PF-04691502, a potent and selective oral inhibitor of PI3K and mTOR kinases with antitumor activity

Jing Yuan¹, Pramod P. Mehta¹, Min-Jean Yin¹, Shaoxian Sun¹, Aihua Zou¹, Jeffrey Chen¹, Kristina Rafidi¹, Zheng Feng¹, Jeffrey Nickel¹, Jon Engebretsen¹, Jill Hallin¹, Alessandra Blasina¹, Eric Zhang², Leslie Nguyen², Minghao Sun³, Peter K. Vogt³, Aileen McHarg⁴, Hengmiao Cheng⁵, James G. Christensen¹, Julie L.C. Kan¹, Shubha Bagrodia¹

¹Oncology Research Unit, ²Pharmacokinetics, Dynamics and Metabolism, ⁴Drug Safety Research and Development and ⁵Cancer Chemistry, Pfizer Worldwide Research and Development, La Jolla Laboratories, San Diego, California.

³Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, California.

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Corresponding Author: Shubha Bagrodia, Phone: 858-526-4828; Fax: 877-481-3086. E-mail: shubha.bagrodia@pfizer.com

Disclosure of Potential conflicts of Interest

Except for MS and PV all other authors are either former or current employees of Pfizer.
Abstract

Deregulation of the PI3K signaling pathway such as by PTEN loss or PIK3CA mutation occurs frequently in human cancer and contributes to resistance to anti-tumor therapies. Inhibition of key signaling proteins in the pathway therefore represents a valuable targeting strategy for diverse cancers. PF-04691502 is an ATP-competitive PI3K/mTOR dual inhibitor, which potently inhibited recombinant class I PI3K and mTOR in biochemical assays and suppressed transformation of avian fibroblasts mediated by wild type PI3K γ, δ or mutant PI3Kα. In PIK3CA-mutant and PTEN-deleted cancer cell lines, PF-04691502 reduced phosphorylation of AKT T308 and AKT S473 (IC₅₀ of 7.5-47 nM and 3.8-20 nM respectively) and inhibited cell proliferation (IC₅₀ of 179-313 nM). PF-04691502 inhibited mTORC1 activity in cells as measured by PI3K-independent nutrient stimulated assay, with an IC₅₀ of 32 nM and inhibited the activation of PI3K and mTOR downstream effectors including AKT, FKHRL1, PRAS40, p70S6K, 4EBP1 and S6RP. Short-term exposure to PF-04691502 predominantly inhibited PI3K, while mTOR inhibition persisted for 24-48 hours. PF-04691502 induced cell cycle G1 arrest, concomitant with upregulation of p27 Kip1 and reduction of Rb. Antitumor activity was observed in U87 (PTEN null), SKOV3 (PIK3CA mutation) and gefitinib- and erlotinib-resistant NSCLC xenografts. In summary, PF-04691502 is a potent dual PI3K/mTOR inhibitor with broad antitumor activity. PF-04691502 has entered phase I clinical trials.
Introduction

The PI3K signaling pathway plays a central role in regulating cell growth, proliferation, survival, angiogenesis, metabolism and motility (1, 2). Inappropriate PI3K signaling, due to genetic aberration, is one of the most frequent occurrences in human cancer (1, 3).

Class I, II and III PI3Ks are lipid kinases, while class IV are Ser/Thr protein kinases including mTOR, DNA-PK, ATM and ATR (4, 5). Following their activation by RTKs, class IA PI3Ks phosphorylate phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-trisphosphate (PIP3), which recruits AKT and PDK1 to the plasma membrane (6). Subsequently, Akt is phosphorylated by PDK1 at Thr308 and by PDK2 (mTORC2 or DNA-PK) at Ser473 (7, 8). Once activated, AKT phosphorylates many target proteins regulating a wide range of cellular functions (1, 2, 9, 10).

Deregulation of the PI3K pathway by gene mutation has been found in ~30% of all human cancers (10). The most frequent genetic alterations in tumors are PTEN loss and PIK3CA mutation or amplification. Several AKT inhibitors are currently in clinical trials (11), but these may be not effective in AKT-independent, PIK3CA-mutant cancers which are dependent on the PDK1 substrate SGK3 (12). Rapamycin analogs inhibit mTORC1 and have been approved for treating renal cell carcinoma and mantle cell lymphoma, but have shown limited efficacy in clinical trials of other solid tumors, with only a small fraction of patients responding (13). Moreover, inhibition of mTORC1 can remove negative feedback from p70S6K-IRS and activate PI3K/AKT and ERK/MAPK signaling. Furthermore, rapamycin analogs cannot inhibit mTORC2, an AKT upstream kinase (3, 11, 13), although prolonged exposure inhibits mTORC2 assembly in some cell types (14). ATP-competitive mTOR kinase inhibitors may therefore have greater efficacy than rapamycin analogs by inhibiting both mTORC1 and mTORC2 (15, 16).
Dual PI3K/mTOR inhibitors may most effectively block the PI3K pathway, overcome feedback loops (3) and block PI3K-independent mTOR activation. A number of PI3K-selective and PI3K/mTOR-dual inhibitors have entered clinical trials (3, 11). PF-04691502, a potent ATP competitive PI3K/mTOR dual inhibitor, was discovered through high-throughput screening and structure-based drug design (17). Here we present its biochemical and cellular profiles, antitumor efficacy and PK/PD correlations.

