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Potent Antineoplastic Effects of Combined PI3K α -MNK Inhibition in Medulloblastoma

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Abstract

Medulloblastoma is a highly malignant pediatric brain tumor associated with poor outcome. Developing treatments that target the cancer stem cell (CSC) population in medulloblastoma are important to prevent tumor relapse and induce long-lasting clinical responses. We utilized medulloblastoma neurospheres that display CSC characteristics and found activation of the PI3K/AKT pathway in sphere-forming cells. Of all class I_A PI3Ks, only the PI3K α isoform was required for sphere formation by medulloblastoma cells. Knockdown of p110 α , but not p110 β or p110 δ , significantly disrupted cancer stem cell frequencies as determined by extreme limiting dilution analysis (ELDA), indicating an essential role for the PI3K α catalytic isoform in medulloblastoma CSCs. Importantly, pharmacological inhibition of the MAPK-interacting kinase (MNK) enhanced the antineoplastic effects of targeted PI3K α inhibition in medulloblastoma. This

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Acquisition of data: FE, JBB, EMB, JC, GTB, EMK, QM, DZC, CH, RH

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indicates that MNK signaling promotes survival in medulloblastoma, suggesting dual PI3K α and MNK inhibition may provide a novel approach to target and eliminate medulloblastoma CSCs. We also observed a significant reduction in tumor formation in subcutaneous and intracranial mouse xenograft models, which further suggests that this combinatorial approach may represent an efficient therapeutic strategy for medulloblastoma.

Implications: These findings raise the possibility of a unique therapeutic approach for medulloblastoma, involving MNK targeting to sensitize medulloblastoma CSCs to PI3K α inhibition.

Keywords

Medulloblastoma; neurospheres; phosphatidylinositol 3-kinase; MAPK-interacting kinase; alpelisib

Introduction

Medulloblastoma is the second most frequent malignant pediatric brain tumor and represents one of the leading causes of tumor-related deaths in children (1). About one third of medulloblastoma patients succumb to the disease due to relapse and leptomeningeal dissemination (2). Recent evidence indicates that medulloblastoma relapse is caused by therapy-resistant cancer stem cells (CSCs) (3). Like most heterogeneous tumors, medulloblastoma exhibits a subpopulation of CSCs that possess a marked capacity for proliferation, self-renewal and multi-lineage differentiation (4). As CSCs are likely responsible for tumor initiation, maintenance and relapse, they have generated increasing interest as putative targets for curative medulloblastoma therapeutic approaches.

Accumulating evidence suggests key properties of CSCs depend on phosphatidylinositol 3-kinase (PI3K) (5), and activation of the PI3K/AKT pathway contributes to the pathogenesis of medulloblastoma (6). PI3Ks are a family of lipid kinases that translate stimuli from growth factors and cytokines into intracellular signals that regulate multiple pathways including AKT and the mammalian target of rapamycin (mTOR) (7). Class I $_A$ PI3Ks are heterodimers composed of a p110 catalytic subunit (p110 α , p110 β or p110 δ , encoded by the genes *PIK3CA*, *PIK3CB* or *PIK3CD*, respectively) and a p85-type regulatory subunit (7). Activation of PI3K signaling either by reduced expression of phosphatase and tensin homolog (PTEN) or activating mutations in *PIK3CA* has been documented in medulloblastoma (6,8), and the p110 α isoform seems to be of particular importance in medulloblastoma biology and progression of the disease (9,10).

Significantly, the PI3K/AKT pathway contributes to oncogenesis and mediates survival and resistance of brain CSCs. For instance, neural progenitors expressing the progenitor/stem cell marker nestin are prone to AKT and Ras driven oncogenic transformation, promoting gliomagenesis (11). Additionally, nestin-positive cells in the perivascular niche show activation of PI3K/AKT, suggesting a role for this pathway in CSCs of the brain (12). Concomitantly, survival and radio-resistance in medulloblastoma mouse models is mediated by selective activation of the PI3K/AKT pathway in CSCs (13). Together, the important roles for PI3K/AKT in medulloblastoma pathogenesis and medulloblastoma CSC function

suggest that pharmacological inhibition of this pathway might be a promising strategy for targeting both tumor cells and CSCs in this tumor. However, pan-PI3K inhibitors show a limited therapeutic window due to adverse drug events arising from a broader spectrum of targets (14). Thus, isoform-selective inhibitors for PI3Ks are emerging clinically and may achieve greater efficacy with fewer toxic effects (15). Alpelisib is a PI3K α specific inhibitor with a favorable safety profile and a wide therapeutic window (16). However, evidence suggests that alpelisib may require combination with other therapeutics due to activation of compensatory pathways or feedback loops resulting in resistance (17–20).

