Targeting Small Cell Lung Cancer Harboring \textit{PIK3CA} Mutation with a Selective Oral PI3K Inhibitor PF-4989216

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Translational Relevance

Small cell lung cancer (SCLC) patients have poor prognosis and response to second-line chemotherapy. Multiple phase III clinical trials have been conducted, however the survival of SCLC patients has not improved over the years. In this study, we characterized a selective oral PI3K inhibitor, PF-4989216, in preclinical SCLC models to investigate the potential benefit of targeting PI3K in SCLC. PF-4989216 inhibits PI3K signaling, cell proliferation and transformation and subsequently leads to inhibition of xenograft tumor growth in SCLCs harboring a PIK3CA mutation. Surprisingly, PF-4989216 did not induce anti-tumor activity in SCLCs with PTEN loss, suggesting there may be different tumorigenesis and apoptosis mechanisms between a PIK3CA mutation and PTEN loss in SCLCs and providing potential clinical patient selection guidance. Therefore, PF-4989216 is a potential cancer drug candidate for small cell lung cancer patients with PIK3CA mutation but not PTEN loss.
Abstract

Purpose: Constitutive activation of PI3K occurs frequently in many human tumors via either gene mutation in the p110α catalytic subunit of PI3K or functional loss of tumor suppressor PTEN. Small cell lung cancer (SCLC) patients have very poor prognosis and survival rates such that an effective targeted therapy is in strong demand for these patients. In this study, we characterized the highly selective oral PI3K inhibitor, PF-4989216, in preclinical SCLC models to investigate whether targeting the PI3K pathway is an effective targeted therapy option for SCLCs that harbor a PIK3CA mutation.

Experimental Design: A panel of SCLC lines with PIK3CA mutation or PTEN loss were treated with PF-4989216 in several in vitro assays including: PI3K pathway signaling, cell viability, apoptosis, cell cycle progression, and cell transformation. SCLC lines that were sensitive in vitro to PF-4989216 were further evaluated by in vivo animal studies to determine the pharmacokinetic/pharmacodynamic relationship and tumor growth inhibition by PF-4989216 treatment.

Results: PF-4989216 inhibited PI3K downstream signaling and subsequently led to apoptosis induction, and inhibition in cell viability, transformation, and xenograft tumor growth in SCLCs harboring PIK3CA mutation. In SCLCs with PTEN loss, PF-4989216 also inhibited PI3K signaling but did not induce BIM-mediated apoptosis nor was there any effect in cell viability or transformation. These results implicate differential tumorigenesis and apoptosis mechanisms in SCLCs harboring PIK3CA mutation versus PTEN loss.

Conclusion: Our results suggest that PF-4989216 is a potential cancer drug candidate for SCLC patients with PIK3CA mutation but not PTEN loss.
Introduction

The class I lipid kinase family of phosphatidylinositol 3 kinase (PI3K) catalytic subunits are divided into class IA (p110α, p110β, and p110δ) and class IB (p110γ), according to both structure and interaction with the p85 and p55 regulatory subunits (1). In response to activation, PI3Ks phosphorylate the D3 position on membrane phosphatidylinositols to generate phosphatidylinositol 3,4,5-triphosphate (PIP3); PIP3 serves as an important secondary messenger by recruiting and activating proteins that contain a pleckstrin homology (PH) domain including AKT and 3’-phosphoinositide-dependent kinase-1 (PDK1). Recruitment of PDK1 to the plasma membrane to phosphorylate AKT at residue threonine-308 (T308) and phosphorylation of AKT at the serine-473 (S473) residue by mTORC2 fully activate the AKT pathway (2). AKT activation is critical in the regulation of various cellular processes including cell growth, proliferation, survival, and metabolism (2, 3), and aberrant PI3K/AKT signaling occurs commonly in cancer (4-6). Gene mutation, amplification, and copy number gains of p110α have been shown in a variety of human cancers such as breast, endometrial, colon, lung and many others (5-7). Cancer specific mutations have not frequently been found in the other isoforms.

Another PI3K/AKT activation pathway is through the tumor suppressor phosphatase and tensin homologue deleted on chromosome 10 (PTEN); PTEN dephosphorylates 3-phosphoinositides and is frequently mutated, deleted, or down-regulated in many human cancers leading to elevated PIP3 levels and further resulting in constitutive activation of the PI3K/AKT pathway (8). PI3K inhibitors have shown anti-tumor activity in PTEN-null preclinical models of non-small-cell lung cancer (NSCLC).
and prostate tumor cells (9, 10). However, tumor suppressor functions of PTEN have been expanded and include mechanisms that are PI3K/AKT independent (11). These functions include regulation of SRC through its protein phosphatase activity (12), a crucial role in p53-mediated cellular senescence (13), and the participation of nuclear PTEN in controlling genomic stability and cell cycle progression independent of phosphatase activity (14, 15). Furthermore, it has not been well investigated whether PTEN loss would specifically function through the PI3K/AKT pathway to regulate tumor progression in various subsets of tumor types.

Several inhibitors targeting the PI3K pathway have been developed in preclinical discovery programs or clinical trials (16-20), however, there has not yet been a small molecular weight inhibitor of the PI3K pathway that is approved for cancer treatment. Inhibitors in this pathway often have inhibitory activity against both PI3Ks and mTOR kinases, potentially leading to greater combined toxicity than either target alone. The mTORC1 and mTORC2 complexes control critical pathways regulating cell proliferation, apoptosis, angiogenesis, and metabolism through AKT-dependent and AKT-independent mechanisms (16-19). Therefore, PI3K inhibitors that also inhibit mTOR kinase activities may carry extra toxicity through the disruption of mTOR function in normal cells. To develop a drug selectively targeting PI3K, we have screened compounds and identified PF-4989216 as a novel potent and selective PI3K inhibitor, as previously reported (20).

