Experimental Studies

Targeting PI3KC2β Impairs Proliferation and Survival in Acute Leukemia, Brain Tumours and Neuroendocrine Tumours

DANIELLE BOLLER[1*], KATHRIN T. DOEPFNER[1*], ANGELA DE LAURENTIIS[1], ANA S. GUERREIRO[1], MARIN MARINOV[1], TAREK SHALABY[2], PAUL DEPLEDGE[3], ANTHONY ROBSON[3], NAHID SAGHIR[3], MASAHIKO HAYAKAWA[4], HIROYUKI KAIZAWA[4], TOMONOBU KOIZUMI[4], TAKAHIDE OHISHI[4], SARAH FATTET[5], OLIVIER DELATTRE[5], ANELIA SCHWERI-OLAC[6], KATRIN HÖLAND[6], MICHAEL A. GROTZER[2], KARL FREI[7], OLIVIER SPERTINI[8], MICHAEL D. WATERFIELD[3,9] and ALEXANDRE ARCARO[1,6]

[1]*Division of Clinical Chemistry and Biochemistry, and*
[2]*Department of Oncology, University Children's Hospital Zurich, Zurich, Switzerland;*
[3]*PIramed, Slough, Berkshire, UK;*
[4]*Drug Discovery Research, Astellas Pharma Inc., Tsukuba, Ibaraki, Japan;*
[5]*INSERM U509, Paris, France;*
[6]*Department of Clinical Research, University of Bern, Bern, Switzerland;*
[7]*Department of Neurosurgery, University Hospital Zurich, Zurich, Switzerland;*
[8]*Service and Central Laboratory of Hematology, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland;*
[9]*Ludwig Institute for Cancer Research, Proteomics Unit, London, U.K.*

Abstract. *Background: Eight human catalytic phosphoinositide 3-kinase (PI3K) isoforms exist which are subdivided into three classes. While class I isoforms have been well-studied in cancer, little is known about the functions of class II PI3Ks. Materials and Methods: The expression pattern and functions of the class II PI3KC2β isoform were investigated in a panel of tumour samples and cell lines. Results: Overexpression of PI3KC2β was found in subsets of tumours and cell lines from acute myeloid leukemia (AML), glioblastoma multiforme (GBM), medulloblastoma (MB), neuroblastoma (NB), and small cell lung cancer (SCLC). Specific pharmacological inhibitors of PI3KC2β or RNA interference impaired proliferation of a panel of human cancer cell lines and primary cultures. Inhibition of PI3KC2β also induced apoptosis and sensitised the cancer cells to chemotherapeutic agents. Conclusion: Together, these data show that PI3KC2β contributes to proliferation and survival in AML, brain tumours and neuroendocrine tumours, and may represent a novel target in these malignancies.*

Phosphoinositide 3-kinases (PI3Ks) play an essential role in the signal transduction events initiated by the binding of extracellular signals to their cell surface receptors (1-3). Cellular responses controlled by PI3Ks are extremely diverse, including mitogenesis and proliferation, protection from apoptosis and cell motility (1-3). There are eight known PI3Ks in humans, which have been subdivided into three classes, based on structural homology and *in vitro* substrate specificity (4-7). Class I_A comprises three highly homologous isoforms, p110α (8), p110β (9) and p110δ (10, 11), which exist as a heterodimeric complex with a regulatory subunit containing two SRC homology-2 (SH2) domains, mediating enzyme association with phosphotyrosine residues in the cytoplasmic domains of activated polypeptide growth factor receptors (12-14). All class I PI3Ks function as PtdIns(4,5)P_2 3-kinase *in vivo*, upon activation by receptor tyrosine kinases or serpentine receptors (15, 16). PtdIns(3,4,5)P_3 serves as a docking site for the serine/threonine protein kinase phosphoinositide-dependent protein kinase-1 (PDK-1), which is activated upon binding (17). Several protein kinases have been identified as downstream targets of PDK-1, such as the serine/threonine protein kinase B (PKB) /AKT, which is a key regulator of cell survival, ribosomal protein S6 kinase (S6K), which

*These Authors contributed equally to this study.

Correspondence to: PD Dr. Alexandre Arcaro, Division of Pediatric Hematology/Oncology, Department of Clinical Research, University of Bern, Tiefenaustrasse 120c, 3004 Bern, Switzerland. Tel: +41 313088029, Fax: +41 313088028, e-mail: alexandre.arcaro@dkf.unibe.ch

Key Words: PI3KC2β, pharmacological inhibition, cell proliferation, migration, acute leukemia, brain tumours, neuroendocrine tumours.

stimulates protein synthesis and cell growth, glycogen synthase kinase-3 (GSK-3), a key regulator of glycogen synthesis, and a subset of protein kinase C (PKC) isoforms (3, 18, 19).

Class II PI3Ks comprise the Drosophila PI3K_68D/Cpk (20, 21), mouse Cpk-m (21), and human PI3KC2α (22), PI3KC2β (23) and PI3KC2γ (24,25). The hallmarks of class II family members are a substrate specificity restricted to PtdIns and PtdIns(4)P *in vitro*, and a conserved C-terminal C2 domain, involved in phospholipid binding. Recent studies have started to investigate the regulation and functions of class II PI3Ks *in vivo*. Indeed, several reports have shown that class II PI3Ks are downstream targets of activated receptor tyrosine kinases (RTKs), such as the epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene-like protein (C-KIT) and insulin receptor (IR) (26-29). Studies in *Drosophila melanogaster* have also revealed a role for the class II PI3K_68D in cell differentiation downstream of the EGFR (30). Furthermore, recent reports have documented a role for PI3KC2β in cell migration in mammalian cells *via* activation of Rho family GTPases and in human cancer cells (28, 31, 32). PI3KC2β was also reported to contribute to cancer cell growth and AKT activation (29, 33-35). Analysis of a conditional knock-out mouse of *PIK3C2B*, however, revealed no obvious phenotype despite a fairly ubiquitous deletion of *PIK3C2B* (36).

The importance of PI3K signalling in cancer is highlighted by the fact that mutations in the tumour suppressor gene phosphatase and tensin homologue (*PTEN*) occur frequently in human tumours. PTEN is a phosphatase that antagonises the action of PI3K by de-phosphorylating the D-3 position of polyphosphoinositides (37-40). Moreover, numerous reports have described activating mutations in the *PIK3CA* gene encoding the catalytic p110α isoform of PI3K in a variety of human cancer types including breast, colon and ovarian cancer (37, 39-44).

In the present study, we evaluated the expression of the class II PI3KC2β isoform in a panel of primary human tumours and cell lines. Furthermore, we used isoform-specific pharmacological inhibitors and RNAi to inhibit this enzyme in human cancer cell lines. We show for the first time that PI3KC2β is overexpressed in acute myeloid leukemia, brain tumours and neuroendocrine tumours, and that inhibiting this class II PI3K reduces proliferation and survival in cell lines from these types of cancer.

Materials and Methods

Reagents and antibodies. The PI3KC2β antibody was described elsewhere (23). Other antibodies and reagents were purchased from the following companies: Caspase-3, poly(ADP-ribose) polymerase (PARP; Santa Cruz Biotechnology, Santa Cruz, CA, USA); activated AKT/PKB (Ser473), activated c-Jun NH2-terminal kinase (JNK) (Thr183/Tyr185), activated S6 protein (Ser235/236) (Cell Signalling Technology, Danvers, MA, USA); β-actin, β-tubulin (Sigma-Aldrich, St Louis, MO, USA); siGENOME™ siRNA (Dharmacon, Lafayette, CO, USA); Etoposide (Calbiochem, La Jolla, CA, USA); doxorubicin (Pfizer AG, Zurich, CH); PI701 (YM185453) and PI702 (YM182832) were provided by PIramed Pharma Limited (Berkshire, UK); PI701 and PI702, isoform-specific inhibitors of PI3KC2β, were synthesised as described in patent no. US 6,770,641 B2. The use of fused heteroaryl derivatives as PI3K isoform inhibitors has been published previously (45-48).

Apoptosis. For detection of apoptosis, cells were incubated for 16-24 h in the presence or absence of inhibitors. The cells were then lysed and caspase-3 activity was measured using the CaspACE Assay System (Promega, Madison, WI, USA) (49, 50). Additionally, samples were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot with antibodies to caspase-3 and PARP.

Cell culture. Acute myeloid leukemia (AML), neuroblastoma (NB), glioblastoma (GBM), medulloblastoma (MB), small cell lung cancer (SCLC) and mammary epithelial cell lines shown in Table I were grown in Roswell Park Memorial Institute medium (RPMI) or Dulbecco's Modified Eagle's Medium (DMEM) (Life Technologies/Invitrogen, Carlsbad, CA, USA) with 10% (v/v) fetal calf serum (FCS) and penicillin/streptomycin/L-glutamine, and passaged every 3-5 days (51-54). DAOY, D341, D425 and D458 medulloblastoma cell lines were grown in MEM Zinc option (Richter's modification) medium supplemented with 10% FCS (49). For growth factor stimulation, cells were incubated overnight in their growth medium with low serum (0.5-1% v/v) or Optimem medium (Life Technologies/Invitrogen) and washed with serum-free medium prior to incubation with growth factors. Heparinized peripheral blood or bone marrow samples were obtained from adult patients with AML. Blast cells were isolated as described previously (51). Type II human lung pneumocytes (52) were maintained in DCCM-1 medium supplemented with 10% new born calf serum.

Cell proliferation. Cell lines (5×10^3 cells/well) were seeded in 96-well plates and grown for 72 h in serum (10%)-containing medium in the presence or absence of inhibitors PI701 or PI702. The number of viable cells was analysed by means of an [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] (MTS) assay using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega) according to the manufacturer's instructions. Data are the mean with SD from 8 repetitions.

