Targeting the Phosphoinositide 3-Kinase p110-α Isoform Impairs Cell Proliferation, Survival, and Tumor Growth in Small Cell Lung Cancer

Anna Wojtalla1, Barbara Fischer2, Nataliya Kotelevets2, Francesco A. Mauri5, Jens Šobek4, Hubert Rehrauer4, Carlos Wotzkow1, Mario P. Tschan1, Michael J. Seckl5, Uwe Zangemeister-Wittke2, and Alexandre Arcaro1,3

Abstract

Purpose: The phosphoinositide 3-kinase (PI3K) pathway is fundamental for cell proliferation and survival and is frequently altered and activated in neoplasia, including carcinomas of the lung. In this study, we investigated the potential of targeting the catalytic class I, PI3K isoforms in small cell lung cancer (SCLC), which is the most aggressive of all lung cancer types.

Experimental Design: The expression of PI3K isoforms in patient specimens was analyzed. The effects on SCLC cell survival and downstream signaling were determined following PI3K isoform inhibition by selective inhibitors or downregulation by siRNA.

Results: Overexpression of the PI3K isoforms p110-α and p110-β and the antipapoptotic protein Bcl-2 was shown by immunohistochemistry in primary SCLC tissue samples. Targeting the PI3K p110-α with RNA interference or selective pharmacologic inhibitors resulted in strongly affected cell proliferation of SCLC cells in vitro and in vivo, whereas targeting p110-β was less effective. Inhibition of p110-α also resulted in increased apoptosis and autophagy, which was accompanied by decreased phosphorylation of Akt and components of the mTOR pathway, such as the ribosomal S6 protein, and the eukaryotic translation initiation factor 4E-binding protein 1. A DNA microarray analysis revealed that p110-α inhibition profoundly affected the balance of pro- and antiapoptotic Bcl-2 family proteins. Finally, p110-α inhibition led to impaired SCLC tumor formation and vascularization in vivo.

Conclusion: Together our data show the key involvement of the PI3K isoform p110-α in the regulation of multiple tumor-promoting processes in SCLC. Clin Cancer Res; 19(1); 96–105. ©2012 AACR.

Introduction

Lung cancer is a major cause of death in the developed world and the commonest cancer killer in men. Small cell lung cancer (SCLC) represents about 13% to 15% of all cases of lung cancer and is strongly associated with cigarette smoking. Combinatorial chemotherapy regimens with etoposide and platinum-based agents, as well as radiotherapy, are commonly used for the treatment of patients with SCLC. However, an initial therapeutic response is usually followed by disease recurrence in less than 1 year, and therefore the overall 5-year survival rate is less than 5%. Consequently, novel therapeutic strategies are urgently required for SCLC. In the past years, an increasing number of molecular alterations involved in SCLC pathogenesis have been reported, including ectopic expression of neuroendocrine regulatory peptides, upregulation of antipapoptotic Bcl-2 proteins, overexpression of myc family onco genes and extracellular matrix proteins, as well as genetic abnormalities in the tumor suppressor genes TP53 and RB (1, 2). In addition, it has been shown that polypeptide growth factors such as hepatocyte growth factor (HGF), fibroblast growth factor-2 (FGF-2), insulin-like growth factor-1 (IGF-1), and stem cell factor (SCF) control key biologic responses in human SCLC cells, including growth and proliferation, chemoresistance, and migration (3–6). Downstream of activated polypeptide growth factor receptors, activation of 2 major intracellular signaling cascades, the phosphoinositide-3 kinase (PI3K)/Akt/mTOR, and the mitogen-activated Erk kinase (MEK)/extracellular signal-regulated kinase (ERK) pathway, have been found to be involved in the survival and proliferation of SCLC (3, 5–8). Furthermore, it has been reported that mTOR and the ribosomal protein S6 kinases (S6K) are
Small cell lung cancer (SCLC) is a common form of lung cancer and is associated with very unsatisfactory survival rates. Novel therapies are urgently needed and will arise from a better understanding of the disease biology. We were interested in gaining further insight into the potential of targeting phosphoinositide 3-kinase isoforms in SCLC. In this report, we show that the catalytic p110-α isoform is overexpressed in a subset of primary SCLC samples. The growth of SCLC cells was impaired on targeting p110-α using RNA interference or specific pharmacologic inhibitors. Inhibition of p110-α also induced apoptosis and autophagy, which was paralleled by a decrease in the expression levels of antiapoptotic Bcl-2 family proteins. Importantly, SCLC tumors treated with p110-α inhibitors displayed reduced proliferation and enhanced apoptosis in vivo. Together, the results presented in this study show that specific p110-α inhibitors may in the future represent new drugs for SCLC.