Small cell lung cancer (SCLC) represents 13% of all newly diagnosed cases of lung cancer worldwide (21). SCLC has a unique natural history with a shorter doubling time, higher growth fraction, earlier development of widespread metastases than other cancers. While SCLC initially responds to chemotherapy and radiation, it recurs rapidly
with only 5% of patients surviving five years. Response to second-line chemotherapy for patients with refractory disease is less than 10%, and survival is three to four months (22, 23). Multiple phase III trials have been conducted, however the survival of SCLC patients has not improved significantly over the years (24, 25). Therefore, a targeted therapeutic approach is in strong demand for SCLC patients. In this study, we characterized a selective PI3K inhibitor in preclinical SCLC models to investigate whether selectively targeting the PI3K pathway may be a potential effective therapy in SCLC.

In this study, we have described the in vitro and in vivo anti-tumor activity of PF-4989216 in a panel of human small cell lung cancer cells (SCLCs). PF-4989216 inhibited the phosphorylation of PI3K downstream molecules and subsequently led to apoptosis induction and inhibition in cell proliferation, transformation, and xenograft tumor growth in SCLCs harboring a PIK3CA mutation. However, in SCLCs with PTEN loss, PF-4989216 inhibited PI3K signaling but did not induce BIM-mediated apoptosis and was not able to inhibit cell proliferation and transformation implicating different tumorigenesis and apoptosis mechanisms between PIK3CA mutation and PTEN loss in SCLCs. In conclusion, our results suggest that PF-4989216 is a potential cancer drug candidate for small cell lung cancer patients with PIK3CA mutation but not PTEN loss.

Materials and Methods

Selective PI3K inhibitor
PF-4989216 was synthesized as previously described (20). Compounds were dissolved in DMSO for the in vitro cellular assays. PF-4989216 was formulated in 0.5% methyl-cellulose as a suspension (v/v) for in vivo animal studies.

**Cell culture, adenovirus infection, cell viability, anchorage independent growth, and ELISA assays**

Small-cell lung cancer cell lines NCI-H69, NCI-H1048, NCI-H1436, NCI-H82, NCI-H254, NCI-H526, NCI-H1963, NCI-H146, and NCI-H841 were obtained from American Type Culture Collection. Lu99A, Lu134B, and Lu134A were obtained from RIKEN Research Institute. All cell lines were cultured at 37°C in 5% CO₂ in supplier-recommended growth media.

Adenovirus containing PTEN coding DNA sequence (Ad-PTEN) and adenovirus containing GFP (Ad-GFP) were purchased from Vector Biolabs (Philadelphia, PA, USA). A total of $2 \times 10^6$ Lu134B and Lu134A cells were infected with a multiplicity of infection of 100. Infection was verified by fluorescent microscopy. The medium was replaced to contain the indicated treatments after 24 hours.

SCLC cells were cultured (5000 cells/well) in a 96-well microtiter plate and compounds were added to each well starting at 10 μM with a three-fold serial dilution. At 72 hours post compound addition, Cell Titer Glo (CTG) Solution (Promega, Madison, WI, USA) was added per manufactures instructions. Luminescence was read on an Envision plate reader. All experiments were run in duplicate and have been repeated at least three times.

SCLC cells were plated for anchorage independent growth with compound in 0.35% BD Difco Noble agar (BD Diagnostic Systems) over a bottom layer of 0.5% BD
Difco Noble agar containing growth medium. Cultures were maintained for a minimum of four weeks by weekly addition of compound in fresh agar (0.35%) containing medium. Microscope images of colony morphology were taken prior to colony visualization by addition of iodonitrotetrazolium chloride (1 mg/ml, Sigma-Aldrich, St Louis, MO, USA) for 18 hours. Colonies were counted using the colony count function on the Fluorchem Q Gel Imaging System (Alpha Innotech/Protein Simple). All assays were run in duplicate, and have been repeated at least twice.

Cells (25,000-200,000 cells/well) were seeded in a 96-well microtiter plate and cultured overnight. The next day, PF-4989216 was added to each well starting at 10 μM with a three-fold serial dilution for two hours. Cells were washed with PBS twice, and cell lysates were prepared and analyzed by pAKT S473 ELISAs following the manufacturer’s instructions (Cell Signaling Technology Inc, Danvers, MA, USA).

The Rat/Mouse Insulin ELISA kit (Cat. # EZRMI-13K, EMD Millipore, St. Charles, MO, USA) was used for the non-radioactive quantification of insulin in mouse serum samples according to the manufacture’s instruction.

Glucose was measured on the ADVIA® 1200 System chemistry analyzer (Siemens Healthcare Diagnostics, Tarrytown, NY, USA) according to the manufacturer’s instructions using the Siemens ADVIA® 1200 Glucose Hexokinase reagent.

**Immunoblotting and FACS**

Cells and tumors were homogenized in lysis buffer (50 mM Tris-HCl, 1% NP-40, 0.5% TX-100, 150 mM NaCl, 1 mM Na₃VO₄, 1 mM NaF, and protease inhibitor cocktail). Protein concentration was determined using the BCA Protein Assay Kit (Pierce/Millipore, Rockford, IL, USA) per the manufacturer’s instructions. Protein (10-
50 μg) was resolved by SDS-PAGE and transferred onto nitrocellulose membrane. Blots were probed with primary antibodies to detect proteins of interest. After incubation with secondary antibodies, membranes were visualized by chemiluminescence (Pierce/Thermo Fisher Scientific). All antibodies were from Cell Signaling Technology, Inc with the exception of GAPDH (Santa Cruz Biotechnology, Inc). Autophagy inducer STF-62247 and cytotoxic agents including paclitaxel, gemcitabine, carboplatin, dexamethasone, etoposide, camptothecin, and cycloheximide were from EMD Millipore.