Resistance mechanisms of malignant cells include gene expression alterations and activation of pro-survival pathways (21). One such mechanism is triggered by mitogen-activated protein kinase (MAPK)-interacting kinase (MNK) mediated phosphorylation of eukaryotic translation initiation factor 4E (eIF4E) on Ser-209, which stimulates changes in protein synthesis by selective mRNA translation that contribute to cancer progression and therapy resistance (22,23). MNKs also promote resistance in glioblastoma (GBM) and glioma stem cells by stimulating production of oncogenic and anti-apoptotic proteins that promote survival in response to cytotoxic agents (22,24,25). Likewise we have previously reported activation of MNK signaling in response to mTOR complex1 (mTORC1) inhibition in medulloblastoma cells, providing evidence for a similar resistance mechanism in medulloblastoma (26).

Here, we explored the roles of PI3K α and MNKs in medulloblastoma. Analysis of gene expression data indicated a positive correlation between expression of stem cell/pluripotency markers with *MKNK1*, *MKNK2* and *PIK3CA* in medulloblastoma. To study the role of PI3K in CSCs we employed 3-D neurosphere assays and found that of all class I $_A$ PI3Ks only the alpha catalytic isoform p110 α is essential for maintenance of medulloblastoma spheres. Importantly, pharmacological MNK inhibition enhanced the antineoplastic effects of PI3K α inhibition or *PIK3CA* knockdown in medulloblastoma stem-like cancer cells. Finally, in two medulloblastoma mouse models, dual inhibition of PI3K α and MNKs significantly inhibited tumor formation.

Materials and Methods

Cell culture and reagents

For conventional 2-D adherent culture, Daoy and D556 cells were propagated in DMEM with FBS (10%) and gentamycin (0.1 mg/ml). D283 cells were maintained in MEM with FBS (10%) and non-essential amino acids. 3-D stem-like cancer cell cultures were described previously (27,28). Cell lines were regularly tested for mycoplasma and underwent short-tandem repeat (STR) analysis (Genetica DNA Laboratories). Cells were authenticated where published reference STR profiles were available. The most recent STR analysis was done in December 2017. Alpelisib (BYL719) was purchased from ChemieTek and the MNK inhibitor CGP57380 (MNKi) from Santa Cruz Biotechnology. For *in vitro* studies, alpelisib or MNKi was dissolved in DMSO.

Cell viability assays

The Cell Proliferation Reagent (WST-1) assay kit (Roche) was used to determine cell viability as described previously (26). Briefly, Daoy and D556 cells were seeded into 96-well plates at a density of 2000 cells/well in the presence of alpelisib (10 μ M) and/or MNKi (10 μ M) using DMSO as vehicle control. After 5 days, WST-1 (10% v/v) was added and absorbance at 450 nm was analyzed (absorbance at 600 nm served as a reference wavelength), using an Epoch microplate spectrophotometer and Gen5 software (BioTek).

Soft agar assays

Daoy or D556 cells were seeded at a density of 2500 cells per well in soft-agar at 37°C in 5% CO₂ in the presence of alpelisib (5 μ M) and/or MNKi (5 μ M) using DMSO as vehicle control. To assess colony formation, the CytoSelect 96-Well Cell Transformation Assay (Cell Biolabs) was used as described previously (26).

Apoptosis assays

Daoy or D556 cells were grown in 6-well plates in the presence of alpelisib (5 μ M) and/or MNKi (5 μ M) using DMSO as vehicle control. After 3 days, cells were processed using the BD Pharmingen FITC Annexin V Apoptosis Detection Kit I (BD Biosciences) as described before (25). Stained samples were subjected to flow cytometry and analyzed using FlowJo 9.

Bioinformatics and statistical analysis

Gene expression data from medulloblastoma patients using the Northcott_2012 dataset (29) were downloaded from the GlioVis website (30). Subsequently, GraphPad Prism 7.0 was used to perform Pearson correlation analysis of *PIK3CA*, *MKNK1* and *MKNK2* with pluripotency/stem cell genes. GraphPad Prism 7.0 was used for statistics. One-way analysis of variance (ANOVA) was used to compare more than two groups followed by Tukey's test. Two-way ANOVA was used to compare more than two groups and multiple time points followed by Tukey's test. Mantel-Cox test was used for survival curves comparisons. Chi square (χ^2) test was used to compare stem cell frequencies of different groups for ELDA.

Confocal Laser Scanning Microscopy

Daoy and D556 cells and neurospheres were fixed in 4% paraformaldehyde (PFA), permeabilized with 0.1% Triton-X 100 in phosphate buffered saline (PBS) and blocked in a solution of 1% BSA in PBS, followed by incubation with mouse anti-nestin antibody (StemCellTechnologies) overnight at 4°C. Subsequently, samples were incubated sequentially with secondary goat anti-mouse-AF546 antibody (Thermo Fisher), phalloidin-AF488 (Thermo Fisher) and 4',6-Diamidino-2-Phenylindole (DAPI) (Thermo Fisher). A Nikon A1plus inverted microscope with a Plan Apo 60x oil objective lens (NA 1.4) was used for microscopy.