**Materials and Methods**

**Antibodies and reagents**

Antibodies: caspase-3, PARP, Akt, PI3K p110-β (Santa Cruz Biotechnology), p-AktSer473, p-Bcl2Ser70, Bcl2, Bcl-XL, Bax, Bad, p-4EBP1Thr37/46, p-S6Ser235/236 or p-S6Ser240/244, S6 protein, PI3K p110-α (Cell Signaling Technology), β-actin (Sigma Aldrich), Mcl-1, and NF-κB (Epitomics). Etoposide, chloroquine, z-VAD-FMK, 1kB-kinase (IKK) inhibitor/wedelolactone (Calbiochem). RAD001 was supplied by the Novartis Institutes for BioMedical Research (Basel, Switzerland). The PI3K inhibitors PIK75, YM024, TGX221, and PI103 were the kind gift of Prof. Shaun Jackson (Australian Centre for Blood Diseases, Monash University, Victoria, Australia).

**Immunohistochemistry**

Immunoreactivity was evaluated on commercial tissue microarray (TMA) sections of SCLC [Biomax LC10010; 2 cores: female 9 (22.5%), female age 32–66 years (mean value 52.5 years); male 31 (77.5%), male age 34–76 years (mean value 53.0 years); stage I 11, stage II 20, stage IIIa 7, stage IIIb 2; node-negative 12, node-positive 28 (22 N1 and 6 N2)] using the PI3K p110-α (Cell Signaling Technology, 4249), p110-β (Abcam, ab55593), and Bcl-2 (Cell Signaling) antibodies in a modification of the antigen retrieval technique (13). The antibodies were used at a 1:200 dilution for 1 hour at room temperature and then processed with Polymer-HPK Kit (BioGenex) with diamobenzidine development and Mayer hematoxylin counterstaining. Breast cancer tissue was used as positive external control. Negative controls were obtained by omitting the primary antibody. A semiquantitative immunohistochemical (IHS) score was used, including assessment of both the percentage of positive cells and the intensity of staining. For the intensity, a score of 0 to 3, corresponding to negative, weak, moderate, and strong positivity, was recorded. The range of possible scores was thus 0 to 300. IHS and similar semiquantitative scoring systems have been successfully used for TMA evaluation. Each core was scored individually. One observer scored all cases, which were rechecked randomly by the same investigator after a period of time.

**Reverse transcription PCR and TaqMan analysis**

Total cellular RNA was extracted using the RNaseasy Mini Kit (Qiagen) according to the manufacturer’s instruction. For each reverse transcription PCR (RT-PCR), 1 µg of total RNA was used with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The reaction conditions were used according to the manufacturer’s protocol. TaqMan Gene Expression Assays for the PI3Ks p110-α and p110-β, as well as for VEGFA were done according to the manufacturer’s instructions (Applied Biosystems). As internal control the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was analyzed in each sample, separately.