**Materials and Methods**

**Compounds**

PF-04691502 [2-amino-8-[trans-4-(2-hydroxyethoxy)cyclohexyl]-6-(6-methoxypyridin-3-yl)-4-methylpyrido[2,3-d]pyrimidin-7(8H)-one] was synthesized at Pfizer Global Research and Development, La Jolla, CA. LY294002 was from Cell Signaling Technology. Rapamycin was from LC Laboratories.

**Cell lines**

293-MSR cells were from Invitrogen and cultured in DMEM with 10% fetal bovine serum (FBS) and 0.5 mg/ml G418. U87MG glioblastoma, BT20 breast, SKOV3 ovarian and non-small-cell lung carcinoma (NSCLC) cell lines A549, NCI-H460, NCI-H1650 and NCI-H1975 were from the American Type Culture Collection and grown in recommended media. For *in vivo* studies, SKOV3 cell line passaged through mice and cultured in McCoy’s 5A media with 10% FBS. All cell lines were tested by the University of Missouri Research Animal Diagnostic Laboratory for known species of murine viruses and mycoplasma contamination.

**Biochemical assays**
The biochemical protein kinase assays for class I PI3K and mTOR were assessed as previously described (17).

The fluorescence polarization assay for ATP competitive inhibition was performed as follows: mPI3Kα dilution solution (90 nM) was prepared in fresh assay buffer (50 mM Hepes pH 7.4, 150 mM NaCl, 5 mM DTT, 0.05% CHAPS) and kept on ice. The enzyme reaction contained 0.5 nM mouse PI3Kα (p110α/p85α complex purified from insect cells), 30 µM PIP2 (Avanti Polar Lipids), PF-04691502 (0, 1, 4 and 8 nM), 5 mM MgCl2, and 2-fold serial dilutions of ATP (0 – 800 µM). Final DMSO was 2.5%. The reaction was initiated by the addition of ATP and terminated after 30 minutes with 10 mM EDTA. In a detection plate, 15 ul of detector/probe mixture containing 480 nM GST-Grp1PH domain (University of Dundee) and 12 nM TAMRA tagged fluorescent PIP3 (Echelon Biosciences Inc) in assay buffer was mixed with 15 ul of kinase reaction mixture. The plate was shaken for 3 minutes, and incubated for 35-40 minutes before reading on an LJL Analyst HT (Molecular Devices).

Transformation assays

Transformation assays with infectious retroviral vectors were performed as described (18, 19). Chicken embryo fibroblasts (CEF) were transfected with avian retrovirus RCAS constructs using the DMSO/Polybrene method and overlaid with nutrient agar every other day for 2-3 weeks until foci were observed. The plates were stained with crystal violet, and foci were counted. To examine inhibition of focus formation by PF-04691502, different concentrations were added to the nutrient agar in every overlay.

Cell proliferation and apoptosis assays
BT20, U87MG and SKOV3 cells were plated at 3000 cell/well in 96-well culture plates in growth medium with 10% FBS. Cells were incubated overnight and treated with DMSO (0.1% final) or serial diluted compound for 3 days. Resazurin (Sigma-Aldrich) was added to 0.1 mg/ml. Plates were incubated at 37°C in 5% CO₂ for 3 hours. Fluorescence signals were read as emission at 590 nm after excitation at 530 nm. IC₅₀ values were calculated by plotting fluorescence intensity to drug concentration in non-linear curves.

U87MG and SKOV3 cells were plated in 96-well plates overnight and caspase-3/caspase-7 activity was assessed with the Caspase-Glo 3/7 assay kit (Promega).

**P-AKT(S473) and P-AKT(T308) cellular ELISA assay**

Cells were seeded in 96-well plates at 2.5 x 10⁴ cells/well in appropriate medium with 10% FBS and incubated overnight. Cells were treated with PF-04691502 at 10 µM to 0.17 nM in 3 fold serial dilutions and incubated for 1 hour at 37°C. The medium was removed and cells were lysed with lysis buffer (Cell Signaling Technologies). Cell lysates were transferred to ELISA plates and the manufacturer’s instructions followed. ELISA kits were from Cell Signaling Technology: PathScan Phospho-AKT (Ser473) and Phospho-AKT (Thr308) Sandwich ELISA Kit.