Bromo-deoxy-uridine (BrdU) incorporation. AML cells were incubated *in vitro* with bromo-deoxy-uridine (BrdU) and, after preparation of cytocentrifuge smears, stained with an anti-BrdU antibody to enable detection of cells in the S phase of the cell cycle (BrdU-labeling index, LI) (BrdU Labeling and Detection Kit; Roche Diagnostics, Rotkreuz, Switzerland) (55).

Combination experiments with chemotherapeutic agents. Cell lines (5×10^3 cells/well) were seeded in 96-well plates and grown for 72 h in serum (10%)-containing medium in the presence of the inhibitor PI701, etoposide, or doxorubicin, as single agents or in combination. The number of viable cells was analysed by means of

Table I. *Half maximal inhibitory concentration (IC_{50}) values (μM) of PI701 and PI702 against a panel of cancer cell lines and primary cultures from neuroblastoma, glioblastoma, medulloblastoma, acute myeloid leukemia, and small cell lung cancer.*

	IC_{50} Values	
Inhibitor	PI701	PI702
Neuroblastoma cell lines		
CHP134	7.4	8.0
LAN1	8.3	10.0
SHSY5Y	15.8	6.6
WAC2	5.2	6.0
Glioblastoma cell lines and *ex vivo* cultures		
T98G	6.9	>20
U251	8.0	>20
LN319	19.6	14
LN229	6.2	14.1
U87	>20	10.2
LO	11.6	ND
RE	13.2	ND
SB	18.2	ND
Medulloblastoma cell lines		
DAOY	10.1	>20
D341	4.5	4.0
AML cell lines, patient blasts and control cells		
U937	3.2	5.5
HL-60	9.6	12.4
NB4	5.3	7.8
THP1	8.4	9.5
Kasumi	>20	ND
KG-1	17.0	8.0
K562	10.4	>20
FIN COS	>20	>20
41b MI	>20	>20
FAB M5	2.5	ND
FAB M1	3.6	ND
FAB M0	ND	6.2
N	ND	12.6
SCLC cell lines and control cells		
H69	4.4	ND
H209	4.3	ND
H510	5.0	ND
SW2	10.2	ND
PN	12.0	ND

LO, RE, SB: Glioblastoma *ex vivo* cultures; FIN COS, 41b MI: immortalised B cells; FAB: French-American-British classification; N: non-leukemic bone marrow cells; PN: immortalised type II pneumocytes; ND: not determined.

an MTS assay using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega) according to the manufacturer's instructions. For detection of apoptosis, cells were incubated for 16-24 h in the presence the inhibitor PI701, etoposide, or doxorubicin, as single agents or in combination. The cells were then lysed and samples were analysed by SDS-PAGE and western blot with antibodies to caspase-3 and PARP.

Dissociation of brain tumours. Human brain tumours were removed from four patients who underwent surgery for tumour resection at the University Hospital Zurich. The procedures were conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Canton Zurich. Following removal, tumour tissue was immediately placed in a petri dish, minced mechanically and digested enzymatically with collagenase D and DNase I (Roche Applied Science) for 1 h at 37˚C while being stirred with a magnetic bar. The dissociated cells were then sequentially filtered through 100 and 70 μm cell strainers (BD Falcon; BD Biosciences, Basel, Switzerland) to remove any tissue debris. Erythrocytes were removed by resuspending and incubating the cells in ice-cold ACK buffer [17 mM Tris–HCl (pH 7.2) containing 144 mM NH4Cl] for 10 min on ice. The cells were washed in PBS and plated in DMEM (Life Technologies/Invitrogen) supplemented with 10% (v/v) heat inactivated FCS and gentamycin (20 mg/ml) and passaged every 3-5 days by trypsinization.

DNA microarray. Total RNA was extracted from 60 MB samples and three cell lines (D283, D341, and DAOY) using Trizol reagent (Invitrogen). After DNAse treatment and RNA purification (RNeasy Micro kit; Qiagen, Santa Cruz, CA, USA), gene expression profiles were obtained on the Affymetrix HG-U133 Plus 2.0 array that contains more than 54000 probe sets for transcripts and variants (49, 50, 56). Expression data for nine normal cerebellum tissue samples analysed on the same Affymetrix array version were obtained from Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/). Gene expression data for the samples were normalized using the GCRMA procedure. PI3KC2β expression levels for the samples are presented (unlogged data).

Isolation of RNA from tumour samples and reverse transcription-polymerase chain reaction (RT-PCR). For primary NB samples, ethical approval to use residual tissue was obtained. RNAlater-preserved tumour tissue was available from the Swiss Pediatric Oncology Group tumour bank. All diagnoses were confirmed by histological assessment of the tumour specimen obtained at surgery. NB tissue was disrupted with a sterile disposable tissue grinder (Sage Products, Cary, IL, USA) and homogenised in guanidinium isothiocyanate-containing buffer (54). Total RNA of cell lines and tumour tissue was isolated using the RNeasy kit (Qiagen) according to the manufacturer's protocol (54). Total RNA (3 μg) from each tumour sample was converted into cDNA using the SuperScript™ First-Strand Synthesis System for PCR according to manufacturer's instructions (Invitrogen). mRNA expression of PI3KC2β and 18S (internal control gene) was measured in tumour samples and cell lines by TaqMan® Assay-on-Demand™ Gene Expression products (Applied Biosystems, Foster City, CA, USA). The following primers were used (gene, assay ID): *PIK3C2B*, Hm00153248_m1; eukaryotic *18S* rRNA, Hs99999901_s1. Three replicates were run for each sample in a 96-well format plate. Gene expression assays consisted of a FAM™ dye-labelled TaqMan® MGB probe and two PCR primers. The thermal cycling conditions consisted of an initial denaturation step at 95˚C for 10 min and a 50-cycle countdown at 95˚C for 15 s and 60˚C for 1 min. Each sample was normalised on the basis of its *18S* rRNA content. Relative mRNA expression levels were calculated using the comparative threshold cycle (CT) method (57).

PI3K assays. Recombinant human PI3KC2β was expressed as a glutathione S-transferase (GST)-fusion protein in SF9 insect cells and purified as described previously (23). Recombinant class I

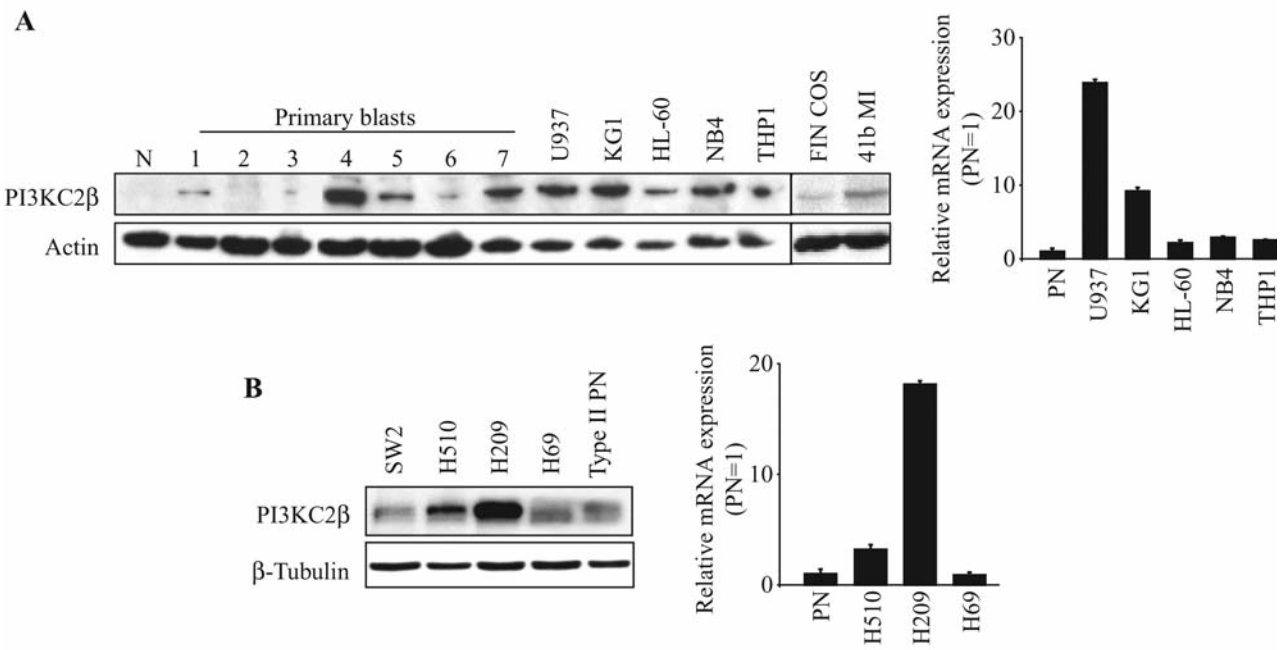

Figure 1. *Phosphoinositide 3-kinase C2β (PI3KC2β) expression in acute myeloid leukemia (AML) and small cell lung cancer (SCLC) cells. Western blot analysis of PI3KC2β expression in A: normal bone marrow cells (N), primary blasts (1-7), AML cell lines and immortalized B cells (FIN COS, 41b MI); and B: type II pneumocytes (PN) and SCLC cell lines (left panel). Relative mRNA expression levels of PIK3C2B in human AML cell lines (A) and SCLC cell lines (B) (right panel). Nontransformed type II human pneumocytes were used as a control.*

PI3K isoforms were expressed and purified likewise. PI3K activity of the different PI3K isoforms was assayed essentially as described (46, 48).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis. Cellular lysates were prepared as previously described (58) separated by SDS-PAGE, transferred to a hydrophobic polyvinylidene difluoride (PVDF) membrane (Hybond-P; Amersham Biosciences, Amersham, UK), and immunoblotted with different antibodies (PI3KC2β, Caspase-3, PARP, activated AKT/PKB (Ser473), activated JNK (Thr183/Tyr185), activated S6 protein (Ser235/236), β-actin, β-tubulin) according to the manufacturer's protocol. Chemiluminescence was used for visualization using the enhanced chemiluminescence (ECL) western blotting detection reagents (Amersham Biosciences, Amersham, UK) according to the manufacturer's protocol.