**Translational Relevance**

Small cell lung cancer (SCLC) is a common form of lung cancer and is associated with very unsatisfactory survival rates. Novel therapies are urgently needed and will arise from a better understanding of the disease biology. We were interested in gaining further insight into the potential of targeting phosphoinositide 3-kinase isoforms in SCLC. In this report, we show that the catalytic p110-α isoform is overexpressed in a subset of primary SCLC samples. The growth of SCLC cells was impaired on targeting p110-α using RNA interference or specific pharmacologic inhibitors. Inhibition of p110-α also induced apoptosis and autophagy, which was paralleled by a decrease in the expression levels of antiapoptotic Bcl-2 family proteins. Importantly, SCLC tumors treated with p110-α inhibitors displayed reduced proliferation and enhanced apoptosis in vivo. Together, the results presented in this study show that specific p110-α inhibitors may in the future represent new drugs for SCLC.
rehydrated through graded dilutions of ethanol. Antigen recovery was conducted with citrate buffer (10 mmol/L) in a microwave (800 W) for 7 minutes. Endogenous peroxidase activity was blocked with 3% H$_2$O$_2$. Sections were incubated in a humid chamber at 4°C overnight with or without (negative controls) active caspase-3 (Abcam, ab2302; recognizes the cleaved active p17 fragment) and Ki67 (Novus Biologicals, NB110-57147) antibodies. Biotin-conjugated goat anti-rabbit (Dako, E0432), and avidin–biotin–horse-radish peroxidase (HRP) complex (Vector Laboratories, Vectastain ABC Kit, PK6100) were used. Bound peroxidase was detected with 3,3′-diaminobenzidine substrate. Sections were subsequently counterstained with hematoxylin and mounted in Aquatex (Merck).

**Transient transfection and stable transduction of SCLC cells**

SCLC cells were transiently transfected with either nontargeting siRNA or SMARTpool siRNA duplexes targeting the PI3K p110-α or PI3K p110-β, using the Amaza Nucleofector system (Amaza Biosystems), according to the manufacturer’s protocol. Briefly, $1.5\times10^8$ SCLC cells grown in RPMI/10% FCS were transfected with 6 μL of 20 μmol/L siRNA in 100 μL of Nucleofector Solution V using the program T-16 on the Amaza Nucleofector. Following transfection, cells were transferred into RPMI/10% FCS overnight, before they were used for experiments.

Lentiviral vectors expressing short hairpin RNA (shRNA) constructs specifically targeting the PI3K p110-α (Sigma-Aldrich) were tested for stable knockdown efficiency. The constructs shPI3KC_2892 and shPI3KC_3433 were chosen for further experiments as they most efficiently silenced p110-α. The nontargeting vector SHC002 was used as a control. Lentivirus production and transduction of cells was done as described earlier (14). The vectors contain a puromycin resistance gene and stably transduced cell populations were selected for 2 weeks using puromycin at the concentration 1.5 μg/mL.

**In vivo experiments**

The effect of the PI3K inhibitor PIK75 on the growth and SCLC tumor formation in vivo were assessed on the CAM of chick embryos (15). Briefly, $3\times10^8$ H69 SCLC cells were placed in RPMI containing 50% BD Matrigel (BD Biosciences) on the CAM on embryonic day 9. Increasing concentrations of the p110-α inhibitors PIK75 were deposited on the SCLC cells. Controls were treated with the solvent of the corresponding drug. CAMs were examined for vessel formation under a stereomicroscope. Tumor size and density of vessels per area around the tumor were determined using the software Vessel_tracer (16). The application of Matrigel without/with PIK75 on the CAM was used as a negative control (Supplementary Fig. S3).

**SDS-PAGE and Western blotting**

The assays were done as described earlier (3).

**DNA microarray**

The cDNA microarray analysis was conducted at the Functional Genomic Center of the University of Zurich (Zurich, Switzerland). Gene expression data were obtained by hybridizing Human Genome U133 Plus 2.0 Affymetrix Gene Chips arrays, on which more than 54,000 transcripts were represented. Each experiment represented a group of 3 independent biologic replicates. Raw data generated by the GCOS Software (Affymetrix) were processed by using the log scale robust multi-array analysis method (17) and further statistically analyzed by using the software R and applying Student t test. The GeneGO MetaCore (GeneGO) was used to define functional annotations for the selected genes, thus assigning them to ontologic categories for association with relevant biologic processes and pathways. The accession number of the data in Gene Expression Omnibus is GSE40564.