Cells were plated in six-well plates (100,000 cells/well) and incubated allowed overnight prior to treatment. The next day, compound was added and cells were incubated for 24, 48, or 72 hours. The caspase inhibitor Z-VAD (OMe)-FMK was purchased from EMD Millipore. At each time point, cells were collected, fixed, and permeabilized using the Cell Cycle Phase Determination Kit (Cayman Chemical) and following the manufacturer’s protocol. Samples were stored at -20°C until stained with propidium iodide prior to sample analysis. All experiments were repeated at least twice, and a minimum of 10000 events were collected per sample on a BD FACSCalibur (BD Biosciences). Data analysis was performed with FCS Express (De Novo Software).

PK sample preparation and LC-MS/MS analysis

Standard stock solutions (STD) of PF-04989216 were prepared in 50:50 DMSO: acetonitrile. The working solution for the internal standard (IS, Terfenadine) was prepared in acetonitrile (10 ng/mL) and stored at -20°C. Samples were prepared by addition of IS solutions to STD and plasma samples and then centrifuged at 3000 Xg for 10 minutes at room temperature. Supernatant from each sample was then run on a Waters Acquity UPLC system (Waters, Milford, MA) and an API 5500 triple-stage
quadrupole mass spectrometer (Applied Biosystems) LC-MS/MS system. The chromatography was performed on a reverse phase column (Phenomenex Kinetex phenyl-hexyl, 50’2 mm 1.7 μm) using a gradient elution method at a flow rate of 500 μL/min. The mobile phase consisted of A = 0.1% formic acid in water and B = 0.1% formic acid in acetonitrile. The gradient starts at 5% B for 0.2 minutes, ramps up to 95% B over 1.3 minutes, is held at 95% B for 0.5 minutes, ramps down to 5% B over 0.1 minutes, and is held at 5% B for 0.5 minutes before the next injection. The mass spectrometer was operated in the positive ionization mode using multiple reaction monitoring (MRM), at specific precursor ion → product ion transition, m/z 380.90→275.90 (CE=50) for PF-04989216 and m/z 472.3→432.6 (CE=30) for IS. The standard calibration curve was constructed using weighted (1/x^2) linear regression. Analyst 1.5.2 software (Applied Biosystems) was used for data acquisition and chromatographic peak integration.

**Animal studies**

Four- to six-week-old SCID female mice were obtained from the Jackson Laboratory and maintained in pressurized ventilated caging at the Pfizer La Jolla animal facility. All studies were approved by the Pfizer Institutional Animal Care and Use Committee. Tumors were established by injecting cells (10x10⁶) suspended 1:1 (v/v) with reconstituted basement membrane (Matrigel, BD Biosciences). For tumor growth inhibition studies, mice with established tumors of ~200-300 mm³ were selected, randomized, and then treated with PF-4989216 using the indicated dose and regimen. Tumor dimensions were measured with vernier calipers and tumor volumes were
calculated using the formula $[\pi/6 \times \text{larger diameter} \times (\text{smaller diameter})^2]$. Tumor growth inhibition percentage (TGI %) was calculated as $[100 \times (1-\Delta T/\Delta C)]$.

**Results**

**PF-4989216 differentially inhibited cell viability in small cell lung cancer cells with a PIK3CA mutation.**

To identify potential targets in SCLCs, mutation frequency was calculated using tumor tissue samples from the Sanger Institute Catalogue of Somatic Mutations in Cancer (COSMIC v67) (26) and SCLC cell line mutation frequency was calculated from the Broad Institute and Novartis Cancer Cell Line Encyclopedia (CCLE) collaboration (CCLE May 7, 2012 hybrid capture release with common SNPs and neutral variants removed) (27). Frequencies excluding intronic, UTR, and synonymous mutations in SCLCs are listed in Table I. The most frequently mutated genes in SCLCs are TP53 and RB1. PIK3CA and PTEN mutations in the PI3K signaling pathway together account for the next highest mutation frequency in cell lines and tumor tissues as shown in Table I. Therefore, we wanted to investigate whether a selective PI3K inhibitor would have anti-tumor activity in small cell lung cancer cell lines and models to provide a preclinical rationale of specifically targeting PI3K in small cell lung cancer patients. A panel of small cell lung cancer cell lines with various genetic mutation status of PIK3CA and PTEN were collected as shown in Table II. PIK3CA mutation in the selected lines are either in the adaptor binding domain (ABD) or in the kinase domain and both result in constitutively active PI3Ka as illustrated in Supplementary Fig. S1A (28, 29). PF-4989216 (Supplementary Fig. S1B) was previously identified and described as a potent inhibitor of
pan-PI3K isoforms and demonstrated excellent selectivity when screened against more than 100 kinases and 50 non-kinases (20). The selectivity profile of PF-4989216 is provided in Supplementary Table SI and Supplementary Fig. S1C. We first determined the ability of PF-4989216 to inhibit cell proliferation in a panel of small cell lung cancer cell lines; the results clearly and not surprisingly indicated that PF-4989216 is significantly more potent in SCLCs with \textit{PIK3CA} mutations than wild-type lines, including cells with \textit{PIK3CA} copy number gain, providing evidence that SCLCs harboring \textit{PIK3CA} mutations are more sensitive to inhibition by PF-4989216 (Fig. 1A). Interestingly, Lu134B cells, which harbor a \textit{PIK3CA} mutation but also have no PTEN protein due to an exon deletion, are not sensitive to a PI3K selective inhibitor in comparison with NCI-H69, NCI-H1048, and Lu99A which also harbor a \textit{PIK3CA} mutation. Similarly, Lu134A and NCI-H1436 cells, which have PTEN protein loss due to a mutation but maintain wild type \textit{PIK3CA}, were also not sensitive to PF-4989216. Moreover, introduction of PTEN protein to Lu134B cells was able to increase the potency of PF-4989216 in cell viability assays and increase the level of cleaved PARP (Supplementary Fig. S2), providing further evidence that functional PTEN is the critical difference between Lu134B and NCI-H69 or NCI-H1048 in mediating the cellular potency of PF-4989216. These results indicate that small cell lung cancer cells with a \textit{PIK3CA} mutation are sensitive to inhibition by a selective PI3K inhibitor, while SCLCs with \textit{PTEN} loss are not responsive to an inhibitor that selectively targets PI3K. Therefore, the cell proliferation data suggest that there may be alternative oncogenic activation pathway(s) independent of the PI3K/AKT pathway in SCLCs with \textit{PTEN} loss whereby inhibition of PI3K activity is not sufficient to inhibit cell proliferation in these cells.
PI3K inhibition blocked cell cycle progression and reduced cell transformation in SCLCs