Cell lysis and immunoblotting

Where indicated, neurospheres were incubated with alpelisib (10 μ M) or MNKi (10 μ M) for 90 minutes. Cells or neurospheres were lysed and processed for SDS-PAGE and western blot as described (25). Antibodies against phospho-AKT (Ser-473), AKT, phospho-eIF4E

(Ser-209), p110 α , p110 β and p110 δ , were purchased from Cell Signaling Technology. Antibodies against GAPDH were purchased from Millipore. Antibodies against HSP90, eIF4E and Actin were purchased from Santa Cruz. Following incubation with primary antibodies, membranes were incubated with anti-rabbit horseradish peroxidase (HRP)-conjugated antibody (GE Healthcare) and anti-mouse-AF488 antibody (Thermo Fisher) simultaneously, and visualized in a ChemiDoc MP Imaging System (BioRad).

Neurosphere assay and extreme limiting dilution analysis (ELDA)

Cells were plated in Ultra-Low Attachment Round Bottom 96-Well Plates (Costar) by flow cytometry as described previously (27). Subsequently, alpelisib (5 μ M) and/or MNKi (5 μ M for neurosphere assay or 10 μ M for ELDA) were added using DMSO as vehicle control. After 7 days, acridine orange staining was performed as previously described (27). For imaging of neurospheres a Cytation 3 Cell Imaging Multi-Mode Reader was used with a 4x objective. Fluorescence signal was analyzed using 469 nm excitation and 525 nm emission filters. The Cytation 3 software was used to measure neurosphere diameters and cross-sectional areas and only neurospheres with a diameter of $\geq 100\mu$ m were scored positive for ELDA analysis (<http://bioinf.wehi.edu.au/software/elda/>) (31).

siRNA-mediated inhibition of gene expression

PIK3CA, *PIK3CB*, *PIK3CD* and control siRNA were from Dharmacon (GE Healthcare). Daoy and D556 cells were transfected with the indicated siRNAs using the Lipofectamine RNAiMAX reagent (Thermo Fisher) and Opti-MEM medium (Thermo Fisher). After 2 days transfection, cells were seeded for ELDA. Knockdown efficiencies were monitored by western blot.

Animal housing

Mouse studies were done in compliance with the Northwestern Institutional Animal Care and Use Committee (IACUC). Five to six-week-old athymic female mice (CrTac:NCr-*Foxn1*^{nu}) were purchased from Taconic Biosciences and housed under aseptic conditions.

Modification of tumor cells with firefly luciferase reporter

Firefly luciferase (Fluc) lentiviral vectors for transduction of D283 cells were described previously (32). Transduction efficiency was monitored *in vitro* using the Xenogen *in vivo* imaging system (IVIS) Lumina System coupled LivingImage software for data acquisition (Xenogen) as described before (33).

In vivo bioluminescence monitoring (BLI)

The Xenogen IVIS System (Xenogen) was used for *in vivo* BLI. Mice anesthetized with isoflurane were imaged 10 minutes after injection of luciferin (150 mg/kg). Signal intensities were quantified within regions of interest defined by the LivingImage software. Bioluminescence measurements for each animal were normalized against their own corresponding baseline bioluminescence measurements, obtained at the initiation of the indicated treatments.

Xenograft studies

For propagation of flank tumors, mice were injected subcutaneously into the left flank with 5×10^6 D556 cells suspended in 100 μ l Matrigel (Corning). Tumors were measured by caliper and tumor volume was calculated using the formula $V = ab^2/2$, where a and b (a > b) are the tumor's length and width. When tumors became palpable, mice were separated into four treatment groups with the same average tumor volume and body weight. For *in vivo* studies, alpelisib was dissolved in Ora-Plus for oral gavage and MNKi was dissolved 10%DMSO/10%Trappsol in water for intraperitoneal (IP) injection. Mice were treated with: 1) Vehicle control (VC); 2) alpelisib (50 mg/kg); 3) MNKi (25 mg/kg); or 4) combination of alpelisib (50 mg/kg) and MNKi (25 mg/kg). Body weight and tumor size were measured every other day. For the orthotopic mouse study, luciferase-expressing D283 (D283-Fluc) cells were implanted into the brains of athymic mice as described (34). Following anesthesia by inhalation of isoflurane, an incision was made to expose the skull and a small hole was drilled with a 25-gauge needle (PrecisionGude) at 3.0 mm to the right of the midline and 6.5 mm posterior from the bregma. At this location, 1×10^5 D283-Fluc cells in 1 μ l Hanks' Balanced Salt Solution without Ca^{2+} and Mg^{2+} (HBSS) were slowly injected (over 1 minute) into the right cerebellum at a depth of 3.0 mm from the underside of the skull. All procedures were carried out under aseptic conditions. After tumor formation was confirmed by BLI, mice were randomized into the same four groups as for the flank tumor model, and treatment was initiated on day 16 after cell implantation. Mice were imaged two times per week by BLI and were monitored daily for the development of neurological symptoms. Mice with intracerebellar injection were maintained until manifestation of neurological or pathological symptoms, including hunched back, body weight loss greater than 20%, reduced food intake, and inactivity. Mice with flank tumors were maintained until tumor size exceeded 1.5 cm^3 or development of pathological symptoms.

