

Therapeutic Potential of Focal Adhesion Kinase Inhibition in Small Cell Lung Cancer

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Abstract

Small cell lung cancer (SCLC) has a poor prognosis. Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase regulating cell proliferation, survival, migration, and invasion, which is overexpressed and/or activated in several cancers, including SCLC. We wanted to determine whether FAK contributes to SCLC aggressive behavior. We first evaluated the effect of FAK small-molecule inhibitor PF-573,228 in NCI-H82, NCI-H146, NCI-H196, and NCI-H446 SCLC cell lines. PF-573,228 (0.1–5 μ mol/L) inhibited FAK activity by decreasing phospho-FAK (Tyr397), without modifying total FAK expression. PF-573,228 decreased proliferation, decreased DNA synthesis, induced cell-cycle arrest in G₂-M phases, and increased apoptosis in all cell lines. PF-573,228 also decreased motility in adherent cell lines. To make sure that these effects were not off-target, we then used a genetic method to inhibit FAK in NCI-H82 and NCI-H446, namely stable

transduction with FAK shRNA and/or FAK-related nonkinase (FRNK), a splice variant lacking the N-terminal and kinase domains. Although FAK shRNA transduction decreased total and phospho-FAK (Tyr397) expression, it did not affect proliferation, DNA synthesis, or progression through cell cycle. However, restoration of FAK-targeting (FAT) domain (attached to focal adhesion complex where it inhibits proliferative proteins such as Rac-1) by FRNK transduction inhibited proliferation, DNA synthesis, and induced apoptosis. Moreover, although FAK shRNA transduction increased active Rac1 level, FRNK reexpression in cells previously transduced with FAK shRNA decreased it. Therefore, FAK appears important in SCLC biology and targeting its kinase domain may have a therapeutic potential, while targeting its FAT domain should be avoided to prevent Rac1-mediated protumoral activity.

Introduction

Lung cancer is the most common cancer and the leading cause of cancer-related death worldwide, with a median 5-year overall survival of 15% (1). Non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) account for 85% and 15% of all lung cancers, respectively (2). SCLC is a neuroendocrine tumor and clinically the most aggressive type of lung cancer, characterized by a tendency for early dissemination and a 5-year overall survival of 5% (3, 4). Unlike NSCLC, there is currently no targeted therapy

validated in SCLC, which is the consequence of a poor understanding of its biology.

Focal adhesion kinase (FAK) is a nonreceptor cytoplasmic tyrosine kinase and scaffold protein localized in focal adhesions, mediating and regulating signals initiated by integrins and G-protein-coupled receptors. FAK plays a role in various cellular functions, including proliferation, survival, adhesion, migration, and invasion. The protein is composed of an N-terminal four-point-one, ezrin, radixin, moesin (FERM) domain, a central kinase domain, and a C-terminal domain (5). Both the N-terminal and C-terminal domains mediate FAK interactions with other proteins critical for its kinase domain's activation and different cellular functions' regulation. FAK is maintained in an inactive state by the binding of the FERM domain to the kinase domain, which blocks access to the key autophosphorylation site tyrosine 397 (Tyr397; ref. 6). Engagement of integrins with the extracellular matrix or stimulation of G-protein-linked receptors following the binding of growth factors leads to signals that displace the FERM domain, resulting in Tyr397 autophosphorylation, changes in conformation of FAK and/or binding partners, binding and/or regulation of downstream effectors such as Src, MAPK, PI3K, paxillin, and Rac (7, 8). The C-terminal domain provides binding sites to proteins such as p130Cas and VEGFR3. It includes the focal adhesion targeting (FAT) sequence responsible for FAK's localization to focal adhesions, promoting its colocalization with integrins through interactions with integrin-associated proteins such as paxillin. The FAT domain also associates with several Rho GTPases, such as p190RhoGEF.

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FAK is overexpressed and activated in several cancers and contributes to cancer progression and metastasis through its important role in cell proliferation, survival, adhesion, spreading, migration, and invasion (5, 9, 10). A role of FAK in evasion of antitumor immunity, angiogenesis, epithelial–mesenchymal transition, regulation of cancer stem cells, DNA damage repair (DDR), and therapy resistance, including radioresistance, has also been described (11–15). This role of FAK in cancer progression stimulated the development of various approaches to inhibit FAK. The first approaches used antisense FAK oligonucleotides, FAK siRNA or shRNA, and overexpression of FAK-Related Non-Kinase (FRNK), a naturally occurring splice variant of FAK that lacks the N-terminal and kinase domains and inhibits FAK phosphorylation in a dominant negative fashion (11, 16). Inhibition of FAK through these approaches induced apoptosis and inhibited migration and angiogenesis. Because these approaches have limitations for clinical applications, small-molecule inhibitors targeting FAK kinase domain have then been developed (11, 16). They decreased Tyr397 phosphorylation and induced antitumoral effects in various cancer types in preclinical studies (17–20). Moreover, some of them (e.g., PF-562,271, VS-6063, and VS-4718) showed promising clinical activities in early-phase clinical trials in patients with selected solid cancers, including NSCLC but not SCLC (5, 16, 21–23). More recently, small-molecule inhibitors targeting different FAK scaffolding protein–protein interactions have been developed, such as inhibitors of FAK and VEGFR-3 interactions, and shown to induce antitumoral effects in preclinical studies (24).