**Leucine stimulation of P-S6RP(S235/236) to measure PI3K-independent mTOR activity**

In Poly D-lysine coated 10 cm dishes, 2.5 x 10⁶ 293-MSR cells were seeded in DMEM with 10% FBS, 0.5 mg/ml G418 and incubated for 24 hours. Then cells were replaced with serum-free DMEM (full amino acids, Invitrogen) and incubated overnight. After
starvation, the medium was replaced with leucine-free, serum–free DMEM (Millipore, catalog # SLM-100). After 1 hour, cells with and without compound (0.1% DMSO) were stimulated with 52 µg/ml leucine for 10 minutes, 20% FBS for 30 minutes or untreated. The medium was discarded and cells were lysed. Western blot analysis was processed as described below. The P-S6RP(S235/236) cellular ELISA plate assay was developed using the PathScan Phospho-S6 ELISA Kit (Cell Signaling Technologies) to determine IC$_{50}$.

**Western blot analysis**

Cells were washed with PBS and lysed with lysis buffer (Cell Signaling Technologies). Proteins were quantitated by the BCA assay (Pierce). Equal amounts of protein were resolved by polyacrylamide gels, Western blotted and probed with the following primary antibodies: P-AKT(Ser473), P-AKT(Thr308), AKT, P-S6RP(Ser235/236), S6RP, P-p70S6K(Thr389), p70S6K, P-4EBP1(Thr37/46), 4EBP1, P-FKHRL1(Thr32), P-ERK(Thr202/Tyr204), ERK, p21 Cip1/Waf1, p27 Kip1, Rb, PARP and GAPDH (Cell Signaling Technologies); FKHRL1(Millipore); α-tubulin (Sigma); P-PRAS40(Thr246) and PRAS40 (Biosource). Secondary antibodies were from Amersham Biosciences. Bands were detected by chemiluminescence and images captured with an AlphalImager system.

**Cell cycle analysis**

Cells were plated in 6-well plates at 0.25-0.5 x 10$^5$ per well for 24 hours. After 24 or 48 hours treatment with PF-04691502 or DMSO, adherent and floating cells were collected, treated with trypsin and RNase A and stained with propidium iodide (PI) according to the
Cycle Test Plus DNA Reagent Kit (BD Biosciences). DNA content was analyzed with FACS Calibur flow cytometer (BD Biosciences). FCS Express program was used for data analysis.

**Tumor growth inhibition in xenograft models**

Female nu/nu mice (6-8 weeks old) were obtained from Charles River Labs. Animals were maintained under standard clean room conditions in accordance with the Pfizer Institutional Animal Care and Use Committee (IACUC). Tumor cells for implantation were harvested and resuspended in serum-free medium mixed with matrigel (1:1, BD Biosciences). SKOV3, U87MG or NSCLC cells (2.5–4 x 10^6) were implanted subcutaneously into the hind flank region. Treatment started when average tumor size was 100-200 mm^3. PF-04691502 was formulated in 0.5% methylcellulose in water suspension and given orally once a day. Animal body weights and tumor volumes were measured every 2 to 3 days. Tumor volume was determined with Vernier calipers and calculated using the formula: length(mm) x width(mm) x width(mm) x 0.5. Percentage of tumor growth inhibition (TGI) was calculated as 100 x \{1 - ([Treated_{Final day} - Treated_{Initial day}] / [Control_{Final day} - Control_{Initial day}])\}. Data are presented as mean ± standard error. Comparisons between treatment groups and vehicle group were done using one-way ANOVA by Dunnett’s tests. Student’s t test was used to determine the P value for the comparison of two groups.

**Immunohistochemistry**

U87MG tumor xenografts were collected and embedded in OCT (Tissue-Tek). Sections were cut at 8 μm and then followed by 4% paraformaldehyde fixation for 10 minutes.
Sections were counterstained with hematoxylin, dehydrated, cleared, and mounted. The primary antibodies used were P-AKT (Ser473), AKT, P-S6RP (Ser235/236), and S6RP (Cell Signaling Technology). All images were visualized using Zeiss Axioplan 2 imaging Microscope and captured through an AxioCam digital camera using Axiovision 4.8.

Results

**PF-04691502 is an ATP-competitive, selective inhibitor of Class I PI3K and mTOR.**

PF-04691502 inhibited human and mouse PI3Kα with Ki of 1.8 and 1.2 nM, respectively, PI3K isoforms β, δ and γ with Ki of 2.1, 1.6 and 1.9 nM, respectively and human mTOR with Ki of 16 nM (Table 1). PF-04691502 inhibited recombinant mouse PI3Kα in an ATP-competitive inhibitor (Fig.1A). No significant inhibitory activity was observed in more than 80 protein kinases at concentration up to 10 µM including the class III PI3K family member hVps34 (12% inhibition at 1 µM), PI3K downstream kinases AKT, PDK1, p70S6K (IC_{50} >10 µM), and MAPK family members such as MEK, ERK, p38 and JNK (IC_{50} >10 µM) (17). Thus, PF-04691502 was shown to be a selective inhibitor of class I PI3K and mTOR.