Next, cell cycle progression and cell transformation assays were performed. NCI-H69, NCI-H1048, Lu134B, and Lu134A cells were treated with PF-4989216 at a variety of concentrations for 24, 48, and 72 hours after which cells were harvested and cell cycle progression was analyzed by FACS. The representative results of the 72 hour treatments are shown in Fig. 1B, and detailed data are included in Supplementary Fig. S3 and Supplementary Table SII. In both NCI-H69 and NCI-H1048 cells, PF-4989216 treatment decreased the cell population of S, G1, and G2/M phases and increased the sub-G1 cell population in a dose-dependent manner (Fig. 1B). Addition of a pan-caspase inhibitor did not significantly change the sub-G1 population (Supplementary Table SIII), suggesting that PI3K inhibition blocked cell cycle progression and induced caspase-independent apoptosis in these SCLC lines. In contrast, PF-4989216 did not have a significant effect on cell cycle progression in either Lu134B or Lu134A cell lines. High concentrations (>1000 nM) of PF-4989216 moderately decreased the G2/M cell population in treated Lu134B cells but did not enhance the sub-G1 population (Fig. 1B). There was no significant observation of cell cycle profile changes in the Lu134A cells treated with any concentration of PF-4989216 (Fig. 1B). Therefore, the cell cycle results indicate that PF-4989216 blocked cell cycle progression and induced an increased sub-G1 cell population in both NCI-H69 and NCI-H1048 cell lines which harbor PI3CA mutation but not in Lu134B and Lu134A cell lines which exhibit functional loss of PTEN.

Next we investigated whether inhibition of PI3K activity by PF-4989216 was able to block cellular transformation of SCLCs. NCI-H69, NCI-H1048, Lu134B and Lu134A
cells were used to perform anchorage independent growth assays by growing cells in soft agar with a variety of concentrations of PF-4989216. All four cell lines were able to form colonies while growing in soft agar, although the rate and efficiency of colony formation were different among the four cell lines. The colony numbers determined after weekly PF-4989216 treatment are shown in Fig. 1C, representative images are detailed in Supplementary Fig. S4, and results indicate that PF-4989216 is able to inhibit colony formation in NCI-H69 and NCI-H1048 cells but not in Lu134B and Lu134A cells, similar to the results obtained from cell proliferation assays. Interestingly, the inhibitory activity of PF-4989216 seems more potent in the soft agar assay than in the cell proliferation assay, suggesting that these PIK3CA mutant cells are highly reliant on PI3K activity to maintain the ability to undergo cell transformation and that inhibition of PI3K activity can very effectively abolish this phenotype.

**PF-4989216 inhibited PI3K downstream signaling in small cell lung cancer cells.**

We measured the IC$_{50}$ values of PF-4989216 inhibition of AKT phosphorylation at S473 by ELISA and results show that PF-4989216 is able to potently inhibit AKT phosphorylation in a variety of the SCLC lines tested (Supplementary Table SIV). PF-4989216 was also added at various concentrations for the indicated times in NCI-H69, NCI-H1048, Lu134B, and Lu134A cells to determine whether the phosphorylation of PI3K downstream molecules was inhibited in these cell lines as shown in Fig. 2. Basal levels of AKT phosphorylation are higher in SCLC lines harboring PIK3CA mutation or PTEN loss in comparison to the wild type cell lines (Supplementary Fig. S5). Phosphorylation of AKT at both S473 and T308 and downstream phosphorylation of S6RP were inhibited by PF-4989216 in a dose dependent manner, and inhibition was
sustained up to 24 hours in all four cell lines tested. Although PF-4989216 did not inhibit cell viability in Lu134B and Lu134A cells (both cell lines harbor PTEN loss), PF-4989216 was able to inhibit the phosphorylation of AKT and S6RP (Fig. 2C and 2D). These results suggest that inhibition of cell proliferation by PF-4989216 in PI3KCA mutant SCLC cells is likely due to inhibition in PI3K signaling, however the inhibition of PI3K activity in PTEN loss SCLC cells is not sufficient to block cell proliferation.