However, FAK has been poorly studied in SCLC. We previously showed that it was amplified and overexpressed in SCLC tumors (25, 26), and activated in SCLC cell lines (25). On the basis of these observations, we hypothesized that FAK activation in SCLC contributes to its aggressive behavior and that FAK may represent a therapeutic target for SCLC. In the current study, we therefore evaluated FAK activity in four SCLC cell lines and evaluated the effects of FAK inhibition by pharmacologic (PF-573,228, PF-562,271, FAK Inhibitor 14) and genetic approaches (FAK shRNA and/or FRNK stable transduction) on cellular functions relevant for cancer progression.

Materials and Methods

Cell culture

Four SCLC cell lines, NCI-H82, NCI-H146, NCI-446, and NCI-H196 (ATCC) were cultured in RPMI (1:1) containing heat-inactivated FCS (10%), L-glutamine (2 mmol/L), penicillin (100 U/mL), and streptomycin (100 µg/mL; Lonza) at 37°C, 5% CO₂. Tetracycline-free FCS (PAN-Biotech GmbH) was used for FRNK transduction experiments. HEK 293FT (ATCC) cell lines were cultured in DMEM supplemented with heat-inactivated FCS (10%), L-glutamine (2 mmol/L), penicillin (100 U/mL), streptomycin (100 µg/mL; Lonza), and neomycin (500 µg/mL; Sigma) at 37°C, 5% CO₂. The experiments were carried out on cells whose passage was between 10 and 25. For the detection of *Mycoplasma* in cell culture, the MycoAlert Mycoplasma Detection Kit (Lonza) was used.

Drugs

PF-573,228 (PF-228; Santa Cruz Biotechnology) and PF-562,271 (PF-271; Sigma) were suspended in DMSO (Sigma), while FAK Inhibitor 14 (Inh14; Sigma) was suspended in water to

get 10, 3, and 35 mmol/L stocks, respectively. These stocks were diluted in culture medium just before experiments to get required concentrations (0.5–10 µmol/L for PF-228, 0.05–3 µmol/L for PF-271, and 3–5 µmol/L for Inh14). In FRNK-transduced cell lines, FRNK expression was induced by doxycycline (1–100 ng/mL; Sigma).

Lentivirus construction

Total RNA was purified using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. RNA was then reverse-transcribed to cDNA using the RevertAid H Minus First Strand cDNA Synthesis Kit with random hexamer primer (Thermo Fisher Scientific). The gateway cloning system (BP and LR) Clonase II enzyme mix (Invitrogen) was used to generate doxycycline-inducible FRNK expression lentivector. To this end, AttB1 and AttB2 sequences were respectively added to FAK's N-terminal and C-terminal by PCR using the KAPA Hifi PCR Kit (KapaBiosystems) according to the manufacturer's instructions. The PCR product was then purified using the Macherey-Nagel PCR Purification Kit (Macherey-Nagel). Purified AttB-FRNK cDNA was cloned into the pJet1.2 vector using the CloneJET PCR Cloning Kit (Thermo Fisher Scientific) and transformed into chemically competent E. Coli-plasmid DNA. The plasmid was isolated using Qiagen Plasmid DNA Mini-Prep Kit (Qiagen) and sequenced in Beckman Coulter Genomics facility (Takeley). BP recombination of pJet AttB-FRNK with donor vector (Addgene Plasmid #29634) was used to generate FRNK-entry vector. This last one was recombined with pCLX-pTF-R1-DEST-R2-EBR65 lentiviral vector (Addgene Plasmid #45952) by LR recombination to generate doxycycline-inducible FRNK expression lentivector (pCLX-pTF-B1-FRNK-B2-EBR65).

Lentivirus production and cell lines' transduction with FAK shRNA and/or FRNK

HEK293T packaging cell lines were transfected with FAK shRNA, no-target (NT) shRNA (Sigma), FRNK plasmid, or empty vector (pCLX) using ProFection Mammalian Transfection System (Promega) in tetracycline-free medium. Virus-containing supernatants were collected 48 and 72 hours posttransfection and immediately used to transduce NCI-H82 and NCI-H446 at a density of 1×10^6 cells/mL for 48 hours in the presence of polybrene (8 µg/mL; Sigma; ref. 27). Transduced cells were selected with puromycin (2 µg/mL) (Invivogen) and/or blasticidin (10 µg/mL; Invivogen) for at least 10 days. The selective pressure with antibiotics was removed 24 hours before each experiment.

Western blot analysis

SCLC cell lines cultured in 12-well flat-bottom plates in 3 mL culture medium were collected (1×10^6 for NCI-H82, NCI-H146, and NCI-446; 1.5×10^5 for NCI-H196) and lysed during 0.5 hours on ice in 150 µL lysis buffer [62.5 mmol/L Tris-HCl (pH 6.8), 2% lauryl sulfate sodium, 10% glycerol 50 mmol/L DTT]. Equal amounts of lysates were separated by 12% SDS-PAGE, electrotransferred onto a nitrocellulose membrane, and immunoprobed with antibodies against phospho-FAK (Tyr397; 1/1,000, rabbit monoclonal; Cell Signaling Technology), total FAK (1/200, rabbit polyclonal; Santa Cruz Biotechnology); PARPp85 (1/1,000, rabbit polyclonal; Promega), phospho-Paxillin (Tyr-118; 1/1,000, rabbit polyclonal; Cell Signaling Technology), total Paxillin (1/1,000, monoclonal mouse; BD Biosciences), and β-actin (1/1,000, mouse monoclonal; Sigma). Secondary antibodies consisted of HRP-conjugated goat anti-rabbit IgG

(1:2,000; Cell Signaling Technology) or HRP-conjugated sheep anti-mouse IgG (1:10,000; Sigma). Immunoreactive bands were developed using chemiluminescence (Amersham ECL; GE Healthcare), detected with a Chemidoc XRS apparatus (Bio-Rad), and densitometrically quantified using Quantity One software (Bio-Rad; results shown in Supplementary Fig. S1).