Hematoxylin and eosin (H&E) staining and immunohistochemistry (IHC)

After overnight fixation in 10% buffered formalin, flank tumors were embedded in paraffin followed by sectioning for H&E staining and IHC. For subsequent processing of sections, the Autostainer Plus and the EnVision+HRP rabbit system from Dako was used. Samples were pretreated with Target Retrieval Solution from Dako for 30 minutes at 100°C followed by incubation with 3% hydrogen peroxide for 10 minutes and incubation with EnVision kit Protein Block. For detection of apoptotic cells, the cleaved caspase-3 antibody (Biocare Medical) was used at a 1:100 dilution for 60 minutes, followed by Dako EnVision System HRP Labelled Polymer Anti-Rabbit for 30 minutes. The Liquid DAB+ Substrate Chromogen System from Dako was added for 10 minutes, followed by counterstaining with Mayer's Hematoxylin for 7 minutes. H&E staining was performed using Program 1 of the Leica Autostainer with Harris Hematoxylin and Eosin Y. Slides were analyzed by light microscopy for mitosis (H&E) and apoptosis (cleaved caspase-3) by a board-certified neuropathologist (C. Horbinski). Images were obtained with a Nikon DS-Ri2 camera, attached to a Nikon Eclipse CI microscope using a 20x objective lens (NA = 0.30). Mitoses and apoptoses were scored per 10 high-power fields.

Results

The PI3K α -selective inhibitor alpelisib exhibits an acceptable toxicity profile, and has shown some promising activity in an early phase clinical trial in solid tumor patients (16). However, activation of resistance pathways suggests targeted combinatorial approaches are needed (17,19,20). In initial studies, we examined the effects of alpelisib, alone or in combination with pharmacological MNK inhibition on medulloblastoma cells. Alpelisib exhibited potent inhibitory effects, but combination of alpelisib and MNKi inhibited cell viability significantly stronger than either agent alone in both Daoy and D556 cells (Fig. 1A). The combination of alpelisib and MNKi also potently inhibited anchorage-independent growth of colonies in soft agar (Fig. 1B) and induced apoptosis (Fig. 1C,D).

We next sought to determine if the enhancing effects of MNK inhibition on alpelisib induced responses, in part, reflect inhibitory responses in medulloblastoma stem-like cancer cells. Most brain tumors are heterogeneous and are composed of different cell types including CSCs (4). Evidence from medulloblastoma mouse models suggests that nestin expressing CSCs activate the PI3K/AKT pathway in response to irradiation and this process seems to play an important role in therapy resistance (13). Consistent with a presumed role in CSCs, we found that *PIK3CA* expression correlates positively with expression of several stem cell/pluripotency markers such as *SOX2*, *NES* (nestin), *KLF4* and *BMI1* in medulloblastoma patients (Fig. 2A, left panel). Expression of these stem cell genes also correlated with expression of *MKNK1* (Fig. 2A, middle panel) and *MKNK2* (Fig. 2A, right panel). To study the role of PI3K α and MNKs in medulloblastoma CSCs, we cultured medulloblastoma cells under serum-free conditions in 3-D. Under these conditions, cells formed neurospheres (Fig. 2B). Stem-like cancer cells grown as 3-D neurospheres exhibited increased expression of nestin, when compared to their 2-D counterparts (Fig. 2B). This indicates that medulloblastoma cells grown as neurospheres adopt CSC characteristics, which is consistent with previous reports (35). Additionally, neurospheres depicted substantial increase in phosphorylation of AKT on Ser-473, indicative of activation of the PI3K/AKT pathway (Fig. 2C).

The increase in AKT phosphorylation in neurospheres was potently inhibited by alpelisib, suggesting an important role for PI3K α in AKT activation in sphere forming cells (Fig. 3A). Additionally, MNKi reduced MNK activity as demonstrated by efficient inhibition of eIF4E phosphorylation on Ser-209, a major target for MNKs (Fig. 3B). Concomitantly, alpelisib and MNKi inhibited neurosphere growth and this effect was even more pronounced when both inhibitors were combined, indicating that dual PI3K α and MNK inhibition potently blocks growth of neurospheres (Fig. 3C, D).