Transient expression in AML cells. AML cells were transfected with small-interfering RNA (siRNA) targeting PI3KC2β using the Amaxa Nucleofector system (Amaxa Biosystems, Gaithersburg, MD, USA) according to the manufacturer's protocol. Cell Line Nucleofector Kit V was used and program V-001 applied (51). After 48 h cells were lysed in cell lysis buffer in order to visualize protein expression by SDS-PAGE and western blotting (51). In addition, cells were analysed for cell proliferation and apoptosis by MTS assay and caspase-3 measurement 72 h after transfection.

Wound-healing assay. EpH4 murine epithelial cells stably transfected with the Ha-*Ras* oncogene and induced by transforming growth factor-β1 (TGF-β1) to undergo epitheliomesenchymal transition (EMT) *in vivo* to establish FibRas cells have been described elsewhere (53) and were obtained from Dr. E. Reichmann (University Children's Hospital Zurich, Switzerland). For wound-healing assays, cells were plated in 12-well culture plates in complete medium and grown to confluency. A wound was created by scraping cells with a 200 μl tip (59). The migration rate was monitored for eight hours by phase contrast microscopy (Leica DM IRBE Inverse, widefield) in the presence or absence of inhibitors (59).

Results

Expression of PI3KC2β in tumour samples and cell lines. Previous reports had documented increased expression of PI3KC2β in leukemia, glioblastoma and lung cancer cell lines and tumours (29,60,61). Therefore, the expression of PI3KC2β was investigated in a panel of tumours and cell lines from AML, neuroendocrine tumours (SCLC and NB) and brain tumours (MB and GBM). In AML, PI3KC2β was highly expressed in a subset of AML blasts and cell lines (Figure 1A, left panel). Interestingly, PI3KC2β expression was much lower in non-leukemic bone marrow cells and immortalised B-cells (Figure 1A) indicating that AML blasts and cell lines overexpress the enzyme. We also reanalysed cDNA microarray data from a previously published study in AML (62). PI3KC2β mRNA expression was found to be higher in certain groups of AML, depending on molecular and cytogenetic abnormalities. AML categories displaying

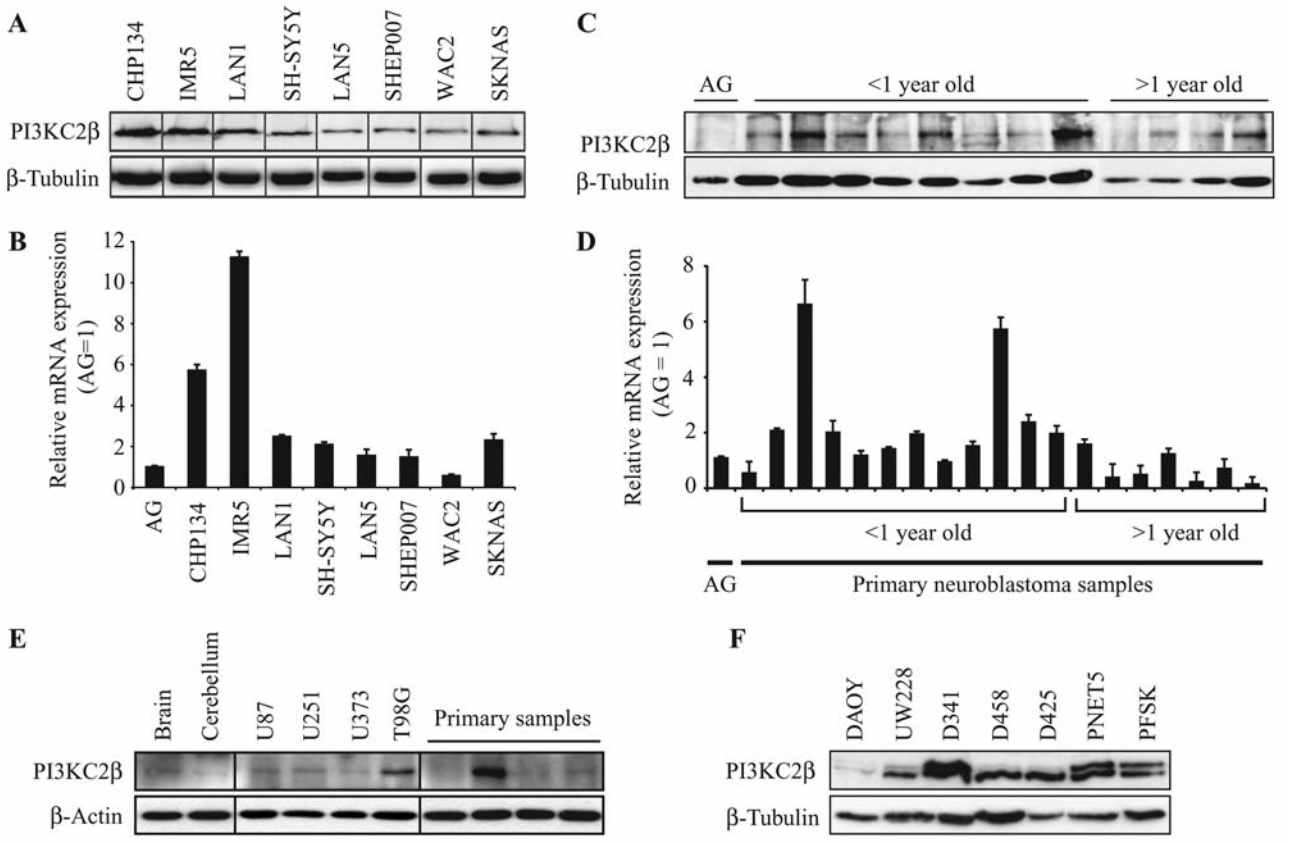

Figure 2. *Phosphoinositide 3-kinase C2β (PI3KC2β) expression in tumours of the central nervous system. In neuroblastoma cell lines (A and B) and primary tumour samples (C and D), PI3KC2β expression was analysed both by western blot analysis (A and C) as well as quantitative reverse transcription-polymerase chain reaction (RT-PCR) (B and D). PI3KC2β protein expression in glioblastoma cell lines and ex vivo cultures (E) and medulloblastoma cell lines (F) by western blot analysis. For neuroblastoma samples, human adrenal tissue (AG) was used as a control. For glioblastoma and medulloblastoma samples, normal human cerebellum served as a control.*

increased expression included FLT3-ITD and EVI1 (levels of expression: 77.37 and 99.21), as compared to FLT3-TKD (level of expression: 41.51). The levels of expression of the PI3KC2β mRNA in every sample were determined by the mean of the hybridization intensities of the *PIK3C2B* probe sets from the patients analysed by cDNA microarray. Increased expression of PI3KC2β was also found in AML with the cytogenetic abnormalities -7 (level of expression: 99.72), when compared to +8, 11q13, t(8;21), idt(16), or NN (levels of expression: 46.86, 39.35, 54.40, 19.80, 60.03). In contrast, there were no significant differences in expression of PI3KC2β mRNA between AML French-American-British (FAB) classes.

Protein expression analysis of SCLC cell lines revealed elevated *PI3KC2β* expression in 2/4 cases when compared to a normal type II pneumocyte cell line (PN) (Figure 1B, left panel). TaqMan analysis of PIK3C2B expression confirmed that mRNA levels are predictive of protein expression (Figure 1B, right panel).

In NB cell lines, western blot analysis revealed broad expression of PI3KC2β (Figure 2A). Upon TaqMan analysis *PIK3C2B* was found to be overexpressed at the mRNA level when compared to normal human adrenal gland (5/8 samples with >2-fold expression) (Figure 2B). In primary tumours from children under the age of one year, PI3KC2β exhibited increased expression (Figure 2C). This distribution was mirrored in data obtained from TaqMan analysis, where increased mRNA expression was found in the same patient subgroup (3/19 samples with >2-fold expression) (Figure 2D).

In GBM cell lines and *ex vivo* cultures PI3KC2β was found to be overexpressed in a subset of samples when compared to normal human brain or cerebellum (Figure 2E).