Cell proliferation

NCI-H82, NCI-H146, NCI-H196, and NCI-H446 were seeded in 96-well plates in antibiotic-free medium at 6×10^4 , 6×10^4 , 4.5×10^4 , and 0.5×10^3 cells per well, respectively. For pharmacologic experiments, PF-228 (0.5–10 $\mu\text{mol/L}$), PF-271 (0.05–3 $\mu\text{mol/L}$), or Inh14 (3–15 $\mu\text{mol/L}$) was added 24 hours after seeding at various concentrations, and cells were cultured for up to 4 days. Every day, WST-1 reagent (Roche) was added to each well and incubated during 3 hours. Wells' absorbance was measured spectrophotometrically at 450nm with iMark microplate absorbance reader (Bio-Rad). Experiments were performed in triplicates.

Cell-cycle analysis

Cells were seeded into 12-well plates at 0.5×10^6 cells per well. After 24 hours, PF-228 (0.5–5 $\mu\text{mol/L}$) or DMSO was added to culture medium. After 24-hour treatment, cells were pulsed with bromodeoxyuridine (BrdU; 10 $\mu\text{mol/L}$) for 0.5 hour, centrifuged, pelleted, and fixed with ice-cold ethanol (70%). DNA denaturation was performed with 2N HCl/0.5% Triton X-100 solution for 30 minutes, followed by quenching with HCl (sodium tetraborate solution 0.1 mol/L pH 8.5). Cells were incubated with FITC-conjugated anti-BrdU antibody (1/20; BD Biosciences), RNaseA (10 $\mu\text{g/mL}$; Sigma), and propidium iodide (PI; BD Biosciences; 20 $\mu\text{g/mL}$). Stained nuclei from 10,000 cells were subjected to flow cytometry. Data were collected on a FACS Canto II flow cytometer (BD Biosciences). Cell-cycle analysis was performed with BD FACS Diva software and FlowJo (FlowJo LLC). Experiments were performed in triplicates.

Apoptosis assay

NCI-H82 and NCI-H446 were seeded in 12-well plates at 0.5×10^6 cells per well. After 24-hour treatment with PF-228 (1, 3, and 5 $\mu\text{mol/L}$) or DMSO, cells were stained with antibodies against cleaved caspase-3 (1:50; Cell Signaling Technology) or, after 48-hour treatment, with BrdU via TUNEL assay (APO-BrdU Kit; BD Biosciences) according to the manufacturer's instructions. Staining was quantified by FACSCanto II. Data acquisition and analysis were performed with FlowJo. Experiments were performed in triplicates.

Wound healing assay associated with time-lapse video recording of cell motility

NCI-H196 and NCI-H446 were grown to confluence in 12-well plates. Cell monolayers were wounded using a micropipette tip and floating cells were washed off with PBS (Lonza). After overnight incubation, PF-228 or DMSO was added to culture medium. Cell movements within wounded area were monitored for 12 hours starting from the time drug was added using a Zeiss Axiovert 200M microscope (Zeiss) at $\times 200$ magnification. Images were captured every 15 minutes from five different fields randomly selected in each well. About 100 individual cells were analyzed using the Tracking Analysis software. Individual cells were tracked manually using MTrackJ, an ImageJ (NIH, Bethesda, MD) plugin. Only nondividing cells were analyzed to exclusively assess motility.

Track's full length (LEN) was determined from the first point to the last point of the track and represented the distance covered by the cell during the experiment. Migration velocity was obtained by dividing LEN with experiment duration (12 hours). Experiments were performed in triplicates.

Matrigel invasion assay

Inserts separating the two chambers of 24-well invasion chambers (Corning) were coated with Matrigel (0.3 g/L) and incubated at 37°C for 2 hours. Lower chambers were filled with RPMI containing 10% FBS. NCI-H196 and NCI-H446 were trypsinized, washed with PBS, suspended in 1% FBS RPMI, plated in the upper chambers (25×10^3 and 10×10^4 cells/well for NCI-H196 NCI-H446, respectively), and incubated at 37°C for 3 hours. PF-228 (3 or 5 $\mu\text{mol/L}$) or DMSO was added in upper chambers 3 hours after seeding. After 12-hour incubation with the drug, cells remaining in the upper chamber were removed with cotton swabs and cells on the lower surface of the insert separating both chambers were fixed and stained with crystal violet. Image acquisition was performed with Axiovert 40 CFL Zeiss microscope (Carl Zeiss Microscopy, LLC). Images were obtained using ImageJ software (NIH, Bethesda, MD). Experiments were performed in duplicate.

Rac pull-down assay for activated GTPases

Active GTPases were pull-downed with Active Rac1 Pull-Down and Detection Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Cells were lysed with a lysis buffer containing a complete protease inhibitor cocktail. Equal amounts of proteins (800 μg) were loaded into kit's pull-down columns. Samples were incubated with Rac/Cdc42 PAK1 PAK-binding domain and rocked for 1 hour. Agarose beads were collected by centrifugation (30 seconds at $6,000 \times g$ and 4°C), washed, resuspended with 50 μL 2 $\times$ SDS sample buffer, and boiled for 5 minutes. Proteins were resolved by 12% SDS-PAGE and electrotransferred onto a membrane probed with mouse anti-Rac1 antibody (Thermo Fisher Scientific). GTP loading controls were incubated with GTP- γS (0.1 mmol/L) for 0.5 hour at 30°C.

