A Sensitized RNA Interference Screen Identifies a Novel Role for the PI3K p110γ Isoform in Medulloblastoma Cell Proliferation and Chemoresistance

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Abstract

Medulloblastoma is the most common malignant brain tumor in children and is associated with a poor outcome. We were interested in gaining further insight into the potential of targeting the human kinome as a novel approach to sensitize medulloblastoma to chemotherapeutic agents. A library of small interfering RNA (siRNA) was used to downregulate the known human protein and lipid kinases in medulloblastoma cell lines. The analysis of cell proliferation, in the presence or absence of a low dose of cisplatin after siRNA transfection, identified new protein and lipid kinases involved in medulloblastoma chemoresistance. PLK1 (polo-like kinase 1) was identified as a kinase involved in proliferation in medulloblastoma cell lines. Moreover, a set of 6 genes comprising ATR, LYK5, MPP2, PIK3CG, PIK4CA, and WNK4 were identified as contributing to both cell proliferation and resistance to cisplatin treatment in medulloblastoma cells. An analysis of the expression of the 6 target genes in primary medulloblastoma tumor samples and cell lines revealed overexpression of LYK5 and PIK3CG. The results of the siRNA screen were validated by target inhibition with specific pharmacological inhibitors. A pharmacological inhibitor of p110γ (encoded by PIK3CG) impaired cell proliferation in medulloblastoma cell lines and sensitized the cells to cisplatin treatment. Together, our data show that the p110γ phosphoinositide 3-kinase isoform is a novel target for combinatorial therapies in medulloblastoma.

Introduction

Medulloblastoma is the most common malignant brain tumour in children and accounts for approximately 20% to 25% of all pediatric central nervous system tumors (1). There are hundreds of new cases worldwide each year, most of which occur at between 5 and 10 years of age (1, 2). Treatment of medulloblastoma involves surgery followed by chemotherapy and, in the case of children older than 3 to 5 years, radiotherapy (3, 4). Although these therapeutic approaches are often effective at shrinking the primary tumor, recurrence, and metastasis frequently occur, so that the 5-year survival rate is below 50% for high-risk patients. Novel therapies are thus urgently required and will arise from a better understanding of the disease biology.

Several reports have documented the involvement of protein and lipid kinases in medulloblastoma biology. Receptor tyrosine kinases (RTKs) such as ErbB-2, ErbB-4, insulin-like growth factor-I receptor (IGF-IR), and platelet-derived growth factor receptor (PDGFR) have been shown to be expressed and to control cell proliferation, survival, and the development of metastasis in human medulloblastoma cells (1, 5). New therapies for medulloblastoma are emerging, which are based on blocking signalling by these RTKs to some of their downstream signalling targets such as phosphoinositide 3-kinase (PI3K), protein kinase B (PKB/Akt), the mammalian target of rapamycin (mTOR), and mitogen-activated extracellular signal-regulated kinase activating kinase (MEK) (6–12).

The ability to use RNA interference (RNAi) as a tool for gene silencing in mammalian cells has opened up the possibility of carrying out high-throughput loss-of-function screens in tissue-culture systems (13–16). To identify new molecular targets for medulloblastoma, we have carried out survival RNA interference (RNAi) screens targeting most of the known human protein and lipid kinases (719 genes) using libraries of either short hairpin RNA (shRNA) in retroviral constructs, or small interfering RNA (siRNA).
The analysis of cell proliferation after RNAi transfection in combination with cisplatin treatment identified new protein and lipid kinases involved in medulloblastoma cell responses. In particular, p110γ and LYK5, two kinases which had not previously been implicated in medulloblastoma biology, were shown to play a role in cell proliferation and chemoresistance by contributing to activation of the mTOR pathway.