In MB cell lines, heterogeneous PI3KC2β expression was observed (Figure 2F). Microarray analysis of a panel of primary MB samples also showed that PI3KC2β was overexpressed in 16/60 samples compared to normal human cerebellum (Figure 3). However, in more than half of the MB

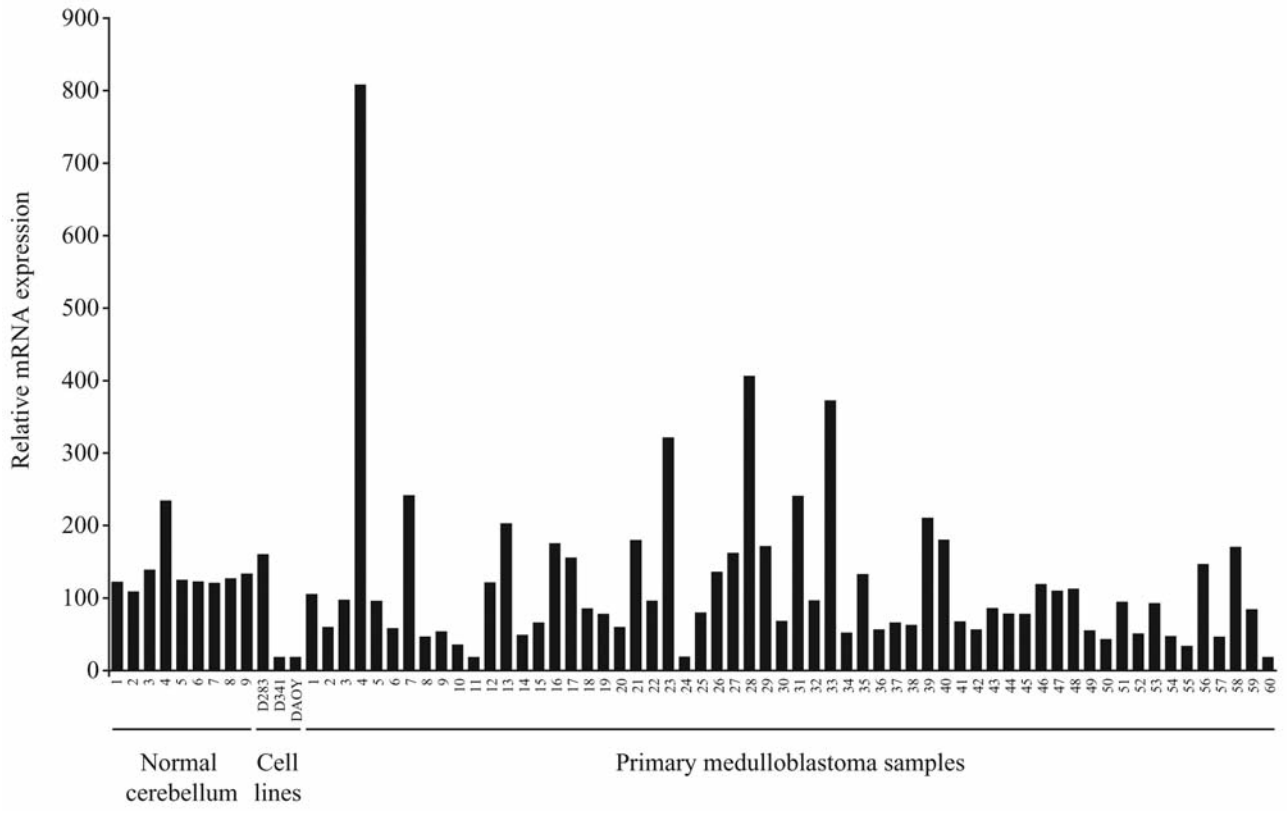

Figure 3. *DNA Microarray analysis of phosphoinositide 3-kinase C2β (PI3KC2β) expression in a panel of primary medulloblastoma samples and cell lines. Gene expression profiles were obtained with the Affymetrix HG-U133 Plus 2.0 array.*

samples PI3KC2β expression was reduced compared to normal human cerebellum (Figure 3).

Together, these data revealed subgroups of tumours and cell lines displaying PI3KC2β overexpression, in which the class II PI3K may play a role in regulating proliferation, survival or migration.

Inhibition of cell proliferation by pharmacological PI3KC2β inhibitors and by siRNA targeting PI3KC2β. To gain insight into the contribution of the class II PI3K isoform PI3KC2β in cell proliferation, two different isoform-specific pharmacological inhibitors (PI701 and PI702) were used. The specificity of these inhibitors was verified by *in vitro* PI3K assays using purified recombinant preparation of various PI3K isoforms. The half maximal inhibitory concentration (IC_{50}) values for specific inhibition of the enzymatic activity of PI3KC2β were 528 nM for PI701 and 632 nM for PI702 while values above 10 μM were observed for the other PI3K isoforms (Table II). Both inhibitors also failed to inhibit the activity of mammalian target of rapamycin (mTOR) and DNA-activated protein kinase (DNA-PK), with IC_{50} values above 100 μM (data not shown). A screen against a panel of 72 protein kinases *in vitro* confirmed that the two compounds are selective for PI3KC2β. Indeed, they only showed some inhibitory activity, at a concentration of 10 μM *in vitro*, against anaplastic lymphoma kinase (ALK), v-raf-1 murine leukemia viral oncogene homolog 1 (c-Raf), p38-regulated/activated kinase (PRAK) and tyrosine protein kinase receptor B (TRKB)* (*PI702 only) (data not shown).

The antiproliferative activity of both inhibitors was investigated in a panel of 28 cell lines and primary cultures from AML, GBM, MB, NB and SCLC. As controls, non-leukemic bone marrow cells (N), two immortalised B cells lines (FIN COS, 41b MI) and an immortalised Type II pneumocyte cell line (PN) were included. Both inhibitors of PI3KC2β inhibited cell proliferation in a dose-dependent manner with IC_{50} values below 10 μM (PI701: 16/30 cases; PI702: 9/25 cases) (Figure 4 and Table I). The lowest IC_{50} values were observed in SCLC cell lines, AML blasts and D341 MB cells (Table I). In contrast, the IC_{50} values observed in control cells (FIN COS, 41b MI, N, PN) were above 10 μM. In several cases, cell lines with high PI3KC2β expression were more sensitive to PI701 and PI702, as was the case for AML (U937 and NB4), MB (D341), GBM (T98G) and SCLC (H-209 and H-510) (Figures 1 and 2, Table II).

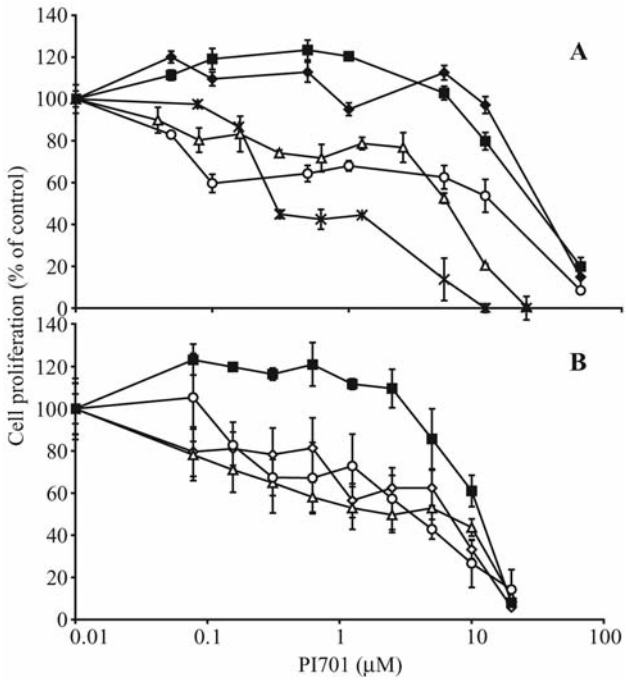

Figure 4. *Titration curve of PI3KC2β-specific inhibitor PI701 in acute myeloid leukemia (AML) and small cell lung cancer (SCLC). A: AML cell lines (U937, triangles; HL-60, circles) and patient blasts (FAB M1, asterisks), and B: SCLC cell lines (H209, triangles; H510, circles) were treated with increasing concentration of the inhibitor for 72 hours. For (A) AML cell lines and blast cells, immortalised B-cells (diamonds and squares) served as a control. For (B) SCLC, non-transformed type II pneumocytes (squares) were used as a control.*

Table II. *Half maximal inhibitory concentration (IC_{50}) values (μM) against isolated enzymes.*

Compound	PI3KC2β	p110α	p110β	p110γ	p110δ
PI701	0.528	>10	>10	>10	>10
PI702	0.632	>10	>10	>10	>10

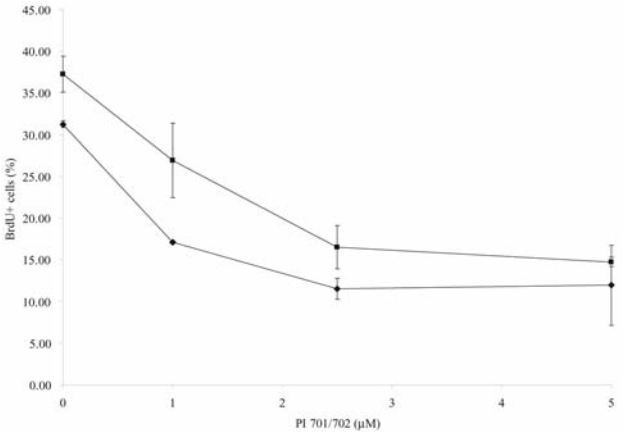

Figure 5. *Phosphoinositide 3-kinase C2β (PI3KC2β)-specific inhibitors impair cell proliferation in acute myeloid leukemia (AML) cells. Titration curves of PI701 and PI702 in AML. NB4 cells were treated with increasing concentrations of the inhibitors (PI701, diamonds; PI702, squares) for 24 hours and BrdU incorporation was measured.*

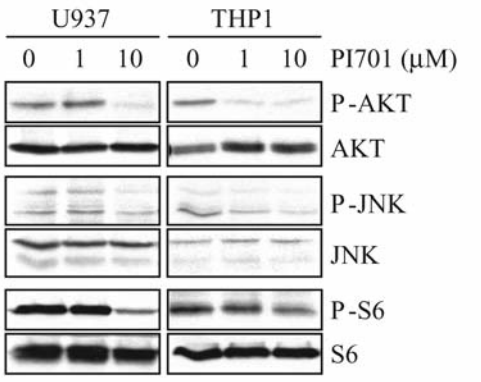

Figure 6. *Effect of PI3KC2β-specific inhibitor PI701 on the phosphorylation of downstream signalling molecules. Acute myeloid leukemia (AML) cell lines (U937 and THP1) were treated with increasing concentrations of PI701 overnight. Pathway activation was visualised by monitoring the phosphorylation status of v-akt murine thymoma viral oncogene homolog (P-AKT^{Ser473}), c-Jun NH_2-terminal kinase (P-JNK$^{Thr183/Tyr185}$) and S6 protein (P-S6 protein$^{Ser235/236}$).*

To confirm that the effects of the inhibitors in the MTS assay were indeed due to reduced cell proliferation, BrdU incorporation assays were performed in AML cell lines. These experiments revealed that PI701 and PI702 indeed reduce BrdU incorporation (Figure 5).