**Results**

Expression of the PI3K p110-α and p110-β and the antiapoptotic protein Bcl-2 in SCLC patient samples

Our previous work in SCLC cell lines had revealed that class Iα PI3K isoforms are overexpressed in comparison with type II pneumocytes, which are one of the precursors of SCLC. To investigate whether this finding could be confirmed in primary tumors, a SCLC TMA was stained with antibodies specific for p110-α or p110-β (Fig. 1A). This analysis revealed that, while normal lung tissue did not express detectable levels of either PI3K isoforms, p110-α and p110-β displayed enhanced expression in subsets of primary SCLC (Fig. 1A and B). Around 25% of SCLC tumors showed overexpression of p110-α, whereas 18% of cases were positive for p110-β (Fig. 1B). In general, SCLC tumors did not display concomitant overexpression of p110-α and p110-β, which was only present in 5% of the tumors (data not shown). In addition, the expression of the antiapoptotic protein Bcl-2 was assessed in SCLC patient samples. The IHC staining of a TMA with a specific antibody revealed a high Bcl-2 overexpression in approximately 90% (35 of 39 cases) of the SCLC patient samples (Fig. 1C and D), compared with normal lung tissue, which did not display detectable levels of Bcl-2.

**Class Iα PI3K p110-α inhibition or silencing blocks cell proliferation and Akt/mTOR signaling**

Previous work had shown that targeting the PI3K/Akt/mTOR pathway by using small-molecule inhibitors impaired SCLC proliferation in vitro and in vivo. Therefore, we investigated the impact of isoform-specific inhibitors of class Iα PI3K isoforms on the proliferation of SCLC cell lines. The potent p110-α inhibitor PIK75 (IC$_{50}$ in vitro = 7.8 ± 1.7 mmol/L; ref. 18) significantly impaired the proliferation of 4 SCLC cell lines with IC$_{50}$ values in the range of 50 to 100 nmol/L (Fig. 2A). The p110-α inhibitor YM024, which is much less potent against the enzyme in vitro (IC$_{50}$ in vitro = 0.3 μmol/L), also impaired the proliferation of the SCLC cell line panel, but at higher concentrations (Fig. 2A). The potent p110-β inhibitor TGX-221 (IC$_{50}$ in vitro = 8.5 ± 0.3 μmol/L)
0.9 nmol/L; ref. 18) only partially impaired the proliferation of SCLC cell lines, but at high concentrations (Fig. 2B). In contrast, the p110-δ inhibitor IC87114 did not significantly impair the proliferation of the cell lines under study (Fig. 2B). We also evaluated the impact of the dual p110-α/mTOR inhibitor PI103 in the panel of SCLC cell lines. PI103 displayed antiproliferative activity against the SCLC panel in vitro, with IC50 values in the range of 100 to 500 nmol/L (Supplementary Fig. S1A).

The impact of the selective PI3K inhibitors on the activation status of the Akt/mTOR pathway in SCLC cell lines was then investigated by Western blot analysis (Fig. 2C). PIK75 and PI103 strongly affected the activation status of Akt and the phosphorylation of the mTOR downstream targets ribosomal S6 protein and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1; Fig. 2C; Supplementary Fig. S1B). YM024 also impaired Akt activation and partially inhibited mTOR pathway activation (Fig. 2C; Supplementary Fig. S1B). In contrast, TGX221 was less effective at blocking the activation of the Akt/mTOR pathway (Fig. 2C; Supplementary Fig. S1B).

To validate our findings with pharmacologic inhibitors, we used RNA interference (RNAi) to downregulate the expression of p110-α and p110-β, and the antiapoptotic protein Bcl-2, in SCLC patient samples. Transient downregulation of p110-α strongly impaired the activation of Akt and the phosphorylation of the ribosomal S6 protein, whereas p110-β silencing was ineffective (Fig. 3A). In addition, p110-α silencing induced a significant decrease in the proliferation of SCLC cells (40% reduction), whereas p110-β downregulation was less effective (25% reduction; Fig. 3B).
We also used lentiviral delivery of shRNAs targeting p110-α in SCLC cell lines. Also, this approach resulted in stable silencing of p110-α, robust affected PI3K downstream signaling (Fig. 3C), and SCLC cell proliferation was partially impaired by shRNA targeting of p110-α (Fig. 3D).

Together these results show that targeting p110-α selectively impairs cell proliferation and activation of the Akt/mTOR pathway in SCLC cell lines in vitro.