Statistical analysis

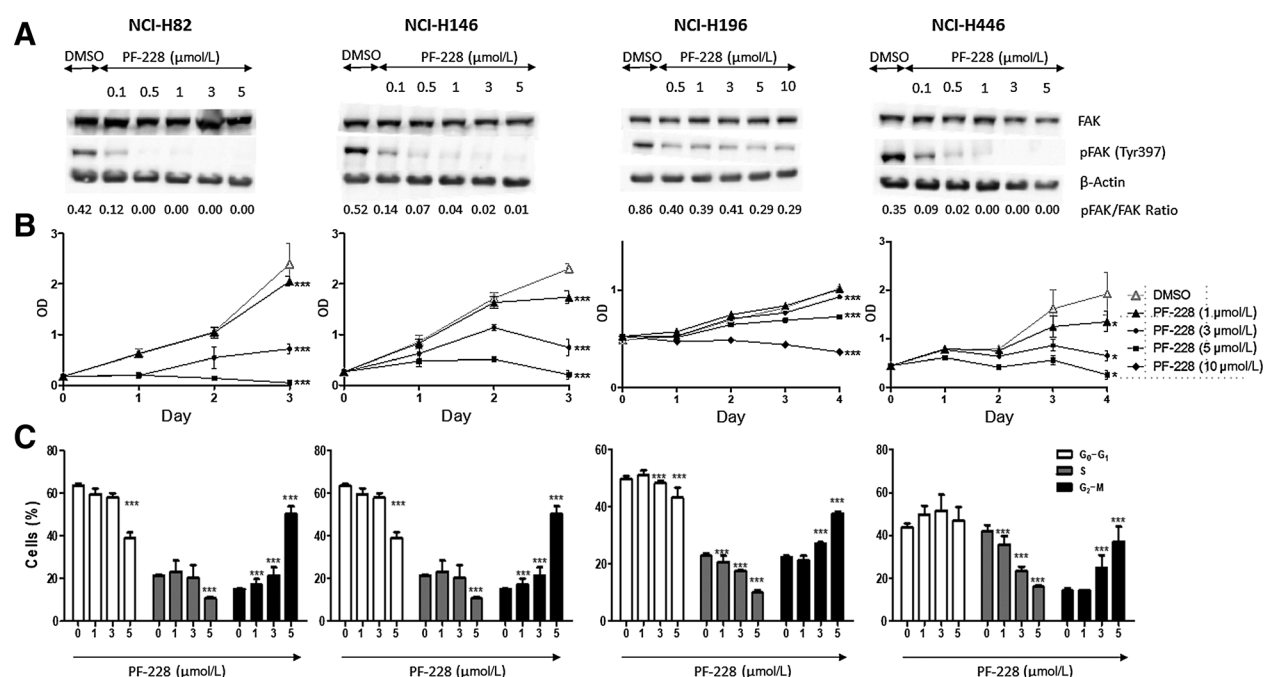
Statistical analyses were performed using the statistical analysis software JMP Pro version 12 (SAS Institute Inc., Cary, NC). Multiple linear regression analysis was used for WST-1 and χ^2 test of independence for cell cycle and apoptosis data. Descriptive statistics were reported as mean $\pm$ SD. Significance level was set at $P < 0.05$ for each analysis.

Results

Pharmacologic inhibition of FAK has several antitumoral effects in SCLC

To investigate whether FAK is involved in the aggressive phenotype of SCLC, we tested the changes of cellular phenotype induced by the FAK small-molecule inhibitor PF-228 in four SCLC cell lines (two growing in suspension: NCI-H82 and NCI-H146, an adherent: NCI-H196, and a mixed-morphology: NCI-H446).

PF-228 inhibits FAK phosphorylation at Tyr397. Treatment with increasing concentrations of PF-228 (0.1–10 $\mu\text{mol/L}$) decreased FAK phosphorylation (Tyr397) in all tested cell lines dose dependently, without modifying total FAK expression (Fig. 1A). Phospho-FAK (Tyr397) decrease was less important in the adherent

**Figure 1.**

PF-573,228 (PF-228)'s effect on FAK expression/activity, cell proliferation, and cell cycle in SCLC cell lines. **A**, FAK expression and phosphorylation evaluation by Western blot analysis. Whole-cell lysates from four SCLC cell lines treated with PF-228 or DMSO control for 90 minutes were resolved by SDS-PAGE, and blots were incubated with antibodies against total FAK (125 kDa), phospho-FAK (Tyr397; 125 kDa), and β -actin (45 kDa) for normalization. Dose-dependent inhibition of FAK phosphorylation (Tyr397) was observed by Western blot analysis in cell lines treated with PF-228 as compared with those treated with DMSO, while total FAK expression was not modified. Western blot densitometric quantification is available in Supplementary Fig. S1. **B**, Cell proliferation evaluation by WST-1 assay. Four SCLC cell lines were cultured for 3 or 4 days in the presence of PF-228 or DMSO. Dose-dependent inhibition of proliferation was observed by WST-1 assay in cells treated with PF-228 as compared with those treated with DMSO. Optical density (OD) in y-axis reflects the proportion of metabolically active cells. Error bars represent mean $\pm$ SD from three independent experiments. All graphs represent one of three independent experiments with similar results. $***, P \leq 0.001$. **C**, Cell-cycle evaluation by flow cytometry. Four SCLC cell lines treated with PF-228 or DMSO for 24 hours were stained with anti-BrdU antibody and propidium iodide (PI), and the staining was quantified by FACS analysis. Dose-dependent inhibition of DNA synthesis and induction of cell-cycle arrest in G_2 -M phase was observed by flow cytometry in cell lines treated with PF-228 as compared with those treated with DMSO. Error bars represent mean $\pm$ SD from three independent experiments. $***, P \leq 0.001$.

cell line NCI-H196, even at higher drug concentrations (0.5–10 μ mol/L vs. 0.1–3 μ mol/L).