Investigation of the effect of PI701 on activation of downstream signalling mediators in AML cell lines showed a dose-dependent decrease in the phosphorylation of key signalling molecules (Figure 6). These include AKT, JNK and ribosomal S6 protein.

Together, these data show that inhibition of PI3KC2β with selective pharmacological inhibitors impairs proliferation in a subset of human cancer cells, with IC_{50} values compatible with inhibition of the enzyme. This is in line with the finding that the phosphorylation of key signalling molecules is decreased upon treatment with PI701 in AML (Figure 6).

To validate the results obtained with pharmacological inhibitors, siRNA targeting PI3KC2β was tested in an AML cell line. The specific down-regulation of PI3KC2β expression by siRNA was verified by western blot analysis

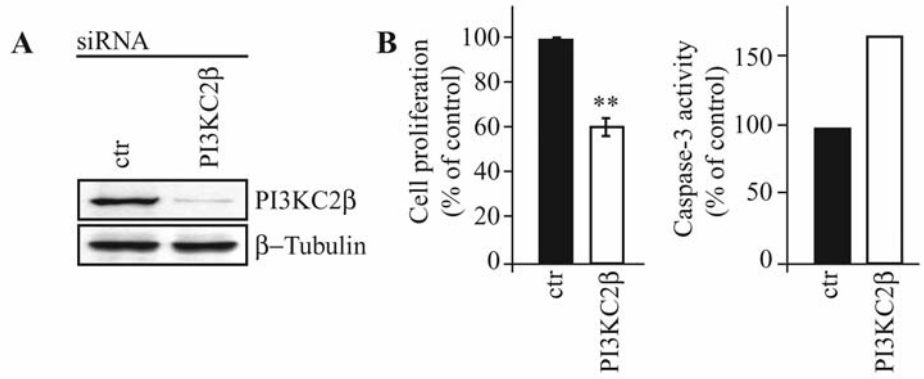

Figure 7. *Inhibition of acute myeloid leukemia (AML) cell proliferation by small-interfering RNA (siRNA) targeting Phosphoinositide 3-kinase C2β (PI3KC2β). A: U937 cells transfected with control (ctr) siRNA or siRNA targeting PI3KC2β were analyzed by western blotting for protein expression. B: Cell proliferation of U937 cells transfected with siRNA targeting PI3KC2β was analysed by MTS assay (left panel) and caspase-3 activity was assessed in parallel. **Significantly different at p<0.01 by analysis of variance test.*

(Figure 7A). Reduced expression of PI3KC2β in U937 cells resulted in a 40% reduction of cell proliferation and was accompanied by increased caspase-3 activity (Figure 7B). The effects of the PI3KC2β siRNA on cell proliferation were less marked in THP1 cells, which displayed lower expression of the enzyme (data not shown).

Targeting PI3KC2β enhances the sensitivity of AML and GBM cells to chemotherapy. We next investigated whether inhibition of PI3KC2β by pharmacological inhibitors could modulate the sensitivity of AML and GBM cells to chemotherapeutic agents. In AML cell lines, combination treatment with PI701 led to markedly increased sensitivity to etoposide (Figure 8A). This effect was also observed in GBM cell lines and *ex vivo* cultures where PI701 combined with doxorubicin had a stronger effect on cell viability than did the chemotherapeutic agent alone (Figure 8B and 8C). Moreover, the combination treatment enhanced the induction of apoptosis as assessed by increased caspase-3 activation and PARP cleavage (Figure 8D and 8E).

In summary, these results highlight the importance of PI3KC2β in regulating the viability and chemoresistance of different types of tumour cells.

PI3KC2β contributes to cell migration in transformed epithelial cells. Previous reports have described a role for PI3KC2β in the migration of cancer cells (28, 31). To investigate the specific contribution of PI3KC2β in the migration of tumourigenic *vs.* non-tumourigenic cells, murine epithelial cells sequentially transformed by oncogenic H-Ras and TGF-β1 were used (53). To analyse the contribution of PI3KC2β to the migratory capacity of cancer cells, murine epithelial cells were treated with PI701. Western blot analysis revealed increased PI3KC2β expression in the FibRas cells, which have previously been shown to display enhanced migration and invasion, as compared to Eph4 and EpRas cells (53) (Figure 9A). Treatment with PI701 impaired migration of FibRas cells but had no significant effect on the migration of Eph4 and EpRas cells (Figure 9B). At the concentrations used, PI701 did not inhibit cell proliferation significantly (Figure 9C). The finding that pharmacological inhibition of PI3KC2β impaired the migratory capacity of highly invasive cells emphasises the importance of PI3KC2β in regulating cancer cell migration.

Discussion

To date, the class IA isoform p110α is the only validated target of the PI3K family in the context of human cancer (6, 39, 40, 63, 64). Other class I_A isoforms have been associated with a role in various malignancies, including p110β in prostate

→

Figure 8. *PI3KC2β-specific inhibitor PI701 sensitizes acute myeloid leukemia (AML) and glioblastoma cells to chemotherapeutical agents. A: Titration curve of U937 (left panel) and THP1 (right panel) cells incubated with increasing concentrations of etoposide in the absence (black circles) or presence (white circles) of PI701 (1 μM). B: Titration curve of T98G (left panel) and U251 (right panel) cells incubated with increasing concentrations of doxorubicin in the absence (black circles) or presence (white circles) of PI701 (1 μM). C: Titration curve of two glioblastoma ex vivo cultures incubated with increasing concentrations of doxorubicin in the absence (black circles) or presence (white circles) of PI701 (1 μM). A representative experiment (out of three) performed with eight repetitions is shown for A-C. *p<0.05 and **p<0.01, significantly different by analysis of variance test. D: T98G cells were treated with PI701 or doxorubicin alone, and in combination, and caspase-3 cleavage was evaluated by means of a western blot. E: U937 cells were treated with increasing concentration of etoposide either alone or in combination with PI701 and poly(ADP-ribose) polymerase (PARP) cleavage was visualised by means of a western blot. The band corresponding to cleaved PARP (85 kDa) is shown.*

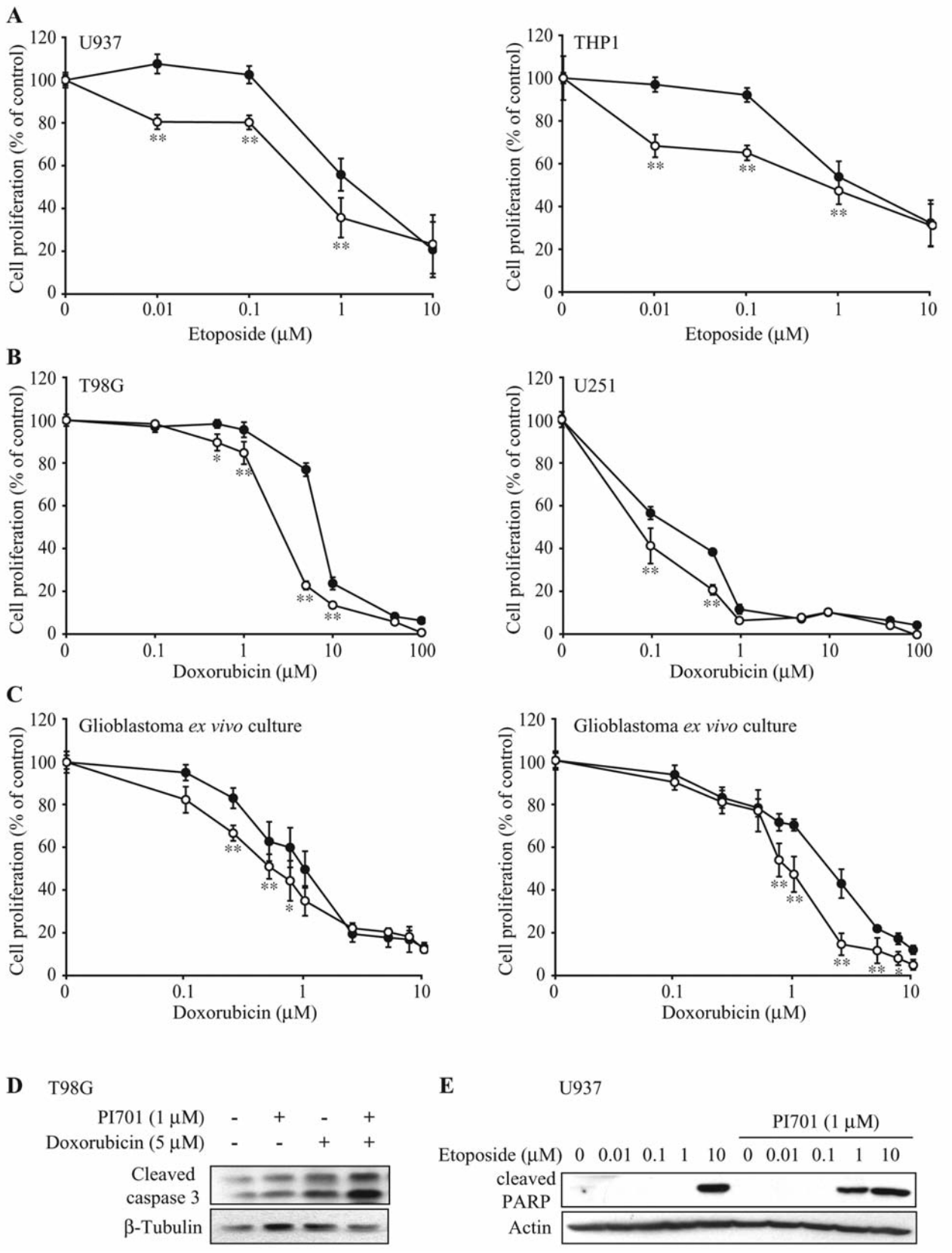
A
U937
THP1
Cell proliferation (% of control)
Etoposide (μM)
B
T98G
U251
Doxorubicin (μM)
C
Glioblastoma ex vivo culture
D
T98G
PI701 (1 μM)
Doxorubicin (5 μM)
Cleaved caspase 3
β-Tubulin
E
U937
PI701 (1 μM)
Etoposide (μM) 0 0.01 0.1 1 10 0 0.01 0.1 1 10
cleaved PARP
Actin