Materials and Methods

siRNA library and screen set up

DAOY medulloblastoma cells were transfected with a 96 well plate arrayed siRNA kinase library (Ambion) containing 2157 unique small interfering RNAs (siRNAs) targeting each of the 719 human kinase genes (3 individual siRNAs per gene). DAOY cells (4500 cells in 100 μl medium/well) were plated in DMEM (Dulbecco’s modified Eagle’s medium; Invitrogen) containing 10% Fetal calf serum (FCS) and 1% L-glutamine and allowed to attach. siRNA transfections (5 pmol siRNA/well) were carried out using Lipofectamine 2000 (Invitrogen) as per manufacturer’s instructions for adherent cell lines, in two identical replica plates. At 24 hours after transfection, one replica plate was treated with 0.5 μmol/L cisplatin (Bristol–Myers Squibb) and one replica plate with vehicle in media. Cell viability was assessed after further 48 hours by MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay using the Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega). On each plate, siRNA for GAPDH (Ambion), siCONTROL Non-Targeting siRNA Pool (Dharmacon), and siCONTROL RISC-Free siRNA (Dharmacon) were used as negative controls (one well each) and siCONTROL TOX was used as positive control for transfection efficiency. The screen was carried out in triplicate and, for each individual siRNA, the mean values of the three independent experiments were calculated. The effect of each siRNA on cell proliferation was assessed dividing the mean of the 3 replica wells by the mean of the 3 negative controls of the corresponding plate, and expressed as a percentage. For determination of the sensitivity to cisplatin treatment, surviving fractions were calculated as follows: Surviving fraction = log2(mean (three replica cisplatin-treated wells)) − log2(mean (three replica vehicle-treated wells)). To allow comparisons, the surviving fraction from all 27 RNAi plates were combined and expressed as a median-centred Z score. Z-score normalization of the data within each well location of the plate was conducted, subtracting the median surviving fraction from the surviving fraction of the individual siRNA and then dividing the difference by the standard deviation. The correction factor 1.4826 for large population size was used. To define effects on cisplatin resistance, a threshold of Z ≤ −2 for sensitization was used.

Screen validation, shRNAs, and plasmid transfections

Two distinct siRNA species, and one shRNA were used to revalidate hits from the screen. The siRNA sequences used for validation were the same as the ones used in the screen. Ambion Catalog numbers of the siRNA sequences were used: PIK3CG#1–ID: 143808, PIK3CG#2–ID: 143807, PLK#1–ID: 103548, PLK#2–ID: 1341, LYK5#1–ID: 43040, LYK5#2–ID: 43116, ATR#1–ID: 103302, ATR#2–ID: 82, PI4KA#1–ID: 1604, PI4KA#2–ID: 144408, WNK4#1–ID: 1105, and WNK4#2–ID: 1106. RNAi-mediated gene silencing was evaluated 48 hours after transfection. mRNA and protein were extracted in order to assess protein expression by western blotting and mRNA expression by quantitative PCR. The shRNA-encoding plasmids used are part of the Human Kinase shRNA Release 1.A2 library (Open Biosystems). The following plasmids were used: v2HS_36776 (MPP2), v2HS_179927 (LYK5), and v2HS_170020 (PIK3CG). The negative control vector (pSM2) containing a nontargeting shRNA sequence was used as shRNA control. To transfect DAOY medulloblastoma cells with plasmids, the Lipofectamine Plus reagent (Invitrogen) was used according to the manufacturer’s recommended protocol.

Clinical samples

Ethical approval to use residual tissue was obtained for all primary medulloblastoma samples used in this study. The samples used in this study and methods for RNA extraction have been previously described (17). Forty-seven medulloblastoma samples collected at diagnosis in a single institution between 1992 and 2004 (Lellouch-Tubiana, Necker Hospital) according to the French law of bioethics (Loi Huriet) were used for gene-expression analyses. An independent set of 10 RNA-later-preserved tumor tissues from the Swiss Pediatric Oncology Group (Tumor Bank was obtained for mRNA and protein-expression analysis.

Expression profiling

Gene-expression profiles were obtained on the Affymetrix HG-U133 Plus 2.0 array, containing 54000 probe sets for transcripts and variants (17). The GC robust multiarray average (GCRMA) procedure was used for normalization of gene-expression data, which were subsequently converted into log2-values. Probes of interest have been considered for analysis and compared with expression levels of 9 normal cerebellum samples (GSM80616, GSM80617, GSM80618, GSM80619, GSM80626, GSM80636, GSM80637, GSM80638, and GSM80639; expression data are available on the public database Gene Expression Omnibus: http://www.ncbi.nlm.nih.gov/geo).

TaqMan real time quantitative reverse transcription-PCR (qRT-PCR)

Total RNA from each tumor sample was converted into cDNA using the SuperScript First-Strand Synthesis System (Invitrogen) for PCR according to manufacturer’s instructions. Assays-on-demand gene-expression products (Applied Biosystems) were used to measure mRNA expression levels of PLK1 (Hs00153444_m1), LYK5 (Hs00405851_m1), MPP2 (Hs00178428_m1), PIK3CG (Hs00114305_m1), PI4KA (Hs01021084_m1), WNK4...
(Hs00260769_m1), ATR (Hs00354807_m1), and GAPDH (Hs9999905_m1; internal control gene). Normal human cerebellum was used as a reference (BioCat GmBh). Relative mRNA expression levels were calculated using the comparative threshold cycle (CT) method (18).