PF-228 inhibits proliferation and progression through cell cycle in SCLC. Inhibition of FAK activity with 1 to 10 μ mol/L PF-228 significantly decreased proliferation of the four SCLC lines, dose dependently ($P < 0.001$ for all tested concentrations beside 1 μ mol/L in NCI-H196; Fig. 1B). The effect was more pronounced in the suspension cell lines NCI-H82 and NCI-H146, which constitutively displayed higher proliferation rates. Cell-cycle analysis showed that PF-228 inhibited progression through cell cycle by significantly reducing the S-phase and inducing cell-cycle arrest in G_2 -M phases in the four cell lines after 24-hour treatment, dose dependently ($P < 0.001$ for all tested concentrations; Fig. 1C).

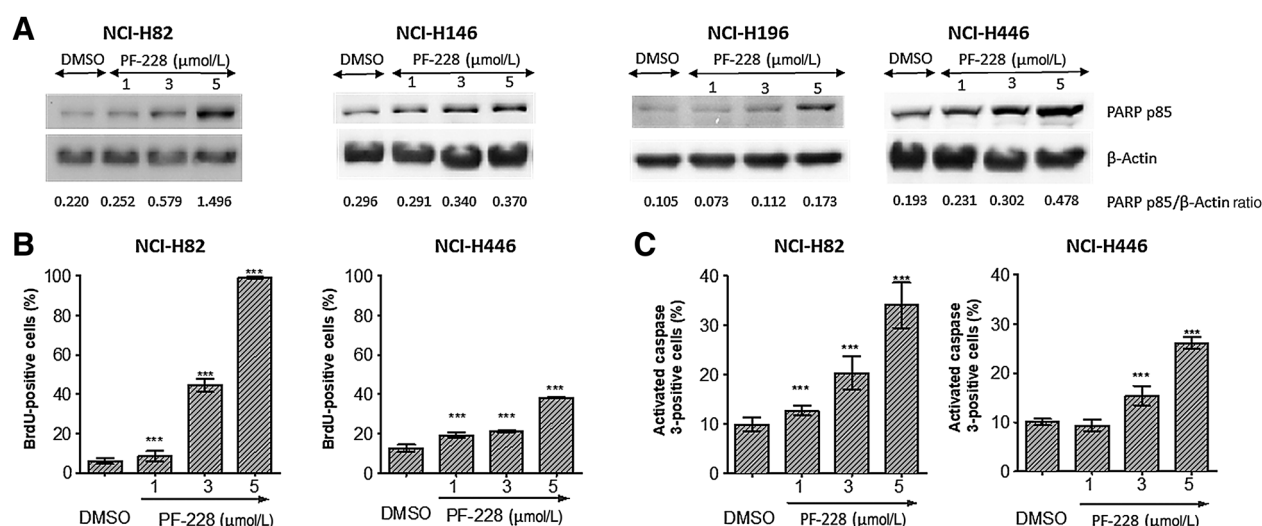
PF-228 induces apoptosis in SCLC. PF-228 at concentrations of 1 to 5 μ mol/L also significantly induced apoptosis in the four cell lines as demonstrated by a dose-dependent increase of PARP p85 expression by Western blot analysis after 48-hour treatment (Fig. 2A). This was confirmed by flow cytometry in NCI-H82 and NCI-H446 cell lines, which showed a significant increase of BrdU-positive and activated caspase 3-positive cells after 48-hour treatment ($P < 0.001$ for all tested concentrations except 1 μ mol/L in NCI-H446 in the caspase-3 assay; Fig. 2C).

PF-228 inhibits migration and invasion in SCLC. Wound healing assay and modified Boyden chambers allowed the investigation of cellular migration and invasion in the two SCLC cell lines with an adherent component. PF-228 at a concentration of 3 μ mol/L tended to decrease migration velocity from 5 to 2.5 μ m/minute in NCI-H196 ($P = 0.0561$) and from 9 to 4 μ m/minute in NCI-H446 ($P = 0.0916$; Fig. 3A; Supplementary Video S1). PF-228 also inhibited invasion after 12-hour treatment at 3 μ mol/L, with the number of invasive cells able to migrate to the lower side of the insert separating the two Boyden chambers decreasing from 150 to 50 per field ($\times 20$ magnification) for NCI-H196 and from 45 to 5 per field for NCI-H446 (Fig. 3B).

Inh14 and PF-271 also inhibit proliferation and induce apoptosis in SCLC. To verify that PF-228's effects were related to FAK, we tested two other FAK inhibitors, Inh14 and PF-271, in NCI-H446. Similarly to PF-228, they both decreased FAK phosphorylation at Tyr397 and proliferation, and increased apoptosis as shown by increased PARP p85 expression (Supplementary Fig. S2).