Extreme limiting dilution analysis (ELDA) is an experimental approach for estimating stem cell frequencies by quantifying the proportion of cells with self-renewal capacity within a cell population (31). We employed RNA interference (RNAi) and ELDA to determine the discrete roles of p110 isoforms on stem cell frequencies and neurosphere growth. Of all class I α catalytic PI3K isoforms, *PIK3CA* knockdown had the most potent inhibitory effect on neurosphere growth (Fig. 4A) and stem cell frequencies (Fig. 4B). These effects were even more pronounced when *PIK3CA* knockdown was combined with MNK inhibition (Fig.

4A, C). Specifically, Daoy stem cell frequencies decreased from 1 in 22.2 cells for controls to 1 in 430.1 cells for *PIK3CA* knockdown (Fig. 4D, upper panel). Similarly, stem cell frequencies for D556 neurospheres decreased from 1 in 25.5 for control cells to 1 in 267.7 for knockdown of *PIK3CA* (Fig. 4D, lower panel). Stem cell frequencies did not substantially drop for knockdown of *PIK3CB* or *PIK3CD* (Fig. 4D). Chi-square analysis revealed highly significant inhibition of stem cell frequencies after knockdown of *PIK3CA* with and without MNKi, while knockdown of other class I_A catalytic PI3K isoforms had only moderate or no effect (Fig. 4E and Supplementary Table S1). Knockdown of p110 isoforms was confirmed by western blot analysis (Fig. 4F). These results indicate a significant disruption of stem cell frequencies after specific knockdown of the *PIK3CA* isoform, and MNK inhibition potentially enhanced these effects. By contrast, knockdown of *PIK3CB* or *PIK3CD* had only minor effects on medulloblastoma stem-like cancer cells (Fig. 4B, D). Taken together, these results strongly suggest a crucial role for PI3K α in medulloblastoma CSCs, and dual inhibition of MNKs and PI3K α , but not PI3K β or PI3K δ may disrupt self-renewal capacities of CSCs in medulloblastoma.

In view of the potent inhibitory effects of alpelisib and MNKi *in vitro*, we sought to evaluate the antitumor activity of dual PI3K α and MNK inhibition *in vivo*. For that purpose, we used a medulloblastoma flank tumor xenograft mouse model. Nude mice were injected subcutaneously with D556 cells in Matrigel. Eight days after injection, mice were treated with vehicle controls (VC), alpelisib, MNKi or the alpelisib/MNKi combination daily for 5 days followed by 2 days rest for 3 weeks. Each drug significantly reduced tumor growth, as compared to vehicle treated control mice (Fig. 5A). The alpelisib-MNKi combination almost eliminated tumor growth (Fig 5A). No significant drug-related adverse reactions were observed (see Supplementary Figure S1), suggesting that combined PI3K α and MNK inhibition by alpelisib and MNKi at concentrations that inhibit tumor growth *in vivo* may be well tolerated. Subsequently, tumors were subjected to IHC analysis. In tumors from mice treated with alpelisib and MNKi, mitotic index indicated a trend toward reduced proliferation (Fig. 5B,C). In addition, cleaved caspase 3 staining of tumors showed a significant increase of apoptosis in tumors from mice treated with both inhibitors as compared to mice treated with either drug alone (Fig. 5D,E). These results indicate that combined PI3K α and MNK inhibition significantly blocks tumor formation *in vivo*, likely through inhibition of tumor cell proliferation and stimulation of apoptosis.

As orthotopic mouse models are more appropriate for preclinical evaluations of new therapies, we next employed an intracerebellar mouse model as described previously (34). D283-Fluc cells were injected into the cerebellum of nude mice and tumor growth was monitored by noninvasive bioluminescence imaging (BLI). After tumor formation was confirmed by BLI, mice underwent treatment with vehicle controls (VC), alpelisib, MNKi or both drugs combined daily for 5 days followed by 2 days rest for two weeks. Again the combination treatment of alpelisib and MNKi significantly decreased tumor growth as compared to vehicle control or MNKi treated animals (Fig. 6A,B). Additionally, mice receiving the combination of alpelisib with MNKi showed significantly prolonged survival as compared to vehicle control or MNKi treated animals (Fig. 6C). Mice treated with vehicles exhibited a median survival of 85 days. Alpelisib or MNKi alone increased the median survival to 109 and 100 days, respectively. Importantly, animals treated with the

drug combination exhibited a median survival of 128.5 days, a 43.5-day increase compared to animals treated with vehicles (Fig. 6C). These studies suggest that dual targeting of PI3K α and MNKs might represent an attractive therapeutic approach for medulloblastoma.