Cell culture and treatments
The cell lines' provenance has been previously described (19). DAOY, UW-228, and PFSK human cell lines were purchased from the American Type Culture Collection. D341 and D425 MB cells were the kind gift of Henry Friedman (Duke University). Cell lines that were not purchased from the American Type Culture Collection in 2009 were tested for their authentication by karyotypic analysis using molecular cytogenetic techniques, such as comparative genomic hybridization. DAOY medulloblastoma cell line was grown in Richter’s MEM Zinc option medium (Invitrogen) with 10% FCS (fetal bovine serum; Sigma) and penicillin/streptomycin (Invitrogen). PFSK primitive neuroectodermal tumor (PNET) cell line was grown in RPMI 1640 (Invitrogen) with 10% FCS and penicillin/streptomycin/L-glutamine. The UW-228 medulloblastoma cell line was grown in DMEM (Dulbecco’s modified Eagle’s medium; Invitrogen) with 10% FCS and penicillin/streptomycin/L-glutamine. All cells were grown in a humidified atmosphere at 37° and 5% CO2. The inhibitors BI2536 (Axon Medchem) and AS 252424 were dissolved in dimethyl sulfoxide (DMSO) at 10 mmol/L and diluted into cell culture medium just prior to use.

Western blotting
Protein expression was analyzed by immunoblotting as previously described (11, 20) using antibodies specific for PI3K p110γ, LYK5 (STRAD N-13), LKB1, MPP2 (Santa Cruz Biotechnology), AMPKα, phospho-Akt (Ser473; Thr308), ATR, phospho-S6 (Ser 235/236 and Ser 240/244), phospho-4E-BP1 (Cell Signaling Technology), and β-Actin (Sigma). The blots were quantified by densitometry using the software TotalLab Quant (TotalLab Limited).

Apoptosis
For detection of apoptosis, cells were either transfected with siRNA as described above or incubated for 16 to 24 hours in the presence or absence of the inhibitors and the cell-death detection ELISA method (Roche Diagnostics) was used for detection of apoptosis. Alternatively, apoptosis was also measured by propidium iodide staining followed by flow cytometry as previously described (21). Caspase 3/7 activity was measured by using the Caspase-Glo 3/7 Assay (Promega).

Statistical analysis
All tests were carried out using GraphPad PRISM 4.03 software. Descriptive statistics including mean and standard error of the mean, one-way ANOVA’s and Student’s two-tailed t-tests were used. P values < 0.05 were considered significant.

Results
Identification of protein and lipid kinases involved in medulloblastoma cell proliferation and cisplatin sensitivity
To identify genes essential for medulloblastoma cell proliferation and involved in cellular responses to cisplatin treatment, we designed a robust, cell-based high-throughput kinome-wide RNA interference screen. We systematically inactivated all human protein and lipid-kinase genes by siRNAs and scored them for inhibition of cell proliferation, in the presence and absence of low-dose cisplatin. DAOY medulloblastoma cells were transfected with a 96-well plate arrayed library of siRNA duplexes targeting each of the 719 human kinase genes. We chose the DAOY cell line for the screen, as well as UW-228 and PFSK cell lines for validation experiments, because our previous published work has characterized these cell lines in terms of response to cisplatin and activation of various survival pathways (11, 19, 22–24). The characteristics of the cell lines under study are described in the Supplementary Table S1. We first assessed viability effects induced by siRNAs, in the absence of cisplatin treatment. In the screen, kinases involved in proliferations were defined as kinases whose downregulation led to a >25% decrease in cell proliferation compared with the scrambled controls, with at least two individual siRNA sequences. In large-scale screens, confirmation of an observed phenotype with redundant silencing reagent offers the most straightforward way of showing RNAi target specificity, allowing exclusion of possible off-target effects (25). Overall, 31 kinases were identified as kinases involved in proliferation (Fig. 1A), with siRNAs targeting MPP2 (membrane protein, palmitoylated 2 (MAGUK p55 subfamily member 2) and PLK1 (polo-like kinase 1) having the most prominent effect (Fig. 1A). One of the kinases involved in proliferation identified, the proto-oncogene PIK3CA, encoding for the PI3K class Iγ catalytic subunit p110α, had been characterized in our previous studies as an important mediator of medulloblastoma cell survival, regulating proliferation, response to polypeptide growth factor stimulation, apoptosis and cell migration (11).