Genetic inhibition of FAK leads to antitumoral effects only in the presence of FRNK

Loss of total FAK following FAK shRNA transduction does not affect proliferation and progression through cell cycle in SCLC. Aiming to

**Figure 2.**

PF-228's effect on apoptosis in SCLC cell lines. **A**, Apoptosis evaluation by PARP p85 Western blot analysis. Whole-cell lysates from four SCLC cell lines treated with PF-228 or DMSO for 48 hours were resolved by SDS-PAGE, and blots were incubated with antibodies against PARP p85 (85 kDa) and β-actin (45 kDa) for normalization. Dose-dependent increase of PARP p85 expression was observed by Western blot analysis in cell lines treated with PF-228 as compared with those treated with DMSO. Western blot densitometric quantification is available in Supplementary Fig. S1. **B** and **C**, Apoptosis evaluation by flow cytometry. Two SCLC cell lines treated with PF-228 or DMSO for 24 hours (**B**) or 48 hours (**C**) were stained with anti-BrdU antibody and PI (**B**) or Pacific-Cleaved Caspase 3 and PI (**C**), and the staining was quantified by FACS. Cells were first gated in PI channel (PI-A and PI-H) to discard cells debris and doublets. Dose-dependent increase of apoptotic cells [BrdU-positive cells (**B**) or activated caspase 3-positive cells (**C**)] was observed by flow cytometry in cell lines treated with PF-228 as compared with those treated with DMSO. Error bars represent mean ± SD from three independent experiments. ***, $P \leq 0.001$.

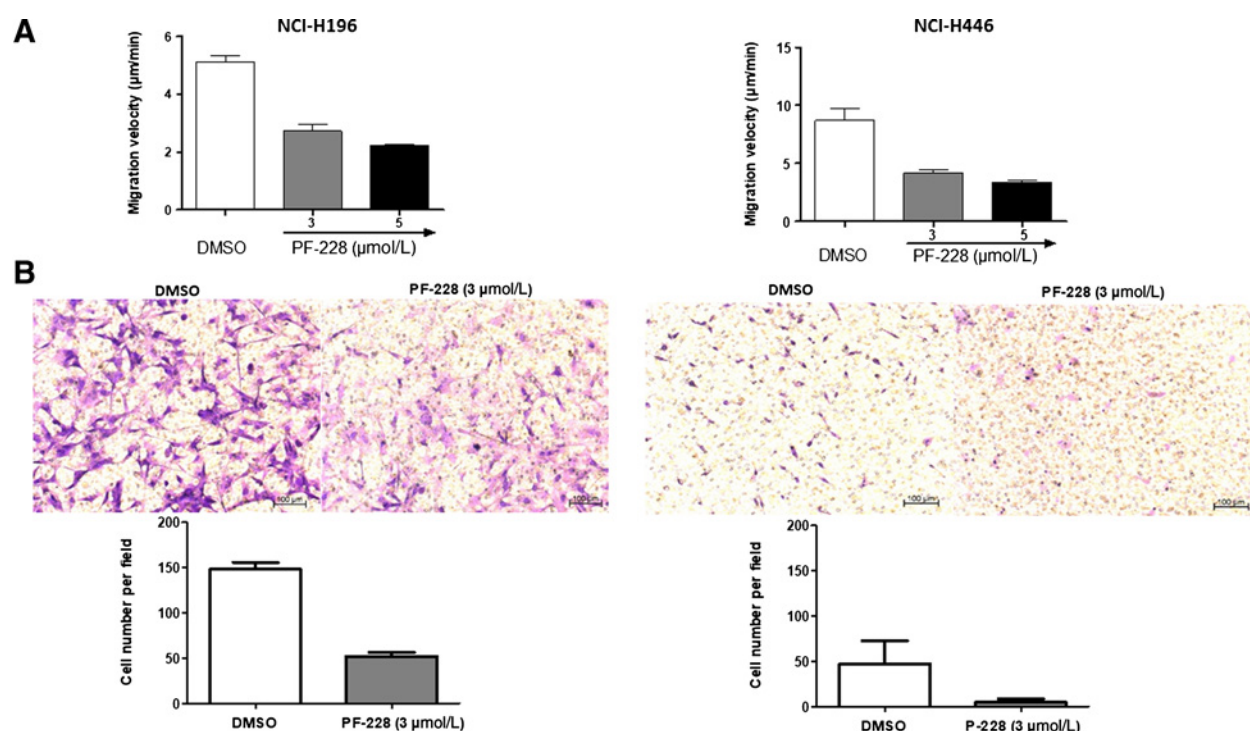
confirm the specificity of PF-228's antitumoral effects in SCLC cell lines, experiments were carried out in NCI-H82 and NCI-H446 cells where FAK was inhibited by a genetic approach, namely the stable transduction of FAK shRNA (five clones). Western blot analysis confirmed the almost complete loss of total FAK and phospho-FAK (Tyr397) expression following transduction with FAK shRNA as compared with NT shRNA (Fig. 4A1). However, FAK shRNA transduction did not modify cell proliferation over 3 days and progression through cell cycle as evaluated by WST-1 and flow cytometry, respectively (Fig. 4A2–4A3).

Once again aiming to evaluate PF-228's specificity, we treated SCLC cell lines transduced with FAK or NT shRNA with PF-228. As expected, we observed a significantly less important inhibition of proliferation in cell lines transduced with FAK shRNA. We also showed that PF-228 induced apoptosis, as demonstrated by increased PARP p85 expression, only in cells transduced with NT shRNA (Supplementary Fig. S3).

FRNK overexpression following transduction inhibits proliferation and survival in SCLC. To address the apparent discrepancy between PF-228's effects and those of FAK shRNA transduction, we used a second genetic approach to inhibit FAK in NCI-H446, namely the stable transduction of a doxycycline-inducible FRNK vector. FRNK, which lacks FAK's N-terminal and kinase domains, is a known physical repressor of FAK signaling (28). Western blot analysis confirmed a significant and dose-dependent (doxycycline) increase of FRNK expression in NCI-H446 transduced with doxycycline-inducible FRNK vector and treated with doxycycline, as compared with those not treated with doxycycline or transduced with pCLX empty vector, while total FAK and phospho-FAK (Tyr397) expression remained unchanged (Fig. 4B1).