Class Iα PI3K p110-α inhibition induces apoptosis and autophagy in SCLC cell lines

Previous work had shown that targeting the PI3K/Akt/mTOR pathway by using small-molecule inhibitors (7, 19) induced apoptosis in SCLC cell lines. Therefore, we investigated the impact of isoform-specific inhibitors of class Iα PI3K isoforms on the survival of SCLC cell lines. Treatment of a panel of SCLC cell lines with PIK75 or YM024 induced cleavage of PARP and reduction of procaspase-3, which are markers of apoptosis induction (Fig. 4A), whereas TGX221 was less efficient at inducing apoptosis (Fig. 4A). The induction of apoptosis upon p110-α inhibition seemed to be stronger in the SCLC cell lines H69 and H209, compared with the effects observed in H510 (intermediate) and SW2 (low; Fig. 4A). The caspase inhibitor zVAD-FMK was able to rescue 20% of the PIK75-induced apoptosis in H69 cells and 10% of the TGX221-induced apoptosis (P < 0.01; Fig. 4B).

Because the strongly affected cell viability in SCLC cells upon p110-α inhibition could not only be explained by the apoptotic cell response, we further investigated other cell death mechanisms, such as autophagy. Although the inhibition of p110-α with PIK75 or YM024 induced an increased conversion of LC3-I to LC3-II, which is indicative for autophagic activity, a comparable response could not be observed in cells in which p110-β was inhibited (Fig. 4C; Supplementary Fig. S2). ATG5, another protein used as a typical marker for autophagy, was also expressed in SCLC cells. Compared with the vehicle-treated control, enhanced ATG5 expression was not, or only slightly, observed in PIK75-treated H69 cells (Fig. 4C). Interestingly, at later time points, a decrease in ATG5 expression was observed (data not shown). Chloroquine is known to inhibit autophagic activity due to deacidification of the lysosome.
The proteins of the Bcl-2 family are downstream targets of p110-α

To validate the Bcl-2 family proteins as targets of p110-α inhibition, in comparison with p110-β, we conducted DNA microarray analysis in H69 cells treated with either vehicle, PIK75 targeting p110-α, or TGX221 targeting p110-β (Fig. 6A). The efficacy of the downregulation of the Akt/mTOR pathway by the respective inhibitors was shown by Western blot analysis, as well as qRT-PCR for VEGFA expression (Fig. 2C and data not shown). Inhibiting p110-α significantly affected the expression of 3,411 genes (P value 0.01; FC ≥ 1.5), whereas inhibiting p110-β resulted in significant changes (P value 0.01; FC ≥ 1.5) in 4 genes, suggesting an important role of p110-α, but not p110-β, for the regulation of the expression of a subset of genes in SCLC (Fig. 6A). We next sought to investigate which genes were selectively affected by p110-α inhibition, in comparison with p110-β. Among these genes, antia apoptotic proteins of the Bcl-2 family of proteins were found to be more significantly downregulated in SCLC cells treated with p110-α inhibitor, than in the case of the p110-β inhibitor (data not shown).

The proteins of the Bcl-2 family are downstream targets of p110-α

To validate the Bcl-2 family proteins as targets of p110-α in SCLC cell lines, we used antibody arrays and Western blot

lysosomes, followed by accumulation of ineffective autophagic vesicles. In H69 cells treated with the PI3K inhibitors PIK75 and TGX221, chloroquine was able to rescue 20% (and 10%, respectively) of the autophagy-induced decrease in cell viability (Fig. 4D), suggesting additive roles, or cross-talk, between autophagy and apoptosis in p110-α-induced cell death.