To identify kinases involved in proliferation that further have a role in cellular responses to cisplatin treatment, we carried out a secondary sensitized screen in the presence of low doses of cisplatin. The dose of cisplatin chosen for the screen was determined to induce 20% killing in DAOY cells. We first analyzed the sensitivity ratios between vehicle- and cisplatin-treated cells. Figure 1B shows all kinases, that when downregulated led to a sensitization to cisplatin treatment, with at least two unique siRNA sequences. Cisplatin is a platinum-based chemotherapeutic drug, which covalently binds to purine DNA bases leading to DNA-platinum adducts formation and cellular apoptosis (26). It is one of first and still most commonly used chemotherapeutic drugs, and much is now understood as to how tumors exhibit or acquire resistant to cisplatin treatment (26). Generally, 24 genes were identified as enhancing cell sensitivity to the drug treatment.
 amongst the top hits of our sensitized screen were kinases known to be involved in cellular response to cisplatin treatment, such as the checkpoint kinases ATR, ATM, and CHK1, from the double strand DNA repair mechanisms (Fig. 1B). Of the remaining 21 hits, 6 had also shown to have a positive regulatory effect on cell proliferation (Fig. 1A) and were reexamined (Fig. 4A). Z-score analysis revealed a normal distribution of the siRNA hits (Fig. 1C).

**PLK1 silencing or inhibition inhibits medulloblastoma cell proliferation**

The protein kinase PLK1, which was identified in the survival screen (Fig. 1A), plays an essential role during mitosis and maintenance of genomic stability (27). Cancer cells, but not normal cells, have been shown to be susceptible to PLK1 depletion (28), particularly p53-defective cell lines (29). The siRNAs targeting PLK1 were reassayed in DAOY, PFSK, and UW-228 medulloblastoma cell lines (Fig. 2A). Silencing of PLK1 strongly impaired DAOY and PFSK cell proliferation, whereas no effect was observed in UW-228 cells (Fig. 2A). The siRNAs to PLK1 were shown to reduce target mRNA and protein levels by more than 60% (Fig. 2B). Greater than two-fold increase in apoptosis was observed upon downregulation of PLK1 in DAOY cell line (Fig. 2C). Recent studies have shown that the small molecule inhibitor BI2536, a potent and selective inhibitor of PLK1, suppressed growth of a panel of human cancers in vivo (30, 31). We then investigated the effects of BI 2536 treatment in medulloblastoma cell proliferation in the cell lines under study. A strong inhibitory effect was observed in the p53-mutant cell line DAOY (IC50 2.5–5 nmol/L), whereas growth of PFSK and UW-228 cells was only partially reduced (Fig. 2D). These results, in line with the observations upon depletion of PLK1 by siRNA, show that PLK1 potently controls cell proliferation in medulloblastoma cells, underscoring the role of PLK1 in oncogenic signalling.
PIK3CG and LYK5 silencing inhibits cell proliferation and increases sensitivity to cisplatin treatment

Two genes identified in the sensitised siRNA screen (Fig. 1) were further validated with two siRNA and one shRNA sequences in three medulloblastoma cell lines, DAOY, PFSK, and UW-228 cells: the phosphoinositide-3-kinase, catalytic, gamma polypeptide (PIK3CG; gene ID 5294), and the protein kinase LYK5 (LYK5; gene ID 92335; Fig. 3, Fig. 4, and Supplementary Fig. S1A). The siRNAs targeting PIK3CG and LYK5 were shown to reduce target mRNA levels by more than 85% (Fig. 3A and Supplementary – Fig. S1). The downregulation of PIK3CG and LYK5 were confirmed by TaqMan-based qRT-PCR confirming downregulation of PIK3CG mRNA 48 hours following transfection (top). Western blotting analysis confirming PLK1 downregulation by the siRNA in the 3 cell lines (mid panel). TaqMan-based qRT–PCR confirmed downregulation of PLK1 mRNA in the 3 cell lines. DAOY (black bars), PFSK (grey bars), and UW-228 (white bars) (bottom), C, downregulation of PLK1 induces apoptosis in DAOY cells. D, PLK1 small molecule pharmacological inhibitor, BI 2536, strongly impaired DAOY cell proliferation, whereas growth of PFSK and UW-228 was only partly inhibited (DAOY; black bars; PFSK; grey bars; UW-228; white bars). * P < 0.05, compared with control.