Isoform specificity of PF-04691502 was evaluated by blocking transformation of avian fibroblast cells exogenously expressing wild-type p110δ, wild-type p110γ, and p110α mutants. Wild-type p110α isoform lacks oncogenic potential, but E542K, E545K and H1047R mutants and wild-type p110δ and p110γ induce transformation (20, 21). Wild-type p110β is a weak transformer and was not included in the experiment. PF-04691502 inhibited oncogenic transformation by these proteins with similar potency (Fig. 1B). Transformation by Src tyrosine kinase was not inhibited by PF-04691502.
The cellular activity of PF-04691502 was characterized using tumor cell lines with a hyperactivated PI3K pathway. The BT20 breast cancer cell line containing both p110α helical and kinase domain mutations (P539R and H1047R) was used for primary compound screening, SKOV3 (p110α H1047R) ovarian and U87MG (PTEN null) glioblastoma cells were used for in vitro and in vivo evaluation. PF-04691502 potently inhibited AKT phosphorylation on S473 and T308 in all the three cancer cell lines with IC₅₀ values of 3.8-20 nM and 7.5-47 nM, respectively (Fig. 2A).

To determine the inhibitory activity of PF-04691502 against mTOR, a nutrient-stimulated assay measuring PI3K-independent mTORC1 activity was developed (Fig. 2B) (22, 23). In 293-MSR cells, leucine treatment results in increased levels of P-p70S6K(T389) (lower band), P-S6RP(S235/236), and P-4EBP1(T37/46) and as expected did not increase P-AKT(S473) levels (Fig 2C compare lanes 1 and 3). Rapamycin, PI103 and PF-04691502 significantly inhibited phosphorylation of S6RP, p70S6K, and 4EBP1 (electrophoresis shift) after leucine supplementation, but rapamycin did not inhibit P-AKT(S473) at 100 nM (Fig. 2C). Using a 96-well plate based P-S6RP(S235/236) ELISA assay, PF-04691502 potently inhibited mTORC1 activity with an IC₅₀ of 32 nM (Fig. 2D).

**PF-04691502 inhibits PI3K/mTOR signaling and induces cell cycle arrest in cancer cells.**

PF-04691502 inhibited cell proliferation of BT20, SKOV3 and U87MG with IC₅₀ values of 313, 188 and 179 nM, respectively (Fig. 2A).

The effect of PF-04691502 on PI3K and mTOR downstream effectors was evaluated in U87MG cells (Fig. 3). PF-04691502 inhibited phosphorylation of AKT at S473 and T308
in a dose-dependent manner after 3-hour treatment, exhibiting a dramatic decrease at 100 nM and a complete reduction at 500 nM (Fig. 3A). Suppression of AKT activity was consistent with the inhibition of phosphorylation of AKT downstream proteins such as FKHRL1 and PRAS40 observed at 500 nM of PF-04691502. Similarly, phosphorylation of the mTORC1 substrates, 4EBP1(T37/46) and p70S6K(T389) and of P-S6RP(S235/236), a substrate of p70S6K, was suppressed at 100 nM. PF-04691502 treatment also resulted in the appearance of cleaved PARP.

To evaluate the kinetic effects of PF-04691502, a time course experiment was performed (Fig. 3B). Peak inhibition of P-AKT(T308) was observed after 0.5 hour treatment. At 100 nM, P-AKT(T308) levels began to climb back after 3 hours but remained below control levels. The mTORC1 substrates, P-4EBP1(T37/46) and P-p70S6K(T389), showed similar kinetic profiles, but were further inhibited at 24 and 48 hours, indicating longer lasting inhibition of mTOR activity. At 500 nM of PF-04691502, there was sustained inhibition of P-p70S6K and P-S6RP (>48 hours), and significant suppression of P-AKT(T308) was observed for 6 hours. P-AKT(T308) returned to above control levels at 24 hours. ERK activity was not induced in U87MG cells treated with PF-04691502 at different doses and time points (Fig. 3A, Fig. 3B and Supplementary Fig. S1). In SKOV3 cells there is also no change in pERK levels except at the 1 uM dose where there is an increase in pERK levels. (Supplementary Fig. S1).