Figure 8

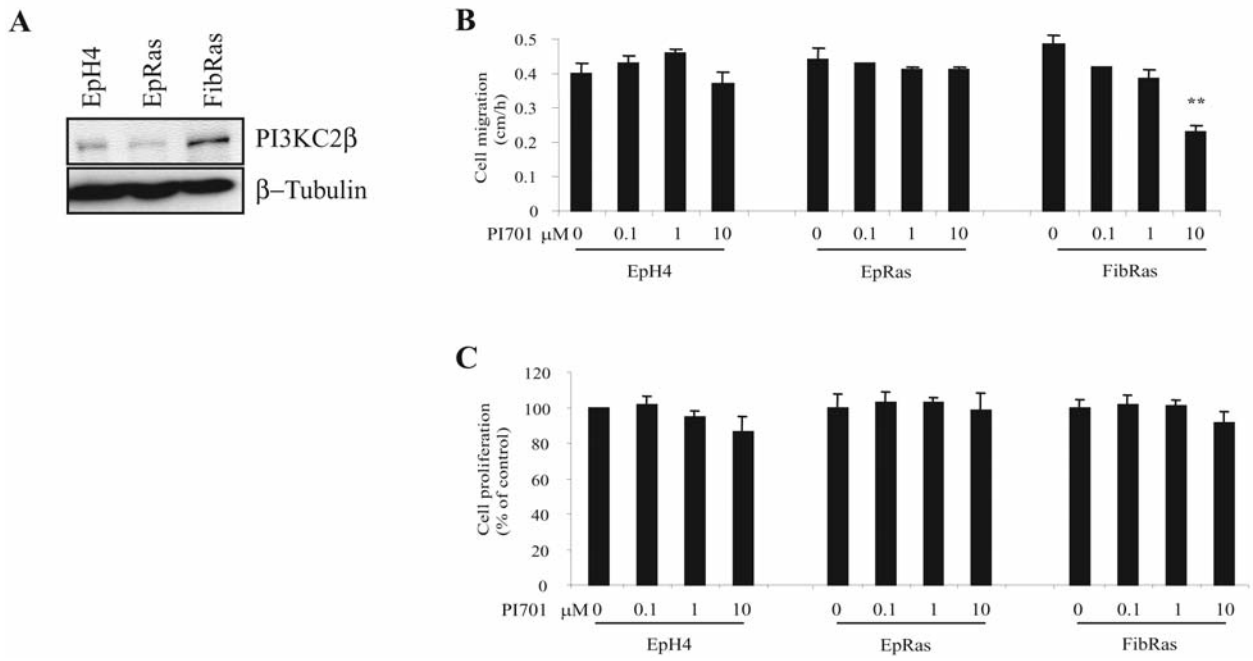

Figure 9. *Role of phosphoinositide 3-kinase C2β (PI3KC2β) expression in mammary epithelial cell lines. A: Western blot analysis of PI3KC2β expression in EpH4, EpRas and FibRas cells. B: Effect of PI3KC2β-specific inhibitor PI701 on migration of mammary epithelial cell lines. Cells were treated with increasing concentrations of PI701 and the distance migrated was measured after 8 hours. C: Cell proliferation was assessed in parallel. **Significantly different at p<0.01 by analysis of variance test.*

cancer (65) and p110δ in leukemia and lymphoma (66-69). Concerning the class II PI3Ks, PI3KC2α has been shown to play a role in survival of HeLa cells (70) and increased expression of PI3KC2β was reported in a subset of tumours and cell lines from AML, GBM and SCLC (29, 60, 61). Furthermore, PI3KC2β has been shown to play a role in the migration of A-431, HeLa and ovarian cancer cells (28, 31), and to contribute to cancer cell growth and AKT activation (29, 33-35). An analysis of single-nucleotide polymorphisms (SNP) in PI3K genes also revealed an association between *PIK3C2B* and prostate cancer risk (71). In the present study, we have extended these studies on PI3KC2β in AML, brain tumours and neuroendocrine tumours.

Here we report that PI3KC2β is overexpressed in a variety of human cancer cell lines and primary cultures compared to control tissue. The growing interest in small molecule inhibitors has led to the development of a plethora of pharmacological PI3K inhibitors whose activity in human cancer remains to be validated. In the present study, the effect of two specific inhibitors of PI3KC2β (PI701 and PI702) on cancer cell responses was investigated. Upon treatment with either compound, a dose-dependent inhibition of cell proliferation was observed in different human tumour cells, while non-tumourigenic cells, such as immortalised B-cells and type II pneumocytes, remained largely unaffected. Moreover, a correlation between protein expression and sensitivity to the inhibitors was observed in a number of cell lines. In AML, siRNA against PI3KC2β also led to reduced proliferation accompanied by an increase in apoptosis. The finding that the activation status of important signalling molecules, including AKT, JNK and S6K, was reduced upon treatment with PI701, is in line with the inhibitory effect of this compound on cell proliferation.

Interestingly, co-treatment of cells with PI701 led to an increased sensitivity to chemotherapeutic agents, such as etoposide and doxorubicin. This supports previous reports showing that the inhibition of important survival pathways, including PI3K/AKT, can enhance the response to cytotoxic reagents (33, 49, 50). In the present study, we have demonstrated for the first time that PI3KC2β inhibitors can sensitize human cancer cells to chemotherapeutic agents such as etoposide and doxorubicin.

Besides its role in regulating cell proliferation, in invasive murine breast cancer cell lines, PI3KC2β was also found to be involved in cell migration. Enhanced expression of PI3KC2β was detected in the most motile cells (FibRas) as compared to non-invasive cells (EpH4) (59). Treatment of these cells with increasing doses of PI701 strongly reduced the migratory capacity in wound-healing assays. This is an interesting observation considering that malignant cells often acquire the ability to migrate, leading to the propagation of metastases throughout the body.

In summary, the present study illustrates that PI3KC2β plays a crucial role in regulating various cellular responses in a broad spectrum of human cancer cell types. Since the pharmacological inhibitor PI701 not only reduced basal cell proliferation, but also had an effect on chemosensitivity and cellular migration, PI3KC2β could prove to be an attractive target for cancer treatment in the future.

Acknowledgements

This work was supported by grants from the Swiss National Science Foundation (Grants 3100A0-105321 and 31003A-120294), Oncosuisse (OCS-01501-02-2004), the Forschungskredit der Universität Zürich, the Stiftung zur Krebsbekämpfung and the Krebsliga Zürich to AA. We thank Dr. E. Reichmann for providing EpH4, EpRas and FibRas cells.

References

1 Katso R, Okkenhaug K, Ahmadi K, White S, Timms J and Waterfield MD: Cellular function of phosphoinositide 3-kinases: implications for development, homeostasis, and cancer. Annu Rev Cell Dev Biol *17*: 615-675, 2001.

2 Vanhaesebroeck B, Leevers SJ, Ahmadi K, Timms J, Katso R, Driscoll PC, Woscholski R, Parker PJ and Waterfield MD: Synthesis and function of 3-phosphorylated inositol lipids. Annu Rev Biochem *70*: 535-602, 2001.

3 Vanhaesebroeck B, Stephens L and Hawkins P: PI3K signalling: the path to discovery and understanding. Nat Rev Mol Cell Biol *13*: 195-203, 2012.

4 Vanhaesebroeck B, Leevers SJ, Panayotou G and Waterfield MD: Phosphoinositide 3-kinases: a conserved family of signal transducers. Trends Biochem Sci *22*: 267-272, 1997.

5 Vanhaesebroeck B, Ali K, Bilancio A, Geering B and Foukas LC: Signalling by PI3K isoforms: insights from gene-targeted mice. Trends Biochem Sci *30*: 194-204, 2005.

6 Vanhaesebroeck B, Guillermet-Guibert J, Graupera M and Bilanges B: The emerging mechanisms of isoform-specific PI3K signalling. Nat Rev Mol Cell Biol *11*: 329-341, 2010.

7 Blajecka K, Borgstrom A and Arcaro A: Phosphatidylinositol 3-kinase isoforms as novel drug targets. Curr Drug Targets *12*: 1056-1081, 2011.

8 Hiles ID, Otsu M, Volinia S, Fry MJ, Gout I, Dhand R, Panayotou G, Ruiz-Larrea F, Thompson A, Totty NF *et al*: Phosphatidylinositol 3-kinase: structure and expression of the 110 kDa catalytic subunit. Cell *70*: 419-429, 1992.