Discussion

CSCs have attracted increased interest because of their central roles in therapy resistance and tumor recurrence (36). In order to study stem-like cancer cells *in vitro*, we propagated established medulloblastoma cell lines, Daoy and D556, as neurospheres in 3-D under stem cell conditions, as described before (27). Resulting neurospheres displayed substantial increase in nestin expression, suggesting they adopted stem-like characteristics, which is consistent with previous reports (35,37,38). When compared to their parental tumor cell lines grown in 2-D, these 3-D stem-like cancer cells exhibited increased phosphorylation of AKT on Ser-473, indicative of PI3K/AKT pathway activation in neurospheres. In solid tumors, activation of PI3K/AKT is frequently found in CSC populations (12), which is consistent with an established role for PI3K signaling as an important mediator of self-renewal in embryonic stem cells (39). This suggests medulloblastoma stem-like cancer cells grown as neurospheres might represent a suitable 3-D model system that recapitulates certain CSC properties, such as expression of progenitor/stem cell markers and activation of PI3K/AKT signaling.

PI3K signaling has emerged as a key pathway in CSC regulation (reviewed in (5)) but little is known about the precise roles of PI3K isoforms in medulloblastoma CSC biology. Using medulloblastoma neurospheres, we found knockdown of *PIK3CA* potently inhibited neurosphere formation and disrupted cancer stem cell frequencies. Importantly, knockdown of *PIK3CB* or *PIK3CD* had minimal effects on CSC frequencies suggesting a functional specificity for the p110 α catalytic isoform in medulloblastoma stem-like cancer cells. Additionally, the PI3K α specific inhibitor alpelisib blocked the elevated AKT phosphorylation observed in neurospheres, indicating a crucial role for PI3K α in the activation of AKT seen in CSCs. It has been suggested that among class I_A PI3Ks the PI3K α isoform is the most promising target in medulloblastoma (9,10). Our study extends this notion by demonstrating essential roles of PI3K α for neurosphere growth and stem cell frequencies, suggesting medulloblastoma CSCs might be particularly vulnerable to PI3K α inhibition. However, resistance to PI3K α inhibitors has been reported in several cancers (17,19,20), indicating PI3K α inhibitors may require targeting of additional pathways.

Accumulating evidence from studies in GBM suggest that PI3K and MAPK are reciprocal bypass pathways that can promote resistance to drugs targeting either pathway alone (40–43). Receptor tyrosine kinase (RTK)/PI3K/MAPK pathways are frequently mutated in solid tumors such as GBM (44), and PI3K and MAPK promote cancer hallmarks, such as uncontrolled growth, survival, and migration (7,45,46). However, combined PI3K and MAPK/ERK Kinase (MEK) inhibition frequently induced systemic toxicity, limiting the effectiveness of these drug combinations (40,47).

MNKs are key downstream effectors of the MEK/MAPK pathway. These kinases have emerged as attractive drug targets, in part, because MNK1/2 double knockout mice exhibit

antitumor effects while lacking adverse effects (48,49). The MNK/eIF4E axis is activated as a resistance mechanism in brain cancers and brain CSCs (22,24,26). Importantly, acquired resistance to PI3K inhibition can be mediated by *EIF4E* amplification, which results in elevated levels of cap-dependent translation (50). Concomitantly, ectopic expression of eIF4E conferred resistance to PI3K inhibitors, while RNAi mediated knockdown of eIF4E increased sensitivity to pharmacological PI3K inhibition (50). Taken together, this represents strong evidence implicating the MNK/eIF4E axis as a stimulator of selective translation to support survival and resistance in response to PI3K inhibition. This raises the possibility of dual inhibition of PI3K and MNK as an attractive strategy for targeted intervention. Here we investigated the therapeutic sensitizing abilities of PI3K α inhibition along with MNK inhibition in medulloblastoma tumor cell lines and stem-like cancer cells. Potent antineoplastic effects were observed in medulloblastoma cells, where the alpelisib/MNKi combination decreased viability and the ability to form colonies in soft agar while also inducing apoptosis. In stem-like cancer cells, dual inhibition of *PIK3CA* along with MNKs significantly inhibited neurosphere growth as compared to either drug alone. Knockdown experiments confirmed a key role for the p110 α catalytic isoform in neurosphere formation. Importantly, disruption of CSC frequencies by p110 α knockdown was strongly enhanced by pharmacologic MNK inhibition. This indicates the MNK pathway may sustain survival signaling after PI3K α inhibition and dual PI3K α and MNK inhibition is required for efficient targeting of medulloblastoma CSCs.