**PF-4989216 induced BIM-mediated apoptosis in SCLCs with PIK3CA mutation**

Since we observed sub-G1 changes in NCI-H69 and NCI-H1048 cells treated with PF-4989216, we next performed an extensive profiling of apoptosis markers after PF-4989216 treatment in these four cell lines. Similar to the cell cycle profiling results, PF-4989216 induced a considerable amount of cleaved PARP in NCI-H69 and NCI-H1048 cells (Fig. 3A and 3B), but the high concentration of PF-4989216 treatment only moderately induced cleaved PARP in Lu134B cells (Fig. 3C) and not at all in Lu134A cells (Fig. 3D). Next Bcl-2 family apoptosis markers were evaluated, and we found that BIM was the most noteworthy marker upon PF-4989216 treatments in NCI-H69 and NCI-H1048 cells in comparison to Lu134B and Lu134A cells. PF-4989216 treatment in NCI-H69 and NCI-H1048 cells induced increased levels of BIM-L and BIM-S; the latter is the shortest splicing form of the BIM protein and is more cytotoxic than the BIM-EL and BIM-L forms (30). BIM-L and BIM-S are pre-existing in non-treated NCI-H69 and NCI-H1048 cells and after PF-4989216 treatment, the levels of both BIM-L and BIM-S were induced to a much higher level (Fig. 3A and 3B). In contrast, while BIM-EL was detected by western analysis in Lu134B and Lu134A cells, only minimal levels of BIM-L and BIM-S were detected at baseline. Treatments with PF-4989216 did not induce any
further increase of either BIM-L or BIM-S in these lines (Fig. 3C and 3D). These results clearly suggest that PF-4989216 was able to induce the more apoptotic BIM-S in NCI-H69 and NCI-H1048 cells which harbor PIK3CA mutation but not in Lu134B and Lu134A cells which harbor PTEN loss. A variety of cytotoxic agents were also used to investigate the production of BIM isoforms in these four cell lines, and results indicates that both BIM-L and BIM-S can be induced by various cytotoxic agents in these four cell lines, although BIM-S induction is relatively more limited to NCI-H1048 and NCI-H69, suggesting that PTEN loss could protect cells from BIM-S mediated apoptosis (Supplementary Fig. S6). These results also imply that a differential mechanism between PIK3CA mutant versus PTEN loss SCLCLs may be driven by BIM-mediated apoptosis. Furthermore, we observed a decrease in pBAD by PF-4989216 in NCI-H1048 cells but the decreased level of pBAD was not very pronounced in the other three cell lines, which may suggest that the pBAD decrease in NCI-H1048 cells may be cell line specific and may help explain why these cells are the most sensitive to PF-4989216 treatment. As autophagy has been implicated in SCLCs via the PI3K/mTOR pathway (31, 32), we also examined autophagy markers in cells treated with PF-4989216. We observed the conversion of LC3-I to LC3-II in NCI-H1048 and NCI-H69 but not in Lu134B or Lu134A cells (Fig. 3), suggesting that autophagy induction may be also playing a role in anti-tumor activity by PI3K inhibition in these SCLC lines.

**PF-4989216 inhibited tumor growth of SCLCs in mice**

Since PF-4989216 was able to block cellular transformation of NCI-H69 and NCI-H1048 cells, we next performed studies to determine the *in vivo* activity of PF-4989216 in these two SCLC models. First, PF-4989216 was administered orally once to
tumor bearing mice and the unbound plasma concentration of PF-4989216 was measured at various time points (Fig. 4A). PF-4989216 exhibited good exposure in mice after oral administration and the unbound drug concentration increased with dose, with the ability to maintain approximately 700 nM, 1900 nM and 2100 nM at the seven hour time point at dosages of 50 mg/Kg, 150 mg/Kg, and 350 mg/Kg, respectively. The unbound plasma drug concentration of PF-4989216 was above the cellular IC$_{50}$ values of pAKT, cell viability and transformation assays such that the relationship of pharmacokinetics and pharmadynmaics (PK/PD) and further tumor growth inhibition were determined next.

PI3K signaling is required for insulin-induced increases in glucose transport via the insulin receptor activation of PI3K and AKT to regulate GLUT4 trafficking (33, 34). Inhibitors of the PI3K/mTOR/AKT pathway have been shown to elevate serum glucose and insulin levels (35, 36); therefore, the serum glucose and insulin levels in mice treated with PF-4989216 were also measured (Fig. 4B). At 350 mg/Kg, PF-4989216 only induced moderate increases of serum insulin or glucose and the levels of both insulin and glucose returned to basal levels after the four hour time point. Therefore, these results suggest that PF-4989216 may have a better therapeutic window in terms of a potential insulin resistance mechanism in comparison to PI3K/mTOR dual inhibitors which do induce higher serum insulin (36).

SCID mice bearing NCI-H69 or NCI-H1048 xenograft tumors were dosed once with PF-4989216 (350 mg/Kg) and tumors were collected and processed to perform western blot analysis to determine the inhibitory activity of PF-4989216 at various time points against phosphorylation of AKT and the downstream molecule S6RP (Fig. 5A and 6A). Similarly, SCID mice bearing NCI-H69 or NCI-H1048 tumors were also dosed
with PF-4989216 at 350 mg/Kg, 150 mg/Kg, and 50 mg/Kg, and tumors were collected at four (NCI-H1048) or two hours (NCI-H69) post-dose to perform western blot analysis (Fig. 5B and 6B). Results from these studies indicate that oral dosing of PF-4989216 is able to inhibit AKT phosphorylation and S6RP downstream signaling, and further induce apoptosis as evidenced by induction of cleaved PARP in a dose- and time-dependent manner. In both the NCI-H69 and NCI-H1048 models, the phosphorylation levels of AKT and S6RP returned to basal levels when the plasma drug concentration of PF-4989216 was low at 24 hours, indicating a good correlation between the observed pharmacodynamic changes and the measured pharmacokinetic drug concentration.