Analysis of the phosphorylation status of downstream molecules following silencing of PIK3CG revealed an inhibition of basal phosphorylation of the S6 ribosomal protein at the Ser 235/236 and at Ser 240/244 sites, as well as 4E-BP1 phosphorylation (Fig. 3C). Silencing of LYK5 induced a marked decrease in LKB1 protein expression, whereas the levels of AMPKα were not affected (Fig. 3C). This downregulation of LKB1 expression upon LYK5 silencing was observed in all three medulloblastoma cell lines under study (Fig. 3C and data not shown). Under these experimental conditions, we did not detect any phosphorylation of AMPKα at Thr172 (data not shown). Silencing of LYK5 also resulted in an inhibition of basal phosphorylation of 4E-BP1 (Fig. 3C). Together these results suggest that PIK3CG and LYK5 are involved in the activation of the mTOR/S6K pathway in medulloblastoma cells.

To confirm the validity of the results from the sensitized siRNA screen, 6 genes that had also been shown to have a positive regulatory effect on cell proliferation (Fig. 1A) were reexamined in proliferation assays in DAOY cells (Fig. 4A) and UW-228 cells (Fig. 4B). Downregulation of target gene expression was validated by quantitative PCR (TaqMan) in the two medulloblastoma cell lines (Supplementary...
that siRNAs for PIK3CG tone-complexed cytoplasmatic DNA fragments confirmed (Fig. 4D). Furthermore, relative quantification of histone-complexed cytoplasmatic DNA fragments confirmed that siRNAs for PIK3CG and LYK5 siRNAs sensitized DAOY cells to cisplatin induced apoptosis (data not shown).

Effects of a PI3K p110γ small molecule inhibitor on medulloblastoma cell proliferation, cell death, and pathway activation

The class IB phosphatidylinositol 3-kinase has been the focus of intensive research and is regarded as a promising therapeutic target, particularly in inflammatory diseases (32). Consequently, a number of small molecule inhibitors have been developed and their potential therapeutic applications extensively studied in preclinical models (32). Using a novel PI3K p110γ small-molecule inhibitor, AS252424, we investigated whether the RNAi-mediated effects observed on cell proliferation and response to cisplatin could be reproduced by means of pharmacological targeting of this kinase. After treatment for 72 hours, AS252424 was shown to decrease the proliferation of 5 medulloblastoma cell lines, including DAOY and PFSK cells in a dose-dependent manner, with IC50 values in the range of 1 to 15 μmol/L (Fig. 5A). Cell lines with low p110γ expression, such as UW-228 and D245, were less sensitive to AS252424 (Fig. 5A and Fig. 6D). However, there was no significant correlation between the IC50 values and the expression of p110γ in the 6 medulloblastoma cell lines under study (Pearson correlation coefficient $R = -0.5721$, $P = 0.2354$). These observations suggest that other factors
modulate the sensitivity of medulloblastoma cells to the pharmacological inhibitor of p110γ. Analysis of the phosphorylation status of downstream molecules following treatment with AS252424 revealed concentration-dependent inhibition of basal phosphorylation of Akt, at the Thr 308 and Ser 437 sites, and S6 ribosomal protein at the Ser 240/244 (Fig. 5B). Furthermore, 24-hour treatment with AS252424 enhanced cisplatin-induced apoptosis in medulloblastoma cells (Fig. 5C and data not shown). Our previous studies have shown that treatment with chemotherapeutic agents such as doxorubicin leads to activation of the PI3K/Akt pathway in medulloblastoma cells (11). It has been shown in several human cancer cells that activation of the PI3K/Akt pathway renders cells resistant to commonly used chemotherapeutic agents, including cisplatin and other platinum-based drugs (33, 34). In line with what was observed upon RNAi-mediated downregulation of p110γ, AS252424 increased the levels of apoptosis induced by cisplatin treatment, sensitizing cells to cisplatin-induced cell death (Fig. 5C). Furthermore, treatment of DAOY cells with the p110γ inhibitor AS 252424 decreased cisplatin-induced phosphorylation of Akt and S6 protein (Fig. 5D). These results unveil a novel role for PI3K p110γ in human medulloblastoma, as a positive regulator of cell proliferation and underline the importance of PI3K p110γ in modulating cellular responses upon cisplatin treatment.