To test whether pharmacological MNK inhibition enhances vulnerability of medulloblastoma to PI3K α inhibition *in vivo*, the combination of alpelisib and MNKi was tested in subcutaneous and intracerebellar xenograft models. Combination treatment significantly suppressed subcutaneous tumor growth, suggesting a promising therapeutic strategy to target medulloblastoma tumors. Immunohistochemistry from subcutaneous tumors revealed a significant induction of apoptosis as compared to either single agent group. In addition, a trend to decreased mitosis could be observed in the combination group. These results are in line with the anti-proliferative and pro-apoptotic effects observed with the alpelisib/MNKi combination *in vitro* (see Fig. 1). A substantial decrease in tumor growth was also evident in the intracerebellar xenograft model, which resulted in significant survival increase for the alpelisib/MNKi combination over the vehicle control group. Thus, combination treatment substantially suppressed tumor growth, suggesting that MNK inhibition might delay or even prevent resistance to PI3K α inhibition. The absence of drug-related adverse reactions in xenograft models suggests that the combination treatment might be well tolerated. As combined PI3K and MEK inhibition is known to induce toxicity (40,47) specific targeting of the alpha isoform of PI3K and the MEK downstream effector MNK might offer a wider therapeutic window while still effectively blocking medulloblastoma growth. CSCs have been implicated in the emergence of resistance and tumor relapse in multiple solid tumors (36). Our study provides important insights into the requirement of specific PI3K isoforms and MNKs in medulloblastoma CSCs. The enhanced effects observed in stem-like cancer cells and mice support the necessity of dual PI3K α and MNK inhibition to disrupt CSCs and block medulloblastoma tumor growth. Altogether, our observations define important roles for the PI3K α isoform in medulloblastoma stem-like

cancer cells and strongly suggest that specific PI3K α inhibition requires additional MNK inhibition to target therapy-resistant CSC populations in medulloblastoma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1: Antineoplastic effects of PI3K α inhibition (alpelisib) and MNK inhibition (MNKi) in medulloblastoma cells.

(A) Daoy (left panel) or D556 (right panel) cells were seeded into 96-well plates at a density of 2,000 cells per well and incubated with alpelisib (10 μ M) and/or MNKi (10 μ M). After 5 days, cell viability was quantified using the Cell Proliferation Reagent, WST-1. Data represent means $\pm$ SEM of 3 independent experiments, each done in triplicates. Unpaired one-way ANOVA, *, P 0.05, ****, P 0.0001. (B) Daoy (left panel) or D556 (right panel) cells were seeded into 96-well plates at a density of 2,500 cells per well in soft agar and incubated with alpelisib (5 μ M) and/or MNKi (5 μ M). After 7 days, colony formation was

quantified using the fluorescent CyQUANT GR Dye. Data represent means $\pm$ SEM of 4 independent experiments, each done in triplicates. Unpaired one-way ANOVA, ****, P 0.0001. (C) Daoy (left panel) or D556 (right panel) cells were treated with alpelisib (5 μ M) and/or MNKi (5 μ M). After 3 days, apoptosis was assessed by co-staining cells with propidium iodide (PI) and Annexin V-FITC followed by flow cytometry analysis. Annexin V positive cells were quantified to determine total apoptosis. Data represent means $\pm$ SEM of 4 independent experiments. Unpaired one-way ANOVA, *, P 0.05, ****, P 0.0001. (D) Representative dot plots from experiment in (C) were generated using FlowJo 9.

Figure 2: Correlation of pluripotency/stem cell markers with PI3K and MNK pathways in medulloblastoma.

(A) Multiple correlation analysis for *PIK3CA* (left panel), *MKNK1* (middle panel) and *MKNK2* (right panel) with pluripotency markers *SOX2* (blue), *NES* (green), *KLF4* (red) and *BMI1* (yellow) in medulloblastoma patients using the Northcott_2012 dataset. Gene expression data from the GlioVis software were analyzed by correlation analysis using GraphPad Prism 7.0. Pearson's correlation analysis, * P 0.05, **** P 0.0001. (B) Medulloblastoma cell lines, Daoy (left panels) and D556 (right panels), were grown in 2-D adherent cell culture (upper panels) or as 3-D neurospheres in cancer stem cell medium (lower panels) and stained for DNA (blue), actin (green) or nestin (red). Corresponding 2-D

and 3-D confocal microscopy images were acquired using identical settings. Scalebar = 50 μm . (C) Daoy or D556 cells were grown as 2-D monolayer (cells) or neurospheres (NS), treated with alpelisib (10 μM , 90 minutes), and subjected to immunoblotting using indicated antibodies.

Figure 3: Effects of dual MNK and PI3K α inhibition in stem-like cancer cells grown as 3-D neurospheres.