Inhibition of the PI3K p110-α impairs SCLC tumor formation and vascularization in vivo

Together the data collected from our experiments and the known literature indicated an important role for p110-α in SCLC cell growth and survival processes in vitro and in vivo. To further investigate its impact on tumor formation and maintenance, an in vivo assay was used to grow vehicle- and PIK75-treated H69 cells on the CAM of chick embryos. Tumor formation was clearly impaired upon PIK75 treatment, which was shown by strongly reduced tumor size and tumor weight compared with the control treatment (Fig. 5A). In addition, IHC staining of PIK75-treated tumor sections showed a decreasing expression of the proliferation marker Ki67 and a rising expression of the apoptotic marker cleaved caspase-3 with increasing PIK75 concentrations (Fig. 5C and D). Finally, p110-α inhibition was able to partially prevent the vascularization on the CAM around the tumorigenic area, as measured by the vessel density (Fig. 5B). Another indication of impaired tumor vascularization upon p110-α inhibition is the reduced VEGFA expression observed in the DNA microarray and quantitative RT-PCR (qRT-PCR) analysis of PIK75-treated H69 cells (data not shown).

DNA microarray analysis of SCLC cells treated with isoform-specific PI3K inhibitors

To investigate whether the class I α PI3K isoform p110-α controls the expression of specific gene subsets in SCLC, we conducted DNA microarray analysis in H69 cells treated with either vehicle, PIK75 targeting p110-α, or TGX221 targeting p110-β (Fig. 6A). The efficacy of the downregulation of the Akt/mTOR pathway by the respective inhibitors was shown by Western blot analysis, as well as qRT-PCR for VEGFA expression (Fig. 2C and data not shown). Inhibiting p110-α significantly affected the expression of 3,411 genes (P value 0.01; FC ≥ 1.5), whereas inhibiting p110-β resulted in significant changes (P value 0.01; FC ≥ 1.5) in 4 genes, suggesting an important role of p110-α, but not p110-β, for the regulation of the expression of a subset of genes in SCLC (Fig. 6A). We next sought to investigate which genes were selectively affected by p110-α inhibition, in comparison with p110-β. Among these genes, antia apoptotic proteins of the Bcl-2 family of proteins were found to be more significantly downregulated in SCLC cells treated with p110-α inhibitor, than in the case of the p110-β inhibitor (data not shown).

The proteins of the Bcl-2 family are downstream targets of p110-α

To validate the Bcl-2 family proteins as targets of p110-α in SCLC cell lines, we used antibody arrays and Western blot

To further investigate its impact on tumor formation and vascularization on the CAM around the tumorigenic area, as measured by the vessel density (Fig. 5C and D). Finally, p110-α inhibition was able to partially prevent the vascularization on the CAM around the tumorigenic area, as measured by the vessel density (Fig. 5B). Another indication of impaired tumor vascularization upon p110-α inhibition is the reduced VEGFA expression observed in the DNA microarray and quantitative RT-PCR (qRT-PCR) analysis of PIK75-treated H69 cells (data not shown).

DNA microarray analysis of SCLC cells treated with isoform-specific PI3K inhibitors

To investigate whether the class I α PI3K isoform p110-α controls the expression of specific gene subsets in SCLC, we conducted DNA microarray analysis in H69 cells treated with either vehicle, PIK75 targeting p110-α, or TGX221 targeting p110-β (Fig. 6A). The efficacy of the downregulation of the Akt/mTOR pathway by the respective inhibitors was shown by Western blot analysis, as well as qRT-PCR for VEGFA expression (Fig. 2C and data not shown). Inhibiting p110-α significantly affected the expression of 3,411 genes (P value 0.01; FC ≥ 1.5), whereas inhibiting p110-β resulted in significant changes (P value 0.01; FC ≥ 1.5) in 4 genes, suggesting an important role of p110-α, but not p110-β, for the regulation of the expression of a subset of genes in SCLC (Fig. 6A). We next sought to investigate which genes were selectively affected by p110-α inhibition, in comparison with p110-β. Among these genes, antia apoptotic proteins of the Bcl-2 family of proteins were found to be more significantly downregulated in SCLC cells treated with p110-α inhibitor, than in the case of the p110-β inhibitor (data not shown).