At both concentrations, PF-04691502 treatment for 24 and 48 hours increased levels of the cyclin dependent kinase inhibitor p27 Kip1 and decreased total Rb protein, while P21 Cip1/Waf1 was not affected by PF-04691502 treatment. Cleaved PARP was detected after treatment with a high dose of PF-04691502 (500 nM) at 3 and 6 hours time points (Fig. 3B).
To further assess the effect of PF-04691502 on apoptosis, caspase 3/7 activity assays were conducted (Fig. 3C). Consistent with detection of PARP cleavage measured by Western blot, PF-04691502 modestly increased caspase3/7 activities (≤3 fold) in a dose-dependent manner in U87MG cells after 3- and 6-hour treatment. No caspase activation was observed at 24 and 48 hour (Fig. 3C). PF-04691502 did not induce caspase activation in SKOV3 cells (data not shown).

In accordance with cell cycle protein regulation, we observed robust cell cycle arrest at the G1 phase after treatment with PF-04691502 for 24 hour (Fig 3D) and 48 hour (data not shown) in a dose-dependent manner in U87MG cells. PI3K inhibitor LY294002 induced G1 cell cycle arrest in U87MG cells as reported (24).

**PF-04691502 exhibits good correlation between pharmacokinetics, target modulation and efficacy in a U87MG xenograft model**

The U87MG xenograft model was used to evaluate the correlation of the pharmacokinetics (PK), pharmacodynamics (PD) and efficacy. Nude mice bearing U87MG tumors were administered orally once a day with PF-04691502 at 0.5, 1, 5, and 10 mg/kg (maximum tolerated dose, MTD). Treatment with 10 mg/kg resulted in a significant reduction of P-AKT(S473) levels at 1 hour post-dosing, and persistent inhibition was observed for 8 hours (Fig. 4A). P-AKT(S473) recovered to above baseline 24 hours after 10 mg/kg treatment (Supplementary Fig. S2). For P-S6RP(S235/236), a similar inhibition time course was observed, but after 24 hours of treatment, P-S6RP levels remained lower than vehicle tumors. Modulation of the AKT downstream effector, P-PRAS40(T246), and mTOR downstream effector, P-4EBP1(T37/46), was observed (Fig. 4A and Supplementary Fig. S2). The PF-04691502 treated tumors were also
evaluated by immunohistochemistry for levels of P-AKT(S473), total AKT, P-S6RP and total S6RP. Phosphorylation of AKT and S6RP were significantly reduced at 4 hours after a single dose of PF-04691502 at 10 mg/kg (Fig. 4B) and as expected there was no change in total AKT and S6RP levels by IHC (data not shown). Dose-dependent tumor growth inhibition (TGI) was obtained in the U87MG xenograft model and ~73% TGI was observed at the MTD dose of 10 mg/kg (Supplementary Table S1). No body weight loss was observed (data not shown).

Oral bioavailability of PF-04691502 in rats following dose of 2 mg/kg in 0.5% methylcellulose suspension is 63% compared to the same dose administration by intravenous route. Time- and dose-dependent plasma concentrations and PD response for P-AKT(S473) (reflecting inhibition of PI3K and mTOR activity) on days 1 and 12 are summarized in supplementary Table S1. Absorption of PF-04691502 was rapid after oral administration, reaching \( C_{\text{max}} \) at 0.5 hour and plasma exposure was linear from 0.5 mg/kg to 10 mg/kg. The apparent half life is 4.4 hours at 5 mg/kg. Modulation of P-AKT(S473) at \( C_{\text{max}} \) dosed from no efficacy (0.5 mg/kg) to maximum efficacy (73% TGI at 10 mg/kg) was saturated. The AUC and \( C_{\text{ss}} \) (steady state concentration) on day 1 and day 12 were comparable, confirming that exposures were constant over time. Robust dose responses were observed for both TGI as well as P-AKT(S473) biomarker suppression at the range of doses studied.

**PF-04691502 shows robust antitumor activity in ovarian cancer and NSCLC models.**

The antitumor effect of PF-04691502 was evaluated in SKOV3 (PI3Kα H1047R kinase domain mutation) ovarian cancer xenograft model at three dose levels (Fig. 5). PF-
PF-04691502 showed dose-dependent TGI on a 16-day once daily dosing schedule. Approximately 72% TGI was observed at the MTD of 10 mg/kg.

The effect of PF-04691502 on phosphorylation of PI3K/AKT/mTOR signaling components was examined by Western blot on day 16 of the TGI study. A dose-dependent decrease in phosphorylation of AKT S473, PRAS40, 4EBP1 and S6RP was observed in tumor lysates collected one hour after treatment (Fig. 5B, left). The effect of PF-04691502 was also measured in a time-dependent manner at 1, 7 and 24 hours post 10 mg/kg dose. Phosphorylation of AKT S473, PRAS40, 4EBP1 and S6 was inhibited up to 7 hours after dosing. P-AKT(S473) increased above vehicle treatment groups 24 hours post dose (Fig. 5B, right).