Interestingly, FRNK overexpression significantly decreased cell proliferation over 5 days ($P < 0.001$) and DNA synthesis after 48-hour treatment with doxycycline ($P < 0.001$) as evaluated by WST-1 and flow cytometry, respectively (Fig. 4B2–4B3). FRNK overexpression also significantly induced apoptosis as shown by increased PARP p85 expression by Western blot analysis after 48-hour treatment with doxycycline (Fig. 4B1). The effects on proliferation, DNA synthesis, and apoptosis were proportional to doxycycline concentrations and FRNK expression levels. As opposed to FAK inhibition by FAK shRNA transduction, FAK inhibition by FRNK overexpression induced antitumoral effects similar to FAK pharmacologic inhibition.

FRNK overexpression following transduction in SCLC cell lines previously transduced with FAK shRNA inhibits proliferation and survival. Facing different results with the two genetic approaches used to inhibit FAK, we wondered whether the loss of FRNK was responsible for the absence of effect of FAK shRNA transduction on survival. To test this, we overexpressed FRNK in NCI-H446 cells stably transduced with FAK shRNA by transducing them with doxycycline-inducible FRNK vector. FRNK overexpression did not modify total FAK and phospho-FAK (Tyr397) expression, which were both downregulated by FAK shRNA transduction (Fig. 5A1). However, in these double-transduced cells, with FAK shRNA and then FRNK, we observed an inhibition of cell growth over 4 days as evaluated by WST-1 ($P < 0.001$; Fig. 5A2), and an induction of apoptosis as shown by increased PARP p85 expression by Western blot analysis (Fig. 5A1). The effects on proliferation and apoptosis were both proportional to doxycycline concentrations and FRNK expression levels.

**Figure 3.**

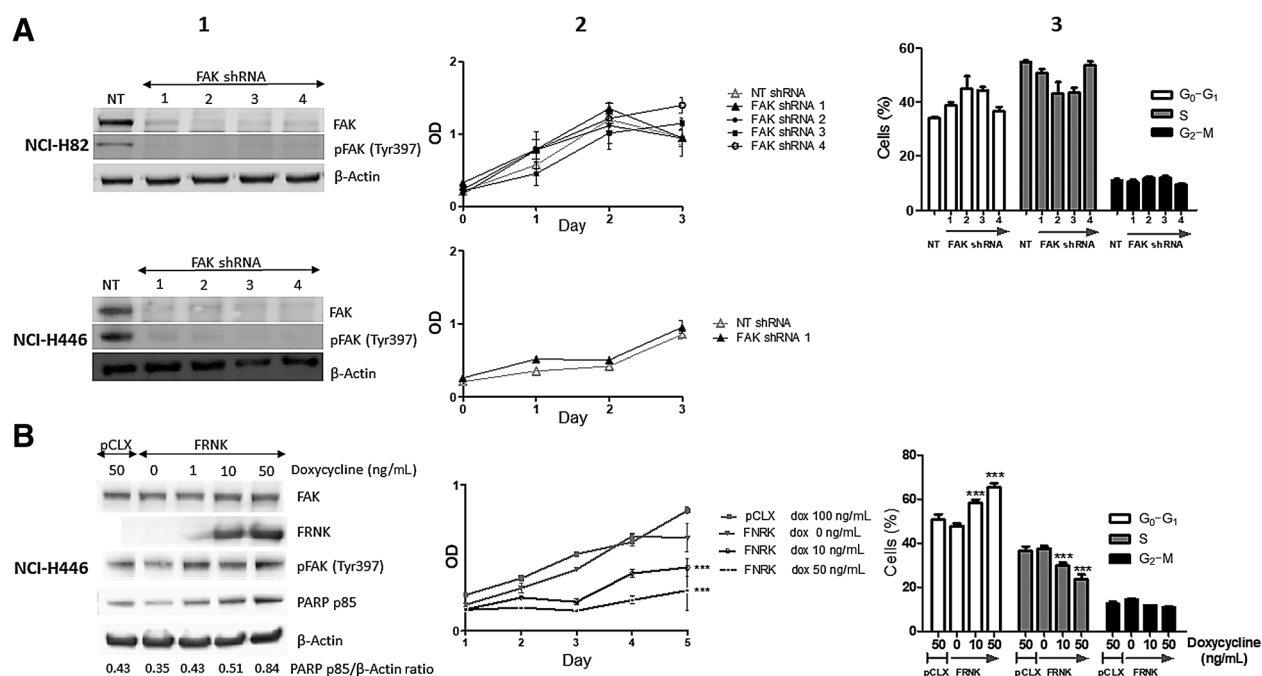
PF-228's effect on migration and invasion in SCLC cell lines. **A**, Migration evaluation by wound healing assay associated with time-lapse microscopy. Two adherent SCLC cell lines were grown to confluence, wounded, incubated overnight with culture medium, and then treated with PF-228 or DMSO for 12 hours. Cells were monitored during these 12 hours using a Zeiss Axiovert 200M microscope (Zeiss). Images were captured every 15 minutes. Velocity of cell migration was measured using ImageJ. Decreased motility was observed in cell lines treated with PF-228 as compared with those treated with DMSO. Error bars represent mean $\pm$ SD from three independent experiments. **B**, Invasion evaluation by modified Boyden Chamber assay. Two adherent SCLC cell lines (one adherent and one with mixed-morphology) were seeded on the top of an insert precoated with Matrigel and separating the two chambers of a transwell. Culture medium containing 1% FBS was placed in the upper chamber and 10% FBS in the lower chamber. After 12-hour treatment with PF-228 or DMSO, cells that moved through the pores toward the bottom of the insert were stained with crystal violet, digitally pictured, and quantified by the free software ImageJ (NIH, Bethesda, MD). Right, SCLC cell lines stained with crystal violet on the lower side of the insert, which are representative of the numerous fields ($\times 10$ magnification) analyzed in two independent experiments performed in duplicate wells. Left, quantification of the number of cells per field on the bottom of the insert. Decreased invasion was observed in cell lines treated with PF-228 for 12 hours as compared with those treated with DMSO. Error bars represent mean $\pm$ SD from two independent experiments.