The proteins of the Bcl-2 family are downstream targets of p110-α

To validate the Bcl-2 family proteins as targets of p110-α in SCLC cell lines, we used antibody arrays and Western blot
analysis to confirm the results obtained by DNA microarrays. An analysis using antibody arrays revealed impaired Akt pathway activation and downregulation of antiapoptotic Bcl-2 family proteins (data not shown). Downregulation of the expression and impaired activation of the antiapoptotic Bcl-2 family proteins could indeed be shown at the protein level upon p110-α inhibition (Fig. 6B). As was already observed previously for the apoptotic response, the SCLC cell lines under study responded varyingly to p110-α inhibition. In particular, the cell lines H69 and H209 displayed a strongly decreased expression of the Bcl-2 family members Bcl-2, Bcl-XL, and Mcl-1, whereas SW2 cells seemed to be more resistant. In addition, p110-α inhibition induced the levels of the proapoptotic family members Bad and Bax (Fig. 6B). In contrast to the inhibitors of p110-α, TGX221 had little effect on the expression levels of Bcl-2 family proteins (Fig. 6B). The antiapoptotic Bcl-2 family proteins Bcl-2 and Bcl-Xl are known targets of the NF-κB transcriptional network, which controls different cell responses including immune response, cell proliferation, and survival (20). To gain insight into the transcriptional networks affected by silencing of p110-α in SCLC cells, we...
conducted a biostatistical analysis of the gene expression data, using GeneGo. The transcriptional networks that were most significantly altered comprised HNF4α, SP1, and c-Myc, the estrogen receptor (ER) and also NF-κB (Supplementary Table S1). We could indeed observe a deregulation of NF-κB expression in H69 cells treated with PIK75 and YM024, whereas p110-β inhibition did not have any effect (Fig. 6C). These results suggested NF-κB to be a downstream target of PI3K p110-α signaling in SCLC, and therefore, we assumed that targeting NF-κB may have an effect on the expression of Bcl-2 and Bcl-xL. This hypothesis was confirmed by the observation that the expression levels of Bcl-2 were indeed decreased in H69 cells treated with wederolactone, an inhibitor of the IKK (Fig. 6D).

Discussion

The PI3K/Akt/mTOR pathway has been shown to play a key role in SCLC cell proliferation, survival, chemoresistance, and migration. Mutations in PIK3CA and gene amplification were reported in primary SCLC, as well as increased expression of PIK3CA at the mRNA and protein level. Broad specificity PI3K/mTOR inhibitors have shown antitumor activity in SCLC models in vitro and in vivo (21). In this report, we have investigated the impact of isoform-specific inhibitors and RNAi targeting class Iα PI3K isoforms on SCLC cell responses. In general, agents targeting p110-α reduced SCLC cell proliferation in vitro, impaired the SCLC tumor formation, which was accompanied by affected vascularization in vivo and decreased the activation status of classical PI3K downstream targets, such as Akt, mTOR, and S6K. Targeting p110-α by RNAi or isoform-specific inhibitors had more pronounced effects on SCLC cell responses than in the case of p110-β or p110-δ, indicating a selective role for p110-α in SCLC. In view of these observations, we hypothesized that p110-α may control the expression of a selective subset of genes implicated in SCLC cell proliferation and/or survival. The comparative DNA microarray analysis of SCLC cell lines in which either p110-α or p110-β were inhibited by selective compounds identified such a gene subset. The Bcl-2 family of proteins was validated as a downstream target of p110-α by a combination of approaches. The observation that Bcl-2 expression was elevated in primary SCLC, in comparison with normal lung tissue further supports this model, in view of the overexpression of p110-α. Importantly, the Bcl-2 family of proteins has been previously shown to play a crucial role in the survival of SCLC cell lines in vitro and in vivo (5, 22–26). The p110-α inhibitors induced increases in both SCLC apoptosis and autophagy, which is consistent with Bcl-2 family proteins being a target of p110-α. Bcl-2 family proteins are key regulators of both apoptosis and autophagy (27) and their reduced expression upon inhibition of the p110-α/NF-κB pathway may play an essential role in the effects of the p110-α inhibitors in SCLC.

We have previously evaluated the mTOR inhibitor everolimus in SCLC cell lines and found that it was effective in a subset of SCLC cell lines characterized by activation of the Akt/mTOR pathway and low expression levels of antia apoptotic Bcl-2 family proteins (24). In view of the results obtained with isoform-selective inhibitors of p110-α, it can be speculated that these agents may be more potent, as they induce a downregulation of antia apoptotic Bcl-2 family proteins.
proteins and of the activity of the Akt/mTOR pathway. This may be particularly relevant for SCLC tumors bearing activating mutations in the PIK3CA gene.

