPK phosphorylation in Res186 and Res259 cells, suggesting that, in the PLGG cell lines used in the present study, modulation of the intracellular effector MAPK may not be the only driver of cell proliferation inhibition following exposure to Selumetinib. When Res186 and Res259 cells were exposed to their respective IC_{30} for an extended period, decreases in the levels of cyclin D1 in both cell lines were observed. However, in Res186 cells, cyclin D1 reduction appeared to be associated with high levels of the cyclin-dependent kinase inhibitor p21, whereas in the sensitive cells, a decrease in cyclin D1 expression appeared to be associated with the p27 rather than the p21 pathway. In order to further elucidate the mechanisms underlying Selumetinib resistance, a cellular model with acquired resistance to Selumetinib, referred to as Res259/R, was established. With the aim of identifying molecular markers of acquired resistance to Selumetinib in PLGG cells, the basal activity of the main intracellular effectors, which were previously observed to be modulated by Selumetinib, was first determined. Hyperactivation of the AKT pathway is a mechanism of resistance to MEK inhibitors in lung cancer, melanoma and colorectal cancer cells (54-56). In addition to high expression levels of AKT, previous studies have demonstrated that resistance to Selumetinib may be either mediated by a strong coincident activation of the PI3K/AKT and ERK1/2 pathways or may be partly attributed to the reactivation of the MEK-ERK signaling pathway despite ongoing treatment with Selumetinib (57,58). In line with these studies, the results of the present study suggested that exposure to Selumetinib for extended periods may lead to positive selection of resistant cells characterized by high levels of the key intracellular regulators for cell proliferation and survival (AKT and MEK). Furthermore, our *in vitro* model of acquired resistance to Selumetinib was observed to express lower levels of the checkpoint proteins for the G_1-S transition of the cell cycle cyclin D1 and p21 compared with those in Res259 cells, which may indicate a more aggressive pattern of proliferation of Res259/R cells compared with that of the parental cell line.

Overactivation of the Ras/Raf/MEK/ERK and PI3K/AKT pathways is also involved in gliomagenesis, and their regulation is dependent on the upstream tumor suppressor protein NF1 (26). NF1 is a Ras GTPase-activating protein, the interaction of which with the Ras proto-oncogene triggers its conversion from the active GTP-bound Ras to the inactive GDP-bound form (26,27). Loss of NF1 or impaired NF1 function leads to uncontrolled activation of Ras, which in turns results in high levels of MEK/MAPK and PI3K/AKT activity (27). As higher levels of phosphorylation of AKT and MEK were observed in Res259/R cells compared with those in the Res259 cell line, NF1 expression levels were next assessed in these cellular models. The results demonstrated that the resistant cell line Res259/R appeared to exhibit lower basal NF1 levels compared with those in the sensitive Res259 cell line, suggesting that low expression of the tumor suppressor protein NF1 may lead to the uncontrolled activation of its downstream effectors in cells, characterized by acquired resistance to Selumetinib. Consistently, NF1 expression levels appeared higher in the sensitive Res259 cells, which may explain the lower AKT and MEK phosphorylation compared with that in the resistant cellular model. Contrasting results have been published regarding a possible association between the sensitivity to MEK inhibitors and NF1 expression in several types of cancer cell lines. For example, NF1-deficient acute myeloid leukemia cells and NF1-deficient glioblastoma cells have been reported to be sensitive to MEK inhibitors (59,60) and Nissan *et al* (61) have reported NF1 loss as a biomarker of sensitivity to the MEK inhibitor in melanoma cells. By contrast, Whittaker *et al* (62) have demonstrated that the loss of NF1 was a mediator of resistance to Selumetinib in melanoma cell lines. Although a complete loss of NF1 expression was not observed in Res259/R cells, it may be hypothesized that there is a possible association between low NF1 levels and Selumetinib resistance in these *in vitro* PLGG models.

In recent years, combination treatments have been proposed for overcoming resistance to MEK inhibitors (63). Since the PI3K/AKT pathway has been demonstrated to be hyperactivated in cancers with acquired resistance (56,64,65), the addition of PI3K, AKT or mTOR inhibitors to MEK inhibitors is currently considered one of the most promising combination strategies for the treatment of MEK inhibitor-resistant tumors (66-69). In addition, a recent study by Arnold *et al* (70) has demonstrated the synergistic effect on PLGG cell proliferation inhibition by combining the mTOR inhibitor TAK228 and the MEK inhibitor Trametinib. In the present study, a cAMP analogue 8-Cl-cAMP was added to Selumetinib in order to evaluate whether this combination treatment may overcome acquired resistance to

Selumetinib in the *in vitro* PLGG models. Although the mode of action of 8-Cl-cAMP has not been completely elucidated, this site-specific cAMP analogue is likely to selectively downregulate the PKA-I isoform, a signaling protein directly involved in cell proliferation, differentiation and neoplastic transformation (34). Since the cAMP/PKA pathway serves a fundamental role in the regulation of several cancer cell functions, drugs targeting this signaling pathway such as 8-Cl-cAMP have been reported to exert antitumor effects in various types of cancer (33,34,71), including gliomas (72-74). In the present study, 8-Cl-cAMP alone induced modestly inhibited the viability of the Selumetinib-resistant and sensitive cell lines. However, the addition of this cAMP analogue to Selumetinib in Res259/R cells exerted a synergist effect on cell viability inhibition (CI<1), suggesting that 8-Cl-cAMP may help reverse acquired Selumetinib resistance in the PLGG cellular model used in the present study.

In conclusion, the present study investigated three PLGG cell lines, including Res259, Res259/R and Res186, which were characterized by sensitivity, acquired resistance and intrinsic resistance to Selumetinib, respectively. In all the evaluated *in vitro* models, Selumetinib exerted a cytostatic effect by inducing G_0/G_1 phase arrest and suppressed MAPK phosphorylation, although at different concentrations. The identification of predictive biomarkers of Selumetinib sensitivity is paramount to selecting the appropriate treatment for patients with PLGG. In this context, the results of the present study demonstrated that the acquired resistance of Res259/R cells to Selumetinib was associated with a higher basal level of p-AKT and lower levels of NF1 compared with those in the parental sensitive cell model. Although further investigations are required, co-treatment with 8-Cl-cAMP and Selumetinib may be an effective combination strategy to overcome acquired resistance to Selumetinib in PLGGs.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LM and DM conceived and designed the study, collected, analyzed and interpreted the data and wrote the manuscript. ABa, AR, MM and GS collected, analyzed and interpreted the data. ABu collected the data. RR conceived and designed the study and provided financial support. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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