Expression of PIK3CG and LYK5 in human medulloblastomas

We next reanalyzed expression profiles obtained from DNA microarray analysis in medulloblastoma (17). From the genes identified in the RNAi screen in medulloblastoma (Fig. 1), PLK1, PIK4CA, and WNK4 showed no significant change in medulloblastoma, compared with normal cerebellum. In contrast, ATR and LYK5 displayed higher expression in medulloblastoma (Supplementary Figs. S2A and S2D), whereas MPP2 expression was lower (Supplementary Fig. S2C). We next carried out additional experiments to verify that PIK3CG and LYK5 are differentially expressed in human medulloblastoma primary tumors. We initially analyzed mRNA expression by quantitative RT-PCR in a set of representative medulloblastoma surgical specimens. LYK5 was found to be commonly overexpressed in medulloblastomas (20/23, 87%) whereas upregulation of PIK3CG was restricted to a subset of tumors (9/17, 53%), compared with normal cerebellum (Fig. 6A and B). Protein-expression levels were further analyzed by western blotting and expression of PI3K p110γ was detected in 13 of 19 of the samples under study (Fig. 6C, 68%). In addition, PI3K p110γ and LYK5 expression was detected in 5 of 7 (71%) of the medulloblastoma cell lines under study (Fig. 6D). In the case of LYK5 there was no significant correlation between mRNA and protein-expression levels in the cell lines (Pearson correlation coefficient $R = 0.6587$, $P = 0.1548$; Fig. 6D and Supplementary Fig. S2F). In the case of
the mRNA and protein levels did not significantly correlate (Pearson correlation coefficient $R = -0.2183$, $P = 0.7817$; Fig. 6A, 6C, 6D, and Supplementary Fig. S2E).

We next reanalyzed a set of Affymetrix Human Genome U133 Plus 2.0 microarray data comprising expression profiles on a series of 47 medulloblastoma patient samples and 9 normal cerebellum samples. This analysis revealed a significant upregulation of the gene encoding for LYK5 in medulloblastomas compared with normal cerebellum (Supplementary Fig. S2A). Interestingly, LYK5 was found to be expressed at significantly higher levels in medulloblastomas presenting typical 17p breakpoints when compared with the group of medulloblastomas without 17p breakpoints (Supplementary Fig. S2B). The analysis of the tumor samples for PIK3CG expression showed variable expression of the gene. A fraction of medulloblastoma tumors also displayed PIK3CG overexpression (data not shown).

**Discussion**

The present report represents one of the first kinome-wide RNAi screens in human brain tumor cell lines and in medulloblastoma. Ideally, such loss-of-function screens should lead to the identification of novel targets for the development of therapeutic strategies for these malignancies, which are associated with a poor outcome in patients (16). Our studies have identified two distinct panels of kinases that contribute to cell proliferation and to the sensitivity of medulloblastoma cells to the commonly used chemotherapeutic agent cisplatin.

As a validation of the survival screen, we carried out additional experiments to assess the contribution of PLK1 to medulloblastoma cell proliferation. A pharmacological inhibitor of PLK1 (BI 2536; ref. 31) reduced cell proliferation in DAOY cells, but was less potent in other medulloblastoma cell lines. In addition, siRNA-mediated downregulation of PLK1 had more pronounced effects than the inhibitor in some cell lines, such as PFSK. These contrasting results could potentially be explained by differences in PLK1 expression between the cell lines, and in p53 status, since it was previously shown that PLK1 depletion has more prominent effects in a mutant p53 background (28), which is the case for DAOY. It is also conceivable that there may be a kinase-independent function of PLK1 in cell proliferation in medulloblastoma.