Since PI3K activity is required for metabolic effects of insulin (25-27), the effect of PF-04691502 on serum glucose and insulin levels was examined in SKOV3 tumor bearing mice after the last drug administration from tumor growth inhibition study. Glucose and insulin levels in serum transiently increased at 0.5 hour while insulin levels returned to baseline 24 hours post repeating dose. Glucose levels were slightly elevated ~1.5-fold from vehicle treated control (Fig. 5C). The whole blood glucose levels after single dose (Day 1) and multiple doses (Day 9) in SKOV3 xenograft model after treatment with PF-04691502 10 mg/kg were comparable, which suggested no accumulation effect of PF-04691502 on glucose at the tolerated dose (Supplementary Table S2).

PF-04691502 showed robust antitumor activity in four NSCLC xenograft models (Fig. 6). TGI was 85-87% in the PTEN deletion line, NCI-H1650 as well as PIK3CA mutant lines, NCI-H1975 and NCI-H460, which also harbor a KRAS mutation. Tumor regression was also observed in the KRAS mutant line A549.

**Discussion**
The PI3K/mTOR axis is one of most frequently disrupted intracellular pathways in human cancer, where it contributes significantly to tumor progression and development of resistance to chemotherapeutic drugs. Inhibitors targeting key proteins within the pathway have shown promise in preclinical and clinical studies. However, due to the complex nature of its feedback regulation and its divergent endpoints, it is widely believed that compounds simultaneously targeting multiple nodes of the pathway will prove most successful in the clinic.

We have shown that PF-04691502 potently inhibits class I PI3K and mTOR in both biochemical and cell-based selectivity assays. PF-04691502 reduced phosphorylation of AKT(T308), AKT(S473) and S6RP in BT20 breast and SKOV3 ovarian cancer cells with a PI3Kα mutation and U87MG glioblastoma cells with a PTEN deletion. Since AKT(S473) is also a substrate of mTORC2 (11), this may explain why the IC₅₀ for P-AKT(S473) reduction is lower than that of P-AKT(T308) after treatment with PF-04691502. Similar levels of reduction in P-AKT(T308), P-4EBP1 and P-p70S6K were seen after 6 hours treatment, but, while strong reductions in P-4EBP1 and P-p70S6K were observed after 24 and 48 hours, P-AKT(T308) levels recovered, suggesting that mTORC1 inhibition by PF-04691502 may persist longer than PI3K inhibition. In fact, P-AKT(T308) levels increased to above control levels, even though P-p70S6K(T389) levels were still reduced. This may reflect disruption of negative feedback on P-AKT by p70S6K-IRS1 and further highlights the importance of a dual inhibition strategy in effectively blocking PI3K signaling.

The differential temporal inhibition of PI3K and mTOR may also explain our observation that PF-04691502 induced some apoptosis in U87MG cells after 3 to 6 hours, but not after 24 to 48 hours treatment. On the other hand, treatment of U87MG cells with
PF-04691502 resulted in pronounced growth arrest in G1 phase, and cell cycle arrest was also observed in SKOV3 and BT20 cells (data not shown). The G1 arrest induced by PF-04691502 correlated well with the up-regulation of the cyclin-dependent kinase inhibitor p27 Kip1, a direct target of forkhead and AKT (1), and a significant reduction of total Rb. Our results are therefore in agreement with previous studies reporting G1 arrest with no apoptosis in U87MG, PC3M and sarcoma cell lines after treatment with the dual PI3K/mTOR inhibitor NVP-BEZ235 (28, 29), the PI3K inhibitor ZSTK474 (30) and the PI3K/mTOR/DNA-PK inhibitor PI103 (31). It has also been reported that most rapamycin analogs induce cytostatic rather than cytotoxic responses (7). NVP-BEZ235 selectively induces apoptosis in either HER2 amplified and/or PIK3CA mutant breast cancer cells through inhibition of PI3K, AKT and mTORC2 (32). In agreement we have observed apoptosis after 16 hours of PF-04691502 treatment in HER2 and/or ER/PR positive breast cancer cell lines (33). Clearly, more studies are needed to fully understand the mechanisms of apoptosis and cell cycle responses to PI3K pathway inhibitors.