Since PF-4989216 inhibited phosphorylation of AKT and S6RP and induced cleaved PARP in both NCI-H69 and NCI-H1048 xenograft tumors, we next determined whether PF-4989216 could induce in vivo anti-tumor activity. NCI-H69 and NCI-H1048 cells were subcutaneously implanted to SCID mice for tumor growth inhibition studies. Daily oral dosing of PF-4989216 started when the tumor size reached an average of 200-300 mm$^3$, and tumor volume was recorded and tumor growth inhibition was calculated as described. Body weight and health observations were recorded daily and indicated PF-4989216 was well tolerated in mice with minimal bodyweight loss (Supplementary Fig. S7). In the NCI-H69 model, dose-dependent tumor growth inhibition (TGI) was observed with PF-4989216, and at 350 mg/Kg induced 99.9 % TGI (Fig. 5C). The mice in the 350mg/Kg group received long term dosing in order to evaluate whether tumors would become resistant. PF-4989216 (350 mg/Kg) was able to maintain tumor stasis for more than 2 months before tumor volumes began to increase, presumably because the xenografts became resistant to PF-4989216 treatment (Fig. 5D).
model, dose-dependent TGI was also observed with PF-4989216, and PF-4989216 induced tumor regression at 350 mg/kg (Fig. 6C). The NCI-H1048 model is also more sensitive to PF-4989216 in vitro as seen in cellular assays, possibly due to the double mutations in PIK3CA that might lead these cells to be more addicted to PI3K activation for cell growth and transformation. Two PTEN loss SCLC models, Lu134A and NCI-H1436, were also tested in TGI studies with PF-4989216, and not surprisingly, PF-4989216 did not induce significant tumor growth inhibition in either models (Supplementary Fig. S8). The in vivo results indicate a good correlation between in vitro and in vivo efficacy, and further confirm that PF-4989216 is an effective drug candidate capable of inducing anti-tumor activity in mice bearing human SCLC tumors with PIK3CA mutation.

Discussion

Novel targeted therapies are urgently demanded in small cell lung cancer (SCLC) patients due to the very unsatisfactory survival rate in this patient population. Mutations in PIK3CA and PTEN occur frequently in the SCLC patient population (Table I), which suggests that targeting the PI3K signaling pathway may provide a mechanism-based targeted therapy opportunity for SCLC patients. We have developed a PI3K selective inhibitor, PF-4989216, with potent and selective inhibition against PI3K kinase activity (20). In this study, we characterized the in vitro and in vivo anti-tumor activity of PF-4989216 in SCLC models, and our results clearly demonstrate that PF-4989216 inhibited PI3K signaling and cell cycle progression, induced cell apoptosis and subsequently
inhibited cell proliferation and transformation and in vivo tumor growth in SCLC models harboring a PIK3CA mutation.

PIK3CA mutation and PTEN loss frequently occur in a variety of human cancers through upregulation of the PI3K/AKT signaling pathway and cell proliferation and transformation (5-8). In this study, however, we observed there may be different tumor progression mechanisms in SCLC harboring PIK3CA mutation versus PTEN loss. The PI3K selective inhibitor, PF-4989216, inhibited PI3K downstream signaling (e.g. pAKT and pS6RP) in all four SCLC lines tested, however, different phenotypic changes were observed in cells harboring PIK3CA mutation versus PTEN loss. PF-4989216 inhibited pAKT and pS6RP, and resulted in cell cycle block, apoptosis, and the subsequent inhibition of cell proliferation and cell transformation in NCI-H69 and NCI-H1048 cells, both which harbor a PIK3CA mutation. Furthermore, PF-4989216 was characterized in animal studies and demonstrated a well correlated PK/PD relationship between inhibition of PI3K signaling and induced tumor stasis (99.9% TGI) or tumor regression in the NCI-69 or NCI-H1048 in vivo models, respectively. These results strongly suggest that PF-4989216 is an effective PI3K inhibitor able to induce in vitro and in vivo antitumor activity in SCLC with PIK3CA mutation. These results are also in line with a recent report that targeting the p110α isoform impaired tumor progression in SCLC (31).

However, in SCLC with PTEN loss, such as the Lu134B and Lu134A cell lines, inhibition of the PI3K signaling pathway (e.g. pAKT and pS6RP) is not sufficient to inhibit either cell viability or cell transformation. Cell cycle profiles of PTEN loss lines are not affected by PF-4989216 treatments, which correlate with the results of cell viability experiments. We also observed a difference in the changes of cell apoptosis
markers between PIK3CA mutated and PTEN loss lines. PF-4989216 induced significant increases in cleaved PARP in NCI-H69 and NCI-H1048 cells which harbor PIK3CA mutation but not in Lu134B and Lu134A cells which harbor PTEN loss. These results explain why a reduced sub-G1 population in cell cycle progression assays and limited sensitivity in the cell viability assays were observed in Lu134B and Lu134A after treatment with PF-4989216. Moreover, we observed a noteworthy difference in BIM-mediated apoptosis between PIK3CA mutant and PTEN loss cell lines. In the NCI-H69 and NCI-H1048 cells, all three isoforms of the BIM family proteins were expressed (BIM-EL, BIM-L, and BIM-S), and BIM-L and BIM-S were significantly increased after PF-4989216 treatments suggesting that the apoptosis mechanism was driven through BIM. However, in Lu134B and Lu134A cells, only BIM-EL could be detected, and there was no change or increase in the detectable BIM-L and BIM-S forms after PF-4989216 treatments. Therefore, the data clearly suggest that PF-4989216 induced cell apoptosis occurs through the BIM-mediated pathway in PIK3CA mutant cell lines (NCI-H69 and NCI-H1048) but not in SCLC cell lines with functional PTEN loss (Lu134B and Lu134A). A number of evidences have shown that PTEN also operates through a PI3K/AKT independent pathway to function as a tumor suppressor (11). In addition, a BIM-mediated deregulated apoptosis mechanism that confers tumor resistance has been reported in non-small cell lung cancers (37, 38) and suppressed expression of the BIM-L and BIM-S forms have been implicated in melanoma cells with PTEN loss that are resistant to a B-Raf inhibitor (39). Results from our study similarly suggest that SCLC lines with PTEN loss may confer resistance to a PI3K inhibitor by decreasing the expression of the more cytotoxic BIM-S form in order to block cell apoptosis. Although
the detailed molecular mechanism still needs to be investigated further to fully understand the differences between PIK3CA mutation versus PTEN loss in the process of tumorigenesis in SCLCs, our study provides the preclinical evidence suggesting that it is preferred to select SCLC patients with PIK3CA mutation for the clinical development of PF-4989216 or other PI3K inhibitors.