When combining the results of the two RNAi screens, a set of 6 genes comprising ATR, LYK5, MPP2, PIK3CG, PIK4CA, and WNK4 were identified as contributing to both cell proliferation and resistance to cisplatin treatment in the DAOY medulloblastoma cell line. It is interesting to note here that several protein and lipid kinases were previously identified in comparable sensitised RNAi screens in different human cancer cell lines, including Akt, ATM, ATR, BUB1, phosphatidylinostiol 4-kinase, and phosphatidylinositol 3-kinase (13, 14). The identities of the PI-kinase isoforms were however different between

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**Figure 5.** Effects of treatment with a novel small kinase inhibitor of PI3K p110γ on medulloblastoma cells. A–C, pharmacological inhibition of the PI3K p110γ isoform, the protein encoded by PIK3CG, led to inhibition of cell proliferation (black diamonds: DAOY; black squares: PFSK; black triangles: UW-228; white squares: D341; black circles: D458; white circles D425). A inhibition of basal phosphorylation of Akt in DAOY cells (B) increased cisplatin-induced apoptosis (black bars: 0 μmol/L AS252424; grey bars: 1 μmol/L AS252424) in PFSK cells (C) and impaired cisplatin-induced phosphorylation of Akt in DAOY cells (D).
our study and previous reports (13, 14). A MAGUK family member, namely MPP7 was also identified in an RNAi screen in lung-cancer cells investigating paclitaxel resistance (15).

We focused our attention on two genes involved in medulloblastoma proliferation and chemoresistance, namely LYK5 and PIK3CG. This was based on an analysis of the expression of the targets identified in the RNAi screens, which revealed an upregulation of the expression of these two kinases in subsets of primary medulloblastoma tumor samples and cell lines.

LYK5 is involved in the regulation, assembly, and localization of LKB1 and the LKB1-dependent AMPK pathway (35). In medulloblastoma cells, silencing of LYK5 induced a pronounced downregulation of LKB1 expression, indicating that LYK5 may be required for LKB1 stability. The AMPK pathway has been studied as a potential target for the development of anticancer drugs in various human malignancies (36). Activation of AMPK suppresses the mTOR pathway directly or indirectly via TSC2. The mTOR pathway has been recognized as an important regulator of the sensitivity of human cancer cells to chemotherapeutic agents (13, 37). Indeed, in a previous study, rapamycin exhibited additive cytotoxicity with cisplatin in DAOY cells (10). Although LKB1 is generally thought to function as a tumor suppressor, recent work has also documented its role in the phosphorylation of proapoptotic proteins by Akt (38). Together with our data, this may indicate that LYK5/LKB1 may function as a positive regulator of the mTOR/4E-BP1 pathway in medulloblastoma.

The present study represents the first report on a role for p110g as a drug target in brain tumors. Genetic targeting of PIK3CG in mice has underlined a central role of this PI3K isoform in inflammation and allergy, as it modulates chemotaxis of leukocytes and degranulation in mast cells (39). In colorectal cancers, PIK3CG was initially proposed to function as a tumor-suppressor gene (40), a finding disputed by a subsequent study (41). An oncogenic potential was then described for p110g, similarly to other class IA PI3K isoforms (42, 43). A recent study described a critical role for p110g in pancreatic cancer, and hypothesized that PI3K/p110gamma overexpression is a key event in the disease progression (44).

The availability of isoform-specific p110g inhibitors allowed the design of validation experiments to assess
the feasibility of targeting this PI3K isoform in medulloblastoma cell lines. Indeed, our results showed for the first time that a pharmacological p110γ inhibitor impaired the proliferation of medulloblastoma cells and the activation of the mTOR/S6K pathway. The Akt/mTOR/S6K pathway was previously shown to be constitutively activated in primary medulloblastoma and cell lines (11). Our data suggest that p110γ and LYK5 contribute to this activation, in view of: (i) the overexpression of p110γ and LYK5 in medulloblastoma; and (ii) the negative impact of targeting these kinases on the basal activation of the pathway in medulloblastoma cell lines.

In addition, the pharmacological p110γ inhibitor sensitized medulloblastoma cells to cisplatin-induced apoptosis. These effects could be explained by the ability of the p110γ inhibitor to impair basal and cisplatin-induced activation of Akt in medulloblastoma cells. Therefore, more work is warranted to validate p110γ as a novel drug target in medulloblastoma. Our previous findings described a role for the class IA PI3K p110α isoform in medulloblastoma proliferation and resistance to doxorubicin (11). However, p110α was not identified in the sensitized RNAi screen with cisplatin, suggesting that different class I PI3K isoforms may contribute to resistance to cisplatin or doxorubicin. Thus, targeting PI3K signalling may represent an attractive novel approach to develop novel therapies for medulloblastoma. Indeed, there now exist different pharmacological inhibitors of PI3K, which have entered clinical trials for adult cancer and could, in the future, be also considered for medulloblastoma (12, 45–47).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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