9 Hu P, Mondino A, Skolnik EY and Schlessinger J: Cloning of a novel, ubiquitously expressed human phosphatidylinositol 3-kinase and identification of its binding site on p85. Mol Cell Biol *13*: 7677-7688, 1993.

10 Vanhaesebroeck B, Welham MJ, Kotani K, Stein R, Warne PH, Zvelebil MJ, Higashi K, Volinia S, Downward J and Waterfield MD: P110delta, a novel phosphoinositide 3-kinase in leukocytes. Proc Natl Acad Sci USA *94*: 4330-4335, 1997.

11 Chantry D, Vojtek A, Kashishian A, Holtzman DA, Wood C, Gray PW, Cooper JA and Hoekstra MF: p110delta, a novel phosphatidylinositol 3-kinase catalytic subunit that associates with p85 and is expressed predominantly in leukocytes. J Biol Chem *272*: 19236-19241, 1997.

12 Otsu M, Hiles I, Gout I, Fry MJ, Ruiz-Larrea F, Panayotou G, Thompson A, Dhand R, Hsuan J, Totty N *et al*: Characterization of two 85 kDa proteins that associate with receptor tyrosine kinases, middle-T/pp60c-SRC complexes, and PI3-kinase. Cell *65*: 91-104, 1991.

13 Inukai K, Anai M, Van Breda E, Hosaka T, Katagiri H, Funaki M, Fukushima Y, Ogihara T, Yazaki Y, Kikuchi, Oka Y and Asano T: A novel 55-kDa regulatory subunit for phosphatidylinositol 3-kinase structurally similar to p55PIK is generated by alternative splicing of the p85alpha gene. J Biol Chem *271*: 5317-5320, 1996.

14 Pons S, Asano T, Glasheen E, Miralpeix M, Zhang Y, Fisher TL, Myers MG Jr., Sun XJ and White MF: The structure and function of p55PIK reveal a new regulatory subunit for phosphatidylinositol 3-kinase. Mol Cell Biol *15*: 4453-4465, 1995.

15 Hawkins PT, Jackson TR and Stephens LR: Platelet-derived growth factor stimulates synthesis of PtdIns(3,4,5)P_3 by activating a PtdIns(4,5)P_2 3-OH kinase. Nature *358*: 157-159, 1992.

16 Stephens LR, Hughes KT and Irvine RF: Pathway of phosphatidylinositol(3,4,5)-trisphosphate synthesis in activated neutrophils. Nature *351*: 33-39, 1991.

17 Alessi DR, Deak M, Casamayor A, Caudwell FB, Morrice N, Norman DG, Gaffney P, Reese CB, MacDougall CN, Harbison D, Ashworth A and Bownes M: 3-Phosphoinositide-dependent protein kinase-1 (PDK1): structural and functional homology with the Drosophila DSTPK61 kinase. Curr Biol *7*: 776-789, 1997.

18 Le Good JA, Ziegler WH, Parekh DB, Alessi DR, Cohen P and Parker PJ: Protein kinase C isotypes controlled by phosphoinositide 3-kinase through the protein kinase PDK1. Science *281*: 2042-2045, 1998.

19 Vanhaesebroeck B and Alessi DR: The PI3K-PDK1 connection: more than just a road to PKB. Biochem J 346 Pt *3*: 561-576, 2000.

20 MacDougall LK, Domin J and Waterfield MD: A family of phosphoinositide 3-kinases in Drosophila identifies a new mediator of signal transduction. Curr Biol *5*: 1404-1415, 1995.

21 Molz L, Chen YW, Hirano M and Williams LT: Cpk is a novel class of Drosophila PtdIns 3-kinase containing a C2 domain. J Biol Chem *271*: 13892-13899, 1996.

22 Domin J, Pages F, Volinia S, Rittenhouse SE, Zvelebil MJ, Stein RC and Waterfield MD: Cloning of a human phosphoinositide 3-kinase with a C2 domain that displays reduced sensitivity to the inhibitor wortmannin. Biochem J *326(Pt 1)*: 139-147, 1997.

23 Arcaro A, Volinia S, Zvelebil MJ, Stein R, Watton SJ, Layton MJ, Gout I, Ahmadi K, Downward J and Waterfield MD: Human phosphoinositide 3-kinase C2beta, the role of calcium and the C2 domain in enzyme activity. J Biol Chem *273*: 33082-33090, 1998.

24 Misawa H, Ohtsubo M, Copeland NG, Gilbert DJ, Jenkins NA and Yoshimura A: Cloning and characterization of a novel class II phosphoinositide 3-kinase containing C2 domain. Biochem Biophys Res Commun *244*: 531-539, 1998.

25 Ono F, Nakagawa T, Saito S, Owada Y, Sakagami H, Goto K, Suzuki M, Matsuno S and Kondo H: A novel class II phosphoinositide 3-kinase predominantly expressed in the liver and its enhanced expression during liver regeneration. J Biol Chem *273*: 7731-7736, 1998.

26 Brown RA, Domin J, Arcaro A, Waterfield MD and Shepherd PR: Insulin activates the alpha isoform of class II phosphoinositide 3-kinase. J Biol Chem *274*: 14529-14532, 1999.

27 Arcaro A, Zvelebil MJ, Wallasch C, Ullrich A, Waterfield MD and Domin J: Class II phosphoinositide 3-kinases are downstream targets of activated polypeptide growth factor receptors. Mol Cell Biol *20*: 3817-3830, 2000.

28 Katso RM, Pardo OE, Palamidessi A, Franz CM, Marinov M, De Laurentiis A, Downward J, Scita G, Ridley AJ, Waterfield MD and Arcaro A: Phosphoinositide 3-kinase C2beta regulates cytoskeletal organization and cell migration *via* Rac-dependent mechanisms. Mol Biol Cell *17*: 3729-3744, 2006.

29 Arcaro A, Khanzada UK, Vanhaesebroeck B, Tetley TD, Waterfield MD and Seckl MJ: Two distinct phosphoinositide 3-kinases mediate polypeptide growth factor-stimulated PKB activation. Embo J *21*: 5097-5108, 2002.

30 MacDougall LK, Gagou ME, Leevers SJ, Hafen E and Waterfield MD: Targeted expression of the class II phosphoinositide 3-kinase in *Drosophila melanogaster* reveals lipid kinase-dependent effects on patterning and interactions with receptor signaling pathways. Mol Cell Biol *24*: 796-808, 2004.

31 Maffucci T, Cooke FT, Foster FM, Traer CJ, Fry MJ and Falasca M: Class II phosphoinositide 3-kinase defines a novel signaling pathway in cell migration. J Cell Biol *169*: 789-799, 2005.

32 Domin J, Harper L, Aubyn D, Wheeler M, Florey O, Haskard D, Yuan M and Zicha D: The class II phosphoinositide 3-kinase PI3K-C2beta regulates cell migration by a PtdIns3P-dependent mechanism. J Cell Physiol *205*: 452-462, 2005.

33 Liu Z, Sun C, Zhang Y, Ji Z and Yang G: Phosphatidylinositol 3-kinase-C2beta inhibits cisplatin-mediated apoptosis *via* the AKT pathway in oesophageal squamous cell carcinoma. J Int Med Res *39*: 1319-1332, 2011.

34 Russo A and O'Bryan JP: Intersectin 1 is required for neuroblastoma tumorigenesis. Oncogene, 2012.

35 Diouf B, Cheng Q, Krynetskaia NF, Yang W, Cheok M, Pei D, Fan Y, Cheng C, Krynetskiy EY, Geng H, Chen S, Thierfelder WE, Mullighan CG, Downing JR, Hsieh P, Pui CH, Relling MV and Evans WE: Somatic deletions of genes regulating MSH2 protein stability cause DNA mismatch repair deficiency and drug resistance in human leukemia cells. Nat Med *17*: 1298-1303, 2011.

36 Harada K, Truong AB, Cai T and Khavari PA: The class II phosphoinositide 3-kinase C2beta is not essential for epidermal differentiation. Mol Cell Biol *25*: 11122-11130, 2005.

37 Yuan TL and Cantley LC: PI3K pathway alterations in cancer: variations on a theme. Oncogene 27: 5497-5510, 2008.

38 Sansal I and Sellers WR: The biology and clinical relevance of the PTEN tumor suppressor pathway. J Clin Oncol *22*: 2954-2963, 2004.

39 Courtney KD, Corcoran RB and Engelman JA: The PI3K pathway as drug target in human cancer. J Clin Oncol *28*: 1075-1083, 2010.

40 Wong KK, Engelman JA and Cantley LC: Targeting the PI3K signaling pathway in cancer. Curr Opin Genet Dev *20*: 87-90, 2010.

41 Samuels Y, Wang Z, Bardelli A, Silliman N, Ptak J, Szabo S, Yan H, Gazdar A, Powell SM, Riggins GJ, Willson JK, Markowitz S, Kinzler KW, Vogelstein B and Velculescu VE: High frequency of mutations of the PIK3CA gene in human cancers. Science *304*: 554, 2004.

42 Weir B, Zhao X and Meyerson M: Somatic alterations in the human cancer genome. Cancer Cell *6*: 433-438, 2004.

43 Broderick DK, Di C, Parrett TJ, Samuels YR, Cummins JM, McLendon RE, Fults DW, Velculescu VE, Bigner DD and Yan H: Mutations of *PIK3CA* in anaplastic oligodendrogliomas, high-grade astrocytomas, and medulloblastomas. Cancer Res *64*: 5048-5050, 2004.