(A, B) Medulloblastoma cell lines Daoy or D556 were grown as 3-D neurospheres in CSC medium and treated with alpelisib (5 μ M) (A) or MNKi (5 μ M) (B) for 90 minutes followed by immunoblotting using indicated antibodies. (C, D) Daoy (C) or D556 (D) cells were grown as spheres in CSC medium for 7 days. Spheres were dissociated and seeded at 500 cells/well into round-bottom 96-well plates in the presence of alpelisib (5 μ M) and/or MNKi (5 μ M). After 7 days, spheres were stained with acridine orange and imaged to determine cross-sectional area. Data represent means $\pm$ SEM of 3 independent experiments, each done in triplicates. Representative images are shown in the upper panels. Unpaired one-way ANOVA, *, P 0.05, **, P 0.01, ***, P 0.001, ****, P 0.0001. Scalebar = 1000 μ m.

Figure 4: Effects of MNK inhibition and knockdown of p110 isoforms on medulloblastoma stem cell frequencies.

Daoy (n = 4) and D556 (n = 3) cells were subjected to *in vitro* limiting dilution assays plating decreasing number of cells after indicated knockdown of p110 isoforms with or without MNK inhibition. Briefly, cells were transfected with control siRNAs or siRNAs targeting *PIK3CA* (siCA), *PIK3CB* (siCB) or *PIK3CD* (siCD). After two days, cells were dissociated with trypsin and seeded in five technical replicates into round-bottom 96-well plates by forward- and side scatter, single-cell sorting at the indicated cell densities. Cells were treated with DMSO or MNKi (10 μ M). After 7 days, neurospheres were stained with

acridine orange and imaged using a Cytation 3 Cell Imaging Multi-Mode Reader with a 4x objective. (A) Images of neurospheres for Daoy (left panels) and D556 (right panels) after knockdown of indicated p110 isoforms (upper panels) or after knockdown of p110 isoforms in combination with MNKi (lower panels). Scalebar = 1000 μ m. (B) ELDA for Daoy (left panel) and D556 (right panel) after knockdown of p110 isoforms using the ELDA software (<http://bioinf.wehi.edu.au/software/elda/>). (C) ELDA as in (B) for Daoy (left panel) and D556 (right panel) after knockdown of p110 α with or without MNKi. (D) Stem cell frequencies of medulloblastoma stem-like cancer cells for Daoy (upper panel) and D556 (lower panel) were estimated as the ratio $1/x$ with the upper and lower 95% confidence intervals, where 1 = stem cell and x = all cells. (E) P -values from chi-square analysis are shown for Daoy (left panel) and D556 (right panel). A complete table with all group comparisons is available in the supplement. (F) Whole cell lysates were subjected to immunoblotting with antibodies against p110 α , p110 β , p110 δ , Actin or GAPDH to monitor knockdown of *PIK3CA*, *PIK3CB* and *PIK3CD*.

Figure 5: Effects of dual PI3K α and MNK inhibition on medulloblastoma flank tumor xenografts.

(A) Tumor volumes from a flank xenograft mouse model are shown. Nude mice were injected subcutaneously into the left flank with D556 cells (5×10^6 cells/mouse). Once mice showed palpable tumors, mice were randomized into vehicle control (VC) ($n = 8$), alpelisib ($n = 7$), MNKi ($n = 8$) or combination (alpelisib and MNKi) ($n = 8$) groups. Mice were treated for 3 cycles (5 days of treatment and 2 days of rest, indicated by light blue boxes). Two-way ANOVA for day 53, **, $P = 0.01$, ****, $P = 0.0001$. (B) Tumors from mice in A were stained with H&E to assess mitotic index. Number of mitoses per 10 high-power fields

(hpf) are shown. One-way ANOVA, *, $P < 0.05$, **, $P < 0.01$. (C) Representative images of H&E stained slides from analysis in B. Arrowheads indicate mitotic nuclei. Scalebar = 50 μm . (D) Tumors from mice in A were stained for cleaved caspase-3 as a marker of apoptosis. Number of cleaved caspase-3 positive cells per 10 high-power fields (hpf) are shown. One-way ANOVA, *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$. (E) Representative images of cleaved caspase-3 stained slides from analysis in D. Arrowheads indicate cells positive for cleaved caspase-3. Scalebar = 50 μm .

Figure 6: Effects of dual PI3K α and MNK inhibition on orthotopic medulloblastoma xenograft mouse models.

(A) Tumor volumes from an intracerebellar xenograft mouse model are shown. Nude mice were injected with D283-Fluc cells (1×10^5 cells/mouse) by intracerebellar injection. Mice were randomized into vehicle control (VC) ($n = 4$), alpelisib ($n = 7$), MNKi ($n = 6$) or combination (alpelisib and MNKi) ($n = 6$) groups. Mice were treated for 2 cycles (5 days of treatment and 2 days of rest, indicated by light blue boxes). Two-way ANOVA for day 74, *, $P = 0.05$, **, $P = 0.01$. (B) Representative images of *in vivo* BLI of medulloblastoma xenografts from mice in (A) on day 74. (C) Survival analysis of mice in (A). Mantel-Cox test, *, $P = 0.05$.