Interestingly, our previous work in neuroblastoma has shown that the class IA PI3K isoform p110-δ contributes to cell proliferation and survival by controlling the activation of the mTOR pathway and the expression levels of antiapoptotic Bcl-2 family proteins. Overexpression of p110-δ was found in a subset of primary neuroblastoma and cell lines, and p110-δ was essential for signal transduction by receptor tyrosine kinases, such as the IGF-1R and EGF receptor (EGFR; ref. 28). In contrast, in SCLC cell lines, p110-α seems to play a more important role in the activation of the Akt/mTOR pathway, which may explain the impact of agents targeting this isoform on SCLC proliferation and survival. A previous report has shown that the activity of any class Iα PI3K isoform can maintain cell survival (29). Therefore, the relative importance of class Iα PI3K isoforms in selected cancer types may be, in part, attributed to differences in expression levels.

Thus, targeting PI3K p110-α signaling may represent an attractive novel approach to develop novel therapies for SCLC. Indeed, different pharmacologic inhibitors of this isoform now exist, which will soon enter clinical trials in oncology, and could, in the future, be developed as new drugs for SCLC.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: A. Wojtalla, B. Fischer, N. Kotelevets, U. Zangemeister-Wittke, A. Arcaro
Development of methodology: A. Wojtalla, N. Kotelevets, U. Zangemeister-Wittke, A. Arcaro
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Wojtalla, B. Fischer, N. Kotelevets, J. Sobek, C. Wotzkow, M.P. Tschan, M.J. Sedl, U. Zangemeister-Wittke, A. Arcaro
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Wojtalla, B. Fischer, N. Kotelevets, F.A. Maurer, J. Sobek, H. Rehrauer, A. Arcaro

Figure 6. The proteins of the Bcl-2 family are downstream targets of p110-α. A, gene expression analysis by DNA microarray: heatmap of gene expression changes caused by p110-α inhibition. B–D, H69, H209, and SW2 (B) or H69 (C and D) cells grown in serum-containing medium were incubated with increasing concentrations of the PI3K p110-α inhibitors PIK75 and YM024, the PI3K p110-β inhibitor TGX221, RAD001, and etoposide/cisplatin or the IKK inhibitor wederolactone (D). After 24 hours, the cells were harvested and cell lysates analyzed by SDS-PAGE and Western blotting for the proteins indicated.
Targeting PI3K Signaling in SCLC

Writing, review, and/or revision of the manuscript: A. Wojtalla, B. Fischer, C. Wotzkow, M.P. Tschan, M.J. Seckl, U. Zangemeister-Wittke, A. Arcaro

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Wojtalla, C. Wotzkow

Study supervision: N. Kotelevets, A. Arcaro

Grant Support

This study was supported by Association for International Cancer Research, Wilhelm Sander-Stiftung, Novartis Stiftung für Medizinisch-Biologische Forschung, and Department of Clinical Research of the University of Bern.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received April 10, 2012; revised October 12, 2012; accepted October 21, 2012; published OnlineFirst November 21, 2012.

References


Published OnlineFirst November 21, 2012; DOI: 10.1158/1078-0432.CCR-12-1138

www.aacrjournals.org

Clin Cancer Res; 19(1) January 1, 2013 105

Downloaded from clincancerres.aacrjournals.org on July 14, 2017. © 2013 American Association for Cancer Research.
Targeting the Phosphoinositide 3-Kinase p110-α Isoform Impairs Cell Proliferation, Survival, and Tumor Growth in Small Cell Lung Cancer

Anna Wojtalla, Barbara Fischer, Nataliya Kotelevets, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-12-1138

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2012/11/21/1078-0432.CCR-12-1138.DC1

Cited articles
This article cites 29 articles, 11 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/19/1/96.full#ref-list-1

Citing articles
This article has been cited by 6 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/19/1/96.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.