44 Arcaro A and Guerreiro AS: The phosphoinositide 3-kinase pathway in human cancer: genetic alterations and therapeutic implications. Curr Genomics *8*: 271-306, 2007.

45 Hayakawa M, Kaizawa H, Kawaguchi K, Ishikawa N, Koizumi T, Ohishi T, Yamano M, Okada M, Ohta M, Tsukamoto S, Raynaud FI, Waterfield MD, Parker P and Workman P: Synthesis and biological evaluation of imidazo[1,2-a]pyridine derivatives as novel PI3 kinase p110alpha inhibitors. Bioorg Med Chem *15*: 403-412, 2007.

46 Hayakawa M, Kaizawa H, Moritomo H, Koizumi T, Ohishi T, Yamano M, Okada M, Ohta M, Tsukamoto S, Raynaud FI, Workman P, Waterfield MD and Parker P: Synthesis and biological evaluation of pyrido[3',2':4,5]furo[3,2-d]pyrimidine derivatives as novel PI3 kinase p110alpha inhibitors. Bioorg Med Chem Lett *17*: 2438-2442, 2007.

47 Hayakawa M, Kaizawa H, Moritomo H, Koizumi T, Ohishi T, Okada M, Ohta M, Tsukamoto S, Parker P, Workman P and Waterfield M: Synthesis and biological evaluation of 4-morpholino-2-phenylquinazolines and related derivatives as novel PI3 kinase p110alpha inhibitors. Bioorg Med Chem *14*: 6847-6858, 2006.

48 Hayakawa M, Kawaguchi K, Kaizawa H, Koizumi T, Ohishi T, Yamano M, Okada M, Ohta M, Tsukamoto S, Raynaud FI, Parker P, Workman P and Waterfield MD: Synthesis and biological evaluation of sulfonylhydrazone-substituted imidazo[1,2-a]pyridines as novel PI3 kinase p110alpha inhibitors. Bioorg Med Chem *15*: 5837-5844, 2007.

49 Guerreiro AS, Fattet S, Fischer B, Shalaby T, Jackson SP, Schoenwaelder SM, Grotzer MA, Delattre O and Arcaro A: Targeting the PI3K p110alpha isoform inhibits medulloblastoma proliferation, chemoresistance, and migration. Clin Cancer Res *14*: 6761-6769, 2008.

50 Guerreiro AS, Fattet S, Kulesza DW, Atamer A, Elsing AN, Shalaby T, Jackson SP, Schoenwaelder SM, Grotzer MA, Delattre O and Arcaro A: A sensitized RNA interference screen identifies a novel role for the PI3K p110gamma isoform in medulloblastoma cell proliferation and chemoresistance. Mol Cancer Res *9*: 925-935, 2011.

51 Doepfner KT, Spertini O and Arcaro A: Autocrine insulin-like growth factor-I signaling promotes growth and survival of human acute myeloid leukemia cells *via* the phosphoinositide 3-kinase/AKT pathway. Leukemia *21*: 1921-1930, 2007.

52 Pardo OE, Arcaro A, Salerno G, Tetley TD, Valovka T, Gout I and Seckl MJ: Novel cross talk between MEK and S6K2 in FGF-2 induced proliferation of SCLC cells. Oncogene *20*: 7658-7667, 2001.

53 Maschler S, Wirl G, Spring H, Bredow DV, Sordat I, Beug H and Reichmann E: Tumor cell invasiveness correlates with changes in integrin expression and localization. Oncogene *24*: 2032-2041, 2005.

54 Boller D, Schramm A, Doepfner KT, Shalaby T, von Bueren AO, Eggert A, Grotzer MA and Arcaro A: Targeting the phosphoinositide 3-kinase isoform p110delta impairs growth and survival in neuroblastoma cells. Clin Cancer Res *14*: 1172-1181, 2008.

55 Hirt A, Schmid AM, Ammann RA and Leibundgut K: In pediatric lymphoblastic leukemia of B-cell origin, a small population of primitive blast cells is noncycling, suggesting them to be leukemia stem cell candidates. Pediatr Res *69*: 194-199, 2011.

56 Fattet S, Haberler C, Legoix P, Varlet P, Lellouch-Tubiana A, Lair S, Manie E, Raquin MA, Bours D, Carpentier S, Barillot E, Grill J, Doz F, Puget S, Janoueix-Lerosey I and Delattre O: Beta-catenin status in paediatric medulloblastomas: correlation of immunohistochemical expression with mutational status, genetic profiles, and clinical characteristics. J Pathol *218*: 86-94, 2009.

57 Giulietti A, Overbergh L, Valckx D, Decallonne B, Bouillon R and Mathieu C: An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. Methods *25*: 386-401, 2001.

58 Guerreiro AS, Boller D, Shalaby T, Grotzer MA and Arcaro A: Protein kinase B modulates the sensitivity of human neuroblastoma cells to insulin-like growth factor receptor inhibition. Int J Cancer *119*: 2527-2538, 2006.

59 De Laurentiis A, Pardo OE, Palamidessi A, Jackson SP, Schoenwaelder SM, Reichmann E, Scita G and Arcaro A: The catalytic class I(A) PI3K isoforms play divergent roles in breast cancer cell migration. Cell Signal *23*: 529-541, 2011.

60 Knobbe CB and Reifenberger G: Genetic alterations and aberrant expression of genes related to the phosphatidyl-inositol-3'-kinase/protein kinase B (AKT) signal transduction pathway in glioblastomas. Brain Pathol *13*: 507-518, 2003.

61 Nobusawa S, Lachuer J, Wierinckx A, Kim YH, Huang J, Legras C, Kleihues P and Ohgaki H: Intratumoral patterns of genomic imbalance in glioblastomas. Brain Pathol *20*: 936-944, 2010.

62 Valk PJ, Verhaak RG, Beijen MA, Erpelinck CA, Barjesteh van Waalwijk van Doorn-Khosrovani S, Boer JM, Beverloo HB, Moorhouse MJ, van der Spek PJ, Lowenberg B and Delwel R: Prognostically useful gene-expression profiles in acute myeloid leukemia. N Engl J Med *350*: 1617-1628, 2004.

63 Liu P, Cheng H, Roberts TM and Zhao JJ: Targeting the phosphoinositide 3-kinase pathway in cancer. Nat Rev Drug Discov *8*: 627-644, 2009.

64 Zhao JJ, Cheng H, Jia S, Wang L, Gjoerup OV, Mikami A and Roberts TM: The p110alpha isoform of PI3K is essential for proper growth factor signaling and oncogenic transformation. Proc Natl Acad Sci USA *103*: 16296-16300, 2006.

65 Jia S, Liu Z, Zhang S, Liu P, Zhang L, Lee SH, Zhang J, Signoretti S, Loda M, Roberts TM and Zhao JJ: Essential roles of PI(3)K-p110beta in cell growth, metabolism and tumorigenesis. Nature *454*: 776-779, 2008.

66 Sujobert P, Bardet V, Cornillet-Lefebvre P, Hayflick JS, Prie N, Verdier F, Vanhaesebroeck B, Muller O, Pesce F, Ifrah N, Hunault-Berger M, Berthou C, Villemagne B, Jourdan E, Audhuy B, Solary E, Witz B, Harousseau JL, Himberlin C, Lamy T, Lioure B, Cahn JY, Dreyfus F, Mayeux P, Lacombe C and Bouscary D: Essential role for the p110delta isoform in phosphoinositide 3-kinase activation and cell proliferation in acute myeloid leukemia. Blood *106*: 1063-1066, 2005.

67 Billottet C, Banerjee L, Vanhaesebroeck B and Khwaja A: Inhibition of class I phosphoinositide 3-kinase activity impairs proliferation and triggers apoptosis in acute promyelocytic leukemia without affecting ATRA-induced differentiation. Cancer Res *69*: 1027-1036, 2009.

68 Meadows SA, Vega F, Kashishian A, Johnson D, Diehl V, Miller LL, Younes A and Lannutti BJ: PI3Kdelta inhibitor, GS-1101 (CAL-101), attenuates pathway signaling, induces apoptosis, and overcomes signals from the microenvironment in cellular models of Hodgkin lymphoma. Blood *119*: 1897-1900, 2012.

69 Lannutti BJ, Meadows SA, Herman SE, Kashishian A, Steiner B, Johnson AJ, Byrd JC, Tyner JW, Loriaux MM, Deininger M, Druker BJ, Puri KD, Ulrich RG and Giese NA: CAL-101, a p110delta selective phosphatidylinositol-3-kinase inhibitor for the treatment of B-cell malignancies, inhibits PI3K signaling and cellular viability. Blood *117*: 591-594, 2011.

70 Elis W, Triantafellow E, Wolters NM, Sian KR, Caponigro G, Borawski J, Gaither LA, Murphy LO, Finan PM and Mackeigan JP: Down-regulation of class II phosphoinositide 3-kinase alpha expression below a critical threshold induces apoptotic cell death. Mol Cancer Res *6*: 614-623, 2008.

71 Koutros S, Schumacher FR, Hayes RB, Ma J, Huang WY, Albanes D, Canzian F, Chanock SJ, Crawford ED, Diver WR, Feigelson HS, Giovanucci E, Haiman CA, Henderson BE, Hunter DJ, Kaaks R, Kolonel LN, Kraft P, Le Marchand L, Riboli E, Siddiq A, Stampfer MJ, Stram DO, Thomas G, Travis RC, Thun MJ, Yeager M and Berndt SI: Pooled analysis of phosphatidylinositol 3-kinase pathway variants and risk of prostate cancer. Cancer Res *70*: 2389-2396, 2010.

Received March 21, 2012
Revised April 20, 2012
Accepted April 23, 2012