Most of the inhibitors targeting the PI3K pathway have inhibitory activity against both PI3Ks and mTOR kinases, and several of these inhibitors are now in the clinical trial phase (19, 36, 40). However, none of these agents has a trial in a SCLC PI3K mutant selected patient population. Since mTOR kinases control multiple tumor-related pathways through AKT dependent and AKT independent mechanisms (16-19), selective PI3K inhibitors without the additional inhibition of mTOR kinase activities may avoid the extra toxicity that would come through the disruption of mTOR function in normal cells and may also have less impact on glucose metabolism in normal cells. Therefore, in this study, we measured the serum glucose and insulin levels in mice treated with PF-4989216. PF-4989216, at a concentration sufficient to inhibit AKT phosphorylation and induce tumor growth inhibition in NCI-H69 and NCI-H1048 xenograft models, did not induce high level increases in serum glucose and insulin in comparison to the levels induced by dual PI3K/mTOR inhibitors (36), suggesting that a selective PI3K inhibitor such as PF-4989216 may have a greater safety window than PI3K/mTOR dual inhibitors in the regulation of glucose metabolism. However, several side effects including hyperglycemia have been reported by selective PI3K inhibitors in clinical trials (41, 42). It is therefore important to evaluate whether PF-4989216 would have a better clinical safety profile in the selected SCLC PIK3CA mutant patient population.
In conclusion, we have developed a selective PI3K inhibitor, PF-4989216, which potently inhibits PI3K signaling as well as *in vitro* and *in vivo* tumor progression in SCLC lines with a *PIK3CA* mutation. Our results also reveal there may be different molecular mechanisms that are likely modulated by BIM-mediated apoptosis between *PIK3CA* mutation and *PTEN* loss that regulate tumor progression in SCLCs. Furthermore, our preclinical data provide insight for clinical development of PF-4989216 in SCLC patients that harbor a *PIK3CA* mutation.

**Acknowledgements**

The authors would like to thank Elizabeth Epps and Stella Chen for their help with cell culture and western blot analysis, and Yi-Zhong Zhang for assistance with PK analysis.

**Conflict of interests**

MW, SMB, PPM, JJZ, MZ, QZ, TS, and MJY are current full time employees of Pfizer, Inc. KKCL is a current employee of Eli Lilly, HE is a current employee of Regulus, and CL is a current employee of Genentech.
Reference:


Figure legends:

Figure 1: Inhibition of the PI3K pathway leads to differential inhibition of cell viability, cell cycle progression and cell transformation in SCLCs with PIK3CA mutation versus PTEN loss. (A) CTG assay results from a variety of SCLC lines with wild type (W), PIK3CA copy number gain (C), PIK3CA mutation (M) or PTEN deletion (D). IC50 values were obtained by incubating cells with a three-fold titration of PF-4989216 (starting at 10 μM) for 72 hours. Data were based on results from at least three repeated experiments. (B) PIK3CA mutant SCLCs (NCI-H69 and NCI-H1048) and PTEN deletion SCLCs (Lu134B and Lu134A) were treated with various concentrations of PF-4989216, harvested for PI staining, and analyzed by flow cytometry to determine cell cycle profile. Representative graphs from the 72 hour treatment are shown. (C) Cells were grown in soft agar with various concentrations of PF-4989216, and the numbers of colonies formed were measured to determine anchorage-independent growth.

Figure 2: PF-4989216 inhibited PI3K downstream signaling in small cell lung cancer cells. SCLC cell lines NCI-H69 (A), NCI-H1048 (B), Lu134B (C), and Lu134A (D) were treated with PF-4989216 (10, 100, 1000, and 10000 nM) for 2, 6, or 24 hours. Cell lysates were prepared and subject to SDS-PAGE, and western analysis was performed with the indicated antibodies. GAPDH was included as a protein loading control.

Figure 3: PF-4989216 induced BIM-mediated apoptosis in SCLCs with PIK3CA mutation but not PTEN loss. PIK3CA mutant SCLC lines, NCI-H69 (A) and NCI-
H1048 (B), and PTEN loss SCLC lines, Lu134B (C) and Lu134A (D), were treated with DMSO, positive control (*PC) which is either 1 μM of staurosporine as an apoptosis positive control, or 1 μM STF-62247 as an autophagy inducer, and 1 μM or 10 μM of PF-4989216 for 24, 48, or 72 hours. Cell lysates were subject to SDS-PAGE. Antibodies against apoptosis markers (PARP, cleaved PARP, phospho-BAD, BAD, BIM), or autophagy marker LC3, and GAPDH (loading control) were used to perform the western analysis. Arrows indicate cleaved PARP bands in western analysis as the antibody recognized total and cleaved PARP. The conversion of LC3-I to LC3-II is also specified by arrows.