Long-term exposure to low concentrations of a PI3K/mTOR dual inhibitor, NVP-BEZ235, has been shown to induce AKT phosphorylation in tumor cells, possibly due to mTOR inhibition (29, 34); a high dose of NVP-BEZ235 was needed to abolish this effect by blocking both PI3K and mTOR (34). In accordance, we found that P-AKT(S473) levels were increased in SKOV3 and U87MG tumors after treatment with PF-04691502 for 24 hours (Fig. 5B and Supplementary Fig. S2). Several labs have reported that PI3K and/or mTOR inhibition relieves feedback inhibition of RTK and consequently activates MAPK pathway (35-37). For PF-04691502, P-ERK induction was observed in SKOV3, which may be related to the feedback upregulation of MAPK signaling pathway. In contrast, increased P-ERK was not seen in U87MG cells suggesting that the activation of
MAPK signaling pathway is cell type-dependent. Further studies are being conducted to determine the patient population for PF-04691502 as single agent or in combination strategies with RTK or MAPK inhibitors to prevent the potential feedback effects.

PF-04691502 exhibited dose-dependent antitumor activity in PTEN-null U87MG and PIK3CA mutant SKOV3 xenografts, which correlated well with plasma concentration, induced time- and dose-dependent target modulation and was well tolerated without body weight loss after oral administration. In NSCLC cells, the PI3K/AKT/mTOR pathway is frequently activated through either amplification/mutation of PIK3CA or functional loss of PTEN. The PI3K/AKT/mTOR pathway also plays a critical role downstream of epidermal growth factor receptor (EGFR) in tumors harboring EGFR mutations. Furthermore, activation of the PI3K/AKT pathway and hyperactive KRAS mutations lead to resistance to EGFR-targeted therapy (38). Dual inhibition of PI3K and mTOR therefore potentially represents an effective therapeutic intervention for NSCLC patients. Our results showed that, PF-04691502 displays robust antitumor efficacies in four NSCLC lines harboring a PIK3CA mutation/PTEN deletion and/or a KRAS mutation which are insensitive to the EGFR inhibitors gefitinib and erlotinib. A549 and NCI-H460 cells have wild type EGFR, while NCI-H1975 [EGFR exon 21 mutation (L858R) and exon 20 mutation (T790M)] and NCI-H1650 [EGFR exon 19 mutation (delE746-A750)] lines have additional mutations in PI3K or PTEN that confer EGFR inhibitor resistance (39). PF-04691502 therefore has potential as an effective anticancer drug for NSCLC patients, including the significant population for whom EGFR tyrosine kinase inhibitors are ineffective.

In conclusion, we have demonstrated that PF-04691502 effectively suppresses PI3K/mTOR signaling pathways, inhibits tumor cell proliferation and shows broad
antitumor efficacy in a variety of xenograft models including EGFR inhibitor-resistant NSCLC models. PF-04691502 is currently in phase I clinical trials.

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References


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Table 1. PF-04691502 chemical structure and its biochemical activity against class I PI3K and mTOR

![Chemical Structure](image)

<table>
<thead>
<tr>
<th>Kinase</th>
<th>Ki (nM)</th>
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<tbody>
<tr>
<td>Mouse PI3Kα</td>
<td>1.2 ± 0.78 (n=4)</td>
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<tr>
<td>Human PI3Kα</td>
<td>1.8 ± 0.86 (n=2)</td>
</tr>
<tr>
<td>Human PI3Kβ</td>
<td>2.1 ± 0.13 (n=2)</td>
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<tr>
<td>Human PI3Kδ</td>
<td>1.6 ± 0.02 (n=2)</td>
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<tr>
<td>Human PI3Kγ</td>
<td>1.9 ± 0.08 (n=2)</td>
</tr>
<tr>
<td>Human mTOR</td>
<td>16 ± 4.9 (n=19)</td>
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</tbody>
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Note: Ki is reported as mean ± SD (nM). Ki = IC_{50}/(1+s/Km)
Figure Legends:

Figure 1. PI3K inhibition by PF-04691502. A, the enzyme rate against 0, 1, 4 and 8 nM of PF-04691502 at various concentrations of ATP was measured to generate the double reciprocal plot. Results are means ± SE from duplicates. B, relative efficiencies of transformation were calculated using the ratio of foci count in the presence of inhibitor to vehicle. Data represent the means of three experiments as a percentage of vehicle control, ± SE.

Figure 2. Cellular profiling of PF-04691502. A, values shown are the mean IC₅₀ ± SD for phosphorylation of AKT S473, AKT T308 and cell proliferation. Student’s t test was used to determine the P value comparing IC₅₀ of P-AKT(Ser473) and P-AKT(T308) in same cell line ( ** P<0.01; *** P<0.001). B, schematic of PI3K independent mTORC1 pathway by nutrient signals, which is adapted from Shaw et al. (22). In response to low nutrient, mTORC1 is inhibited through LKB1-AMPK. AKT, 4E-BP1, p70S6K and S6RP phosphorylation distinguish PI3K-dependent and PI3K-independent mTORC1 activity. C, 293-MSR cells were starved, treated and stimulated as described. Serum, 20% FBS. Leu, 52 µg/ml leucine. Cells were lysed and analyzed by Western blot. GAPDH was used as a loading control. D, PF-04691502 inhibits mTORC1 activity measured by P-S6RP(S235/236) in leucine stimulated PI3K-independent assay.