**Figure 4: PF-4989216 exhibits plasma exposure by oral dosing and does not induce high levels of serum glucose or insulin in mice.** PF-4989216 was orally administrated to tumor-bearing SCID mice at 50 mg/Kg, 150 mg/Kg or 350 mg/Kg. The unbound concentration of PF-4989216 in mouse plasma was analyzed and graphed against the indicated time points post-treatment (A). Serum insulin or glucose levels at various time points were analyzed in mice treated with PF-4989216 at 350 mg/Kg or 150 mg/Kg (B).

**Figure 5: PF-4989216 inhibited PI3K phosphorylation signaling and induced anti-tumor activity in NCI-H69 xenograft models.** (A) NCI-H69 tumor bearing mice were treated with one oral dose of PF-4989216 (350 mg/Kg), and tumors were harvested at 2, 6, and 24 hours post-dose. (B) NCI-H69 tumor bearing mice were treated with PF-4989216 at 350, 150, or 50 mg/Kg, and tumors were harvested at the 2 hour post-dose time point. (C) NCI-H69 cells were subcutaneously implanted in mice; when tumors
reached an average size of 200-300mm$^3$, mice were randomized and treated once daily PO with PF-4989216 at 350, 150, and 50 mg/Kg. (D) Mice from the 350 mg/Kg group from C were subject to long term treatment with PF-4989216 (350 mg/Kg) until day 90. Tumor volumes were measured and recorded, and tumor growth inhibition percentages were calculated and are presented in the graphs.

**Figure 6: PF-4989216 inhibited PI3K phosphorylation signaling and induced anti-tumor activity in NCI-H1048 xenograft models.** (A) NCI-H1048 tumor bearing mice were treated with one oral dose of PF-4989216 (350 mg/Kg), and tumors were harvested at 2, 6, and 24 hours post-dose. (B) NCI-H1048 tumor bearing mice were treated with PF-4989216 at 350, 150, or 50 mg/Kg, and tumors were harvested at the 4 hour post-dose time point. (C) NCI-H1048 cells were subcutaneously implanted in mice; when tumors reached an average size of 200-300mm$^3$, mice were randomized and treated once daily PO with PF-4989216 at 350, 150, and 50 mg/Kg. Tumor volumes were measured and recorded, and tumor growth inhibition percentages were calculated and are presented in the graphs.

**Table I: Mutation frequencies of SCLC cell lines and tumor tissues.** Mutation frequencies for cell lines were calculated from the Broad Institute and Novartis Cancer Cell Line Encyclopedia data set (CCLE, May 7, 2012 hybrid capture release with common SNPs and neutral variants removed), excluding intronic, UTR, and synonymous mutations. Tissue mutation frequencies were calculated as reported by Sanger Institute.
Catalogue of Somatic Mutations in Cancer (COSMIC v67) excluding synonymous mutations.

**Table II: Genetic background of selected SCLC lines.** Mutation information and gene copy number gain were derived from CCLE.
Table I: Mutation frequencies of SCLC cell lines and tumor tissues

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Figure 1

A. Cell viability inhibition IC50 (nM)

B. H69 H1048 Lu134B Lu134A

C. NCI-H69 NCI-H1048

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Figure 3

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I

II

Author Manuscript Published OnlineFirst on November 15, 2013; DOI: 10.1158/1078-0432.CCR-13-1663
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
Figure 4

A.

Free Plasma Concentration (nM)

Time (hr)

PO
n=4/time point

50 mg/Kg
150 mg/Kg
350 mg/Kg

B.

Insulin
Glucose

PF-4989216 at 350 mg/Kg

PF-4989216 at 150 mg/Kg
Figure 5

A. NCI-H69

- Vehicle
- 2 hrs
- 6 hrs
- 24 hrs

- p-AKT (S473)
- p-AKT (T308)
- Total AKT
- p-S6RP
- Total S6RP
- cleaved PARP
- GAPDH

B. NCI-H69

- Vehicle
- 350 mg/Kg
- 150 mg/Kg
- 50 mg/Kg

- p-AKT (S473)
- p-AKT (T308)
- Total AKT
- p-S6RP
- Total S6RP
- cleaved PARP
- GAPDH

C. NCI-H69

- Vehicle
- 350 mg/Kg
- 150 mg/Kg
- 50 mg/Kg

- Study Day
- Tumor volume (mm$^3$)

D. NCI-H69

- Vehicle
- 350 mg/Kg
- 150 mg/Kg
- 50 mg/Kg

- Study Day
- Tumor volume (mm$^3$)

n=7/group PO, QD

99.9 % Inh

28.1 % Inh

64.6 % Inh
Figure 6

A. NCI-H1048

<table>
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<tr>
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<th>Vehicle</th>
<th>1 hrs</th>
<th>4 hrs</th>
<th>7 hrs</th>
<th>24 hrs</th>
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<td>Total AKT</td>
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<td>p-S6RP</td>
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<td>Total S6RP</td>
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<td>cleaved PARP</td>
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<td>GAPDH</td>
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B. NCI-H1048

<table>
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<th></th>
<th>Vehicle</th>
<th>350 mg/Kg</th>
<th>150 mg/Kg</th>
<th>50 mg/Kg</th>
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<tr>
<td>GAPDH</td>
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</tbody>
</table>

C. NCI-H1048

- Vehicle
- 350 mg/Kg
- 150 mg/Kg
- 50 mg/Kg

Tumor volume (mm³)

- Vehicle: 60.6 %
- 350 mg/Kg: 92.9 %
- 150 mg/Kg: R 47.2 %

Study Day

n=8/group
PO, QD
Targeting Small Cell Lung Cancer Harboring *PIK3CA* Mutation with a Selective Oral PI3K Inhibitor PF-4989216

Marlena Walls, Sangita M Baxi, Pramod P. Mehta, et al.

*Clin Cancer Res* Published OnlineFirst November 15, 2013.

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