Figure 3. PF-04691502 inhibits PI3K/mTOR pathway in U87MG cells and induces cell cycle arrest. A, U87MG cells were treated with PF-04691502 for 3 hours. Cell lysates were analyzed by Western blot with indicated antibodies. α-tubulin was used as a
loading control. B, U87MG cells were incubated with 100 nM and 500 nM PF-04691502 in growth medium. C, U87MG cells were treated with PF-04691502 at indicated concentrations and time points. Caspase 3/7 activities were measured. Results represent the mean ± SD from triplicate wells, one of two independent experiments. D, cell cycle analysis after 24 hours of treatment in U87MG cells. The percentages of cells in each phase are indicated in the graphs. The results represent one of three independent experiments.

Figure 4. Pharmacodynamic profile of PF-04691502 in U87MG glioblastoma xenograft model. A, nude mice were treated 10 mg/kg once per day with PF-04691502 or with vehicle for 11 days. Tumors were harvested at 1, 3, or 8 hours after the last dose. Phosphorylated and total AKT, PRAS40, 4EBP1, and S6RP were assessed from tumor protein lysates by Western blot. B, immunohistochemistry staining of P-AKT(S473), P-S6RP(S235/236) in vehicle or in tumor derived from mice treated with a single dose of PF-04691502 at 10 mg/kg for 4 hours. Representative figures are shown (magnification X20. X63 for inserted small figures). Scale bar=100 μM.

Figure 5. In vivo antitumor activity of PF-04691502 in SKOV3 ovarian xenografts. A, nude mice bearing SKOV3 tumors were dosed orally with PF-04691502 or vehicle once daily for 16 days. Mean tumor volume ± SE was plotted for groups of 11 to 12 mice. Percentage of TGI is shown to the right. *, P<0.05, and **, P<0.01 versus vehicle tumor volume by one way ANOVA. B, tumor samples were harvested one hour after final dose from A, tumor was removed from vehicle, 2.5, 5 and 10 mg/kg dose groups (left). Tumor lysates were collected 1, 7 and 24 hours post 10 mg/kg dose on the last day from the same efficacy study as shown in A (right). C, effects of PF-04691502 on
glucose and insulin levels. Serum samples were collected at indicated time points after last drug administration from A. Glucose and insulin levels (mean ± SE) were determined by ELISA assay.

Figure 6. In vivo antitumor activity of PF-04691502 in non-small-cell lung cancer cells (NSCLCs) xenografts. The nude mice were subcutaneously implanted with A, NCI-H460; B, A549; C, NCI-H1650; D, NCI-H1975. Data are the mean tumor volume ± SE from six to eight mice. Student’s t test was used to determine the P value (*, P<0.05; **, P<0.01, ***, P<0.001).
Figure 1

A

B

Transfection (% of vehicle controls)

PF-04691502 (nM)

myr-Akt
Src
p110α E542K
p110α E545K
p110α H1047R
p110δ
p110γ
Table A: Cellular IC_{50} of PF-04691502

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cancer type</th>
<th>Genetic mutation</th>
<th>P-AKT(S473) IC_{50} (nM)</th>
<th>P-AKT(T308) IC_{50} (nM)</th>
<th>Proliferation IC_{50} (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BT20</td>
<td>Breast</td>
<td>PKCaCA mutation</td>
<td>20 ± 8.4 (n=77)</td>
<td>47 ± 20.8 (n=14)**</td>
<td>313 ± 166 (n=4)</td>
</tr>
<tr>
<td>SKOV3</td>
<td>Ovary</td>
<td>PKCaCA mutation</td>
<td>7.4 ± 0.58 (n=2)</td>
<td>13 ± 2 (n=2)**</td>
<td>186 ± 81 (n=4)</td>
</tr>
<tr>
<td>U87MG</td>
<td>Glioblastoma</td>
<td>PTEN deletion</td>
<td>3.8 ± 0.11 (n=2)</td>
<td>7.5 ± 3.39 (n=2)</td>
<td>179 ± 102 (n=6)</td>
</tr>
</tbody>
</table>

Figure B: Diagram of the signaling pathway involving mTORC1 and mTORC2.

Figure C: Western blot analysis showing the effects of PF-04691502 on various proteins.

Figure D: Dose-response curve showing the % of inhibition at IC_{50}=32 nM.
Figure 6

A. NCI-H460 (PIK3CA and KRAS mutation)

B. A549 (KRAS mutation)

C. NCI-H1650 (PTEN deletion and EGFR mutation)

D. NCI-H1975 (PIK3CA and EGFR mutation)
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Jing Yuan, Pramod P. Mehta, Min-Jean Yin, et al.

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