FRNK keeps Rac1 GTPase inactivated in SCLC. On the basis of a previous report in endothelial cells, which also showed that different methods of FAK inhibition result in different functional outcomes and that this occurs through the regulation of Rac activation (29), we evaluated activated Rac1 level in NCI-H446 cell lines double-transduced with FAK shRNA and doxycycline-inducible FRNK vector using Rac pull-down assay for activated GTPases (Fig. 5B1). In NT shRNA and pCLX double-transduced cells used as control, with no FRNK expression, activated Rac1 level was low at baseline, while treating them with GTP significantly increased it as expected (Fig. 5B2). In cells transduced with FAK shRNA and doxycycline-inducible FRNK vector, activated Rac1 was present at baseline in cells without FRNK expression, while FRNK overexpression significantly decreased activated Rac1 level (Fig. 5B2). These results indicate that the loss of FRNK following the physical loss of total FAK increases activated Rac1 level. As Rac1 is a pro-proliferative protein (30, 31), we have an explanation to why SCLC cell lines transduced with FAK shRNA remain proliferative (Fig. 4A2): the pro-proliferative effect of Rac1 activation counterbalances the antiproliferative effect induced by the absence of FAK phosphorylation at Tyr397.

Because FAK is known to phosphorylate Paxillin and phospho-Paxillin to activate Rac1 via the adaptor protein CrkII (32), we evaluated phospho-Paxillin (Tyr118) expression in NCI-H446 by Western blot analysis and immunofluorescence. The two methods showed that FAK inhibition, either with PF-228 or double-transduction with FAK shRNA and FRNK, did not modify phospho-Paxillin (Tyr118) expression, which was however low even at baseline (Supplementary Fig. S4).

Discussion

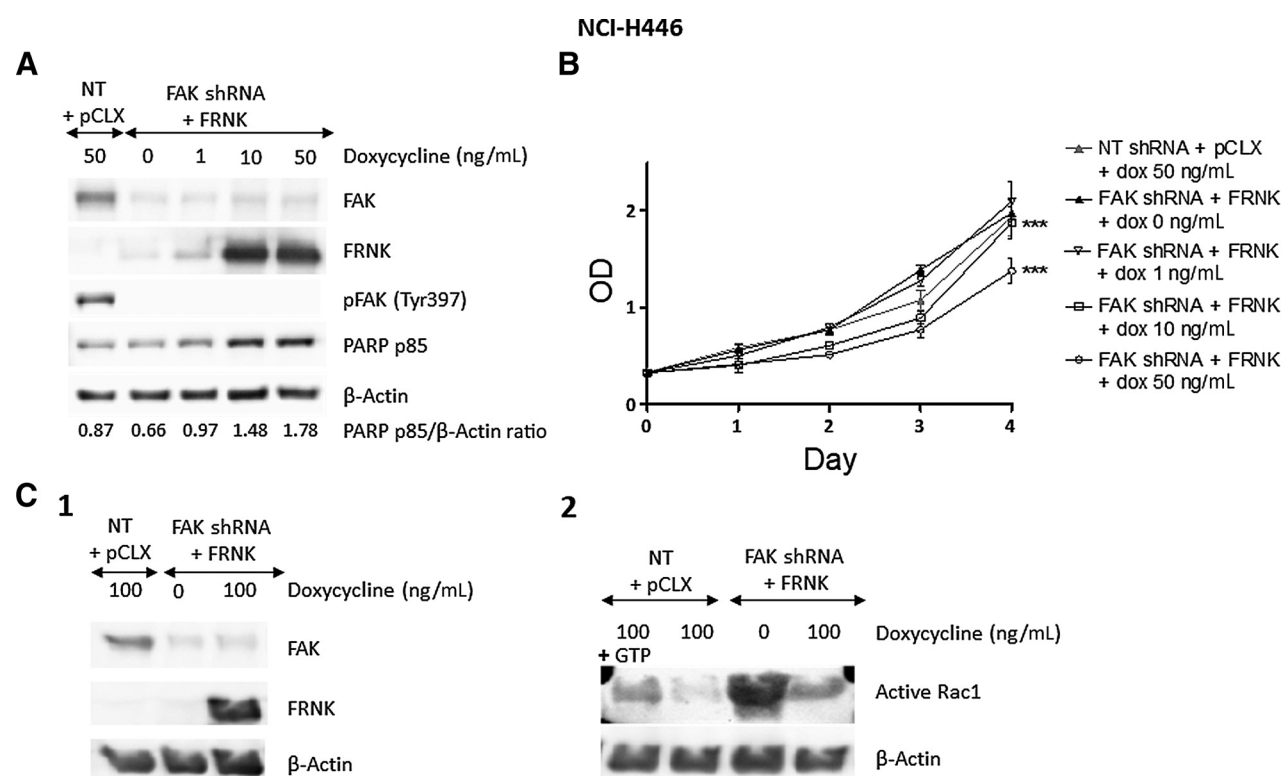
In this study, we evaluated whether FAK, known to be over-expressed in SCLC tumors (25, 26) and activated in SCLC cell lines (25), contributes to the aggressive behavior of SCLC and is a potential therapeutic target in SCLC. In a previous study, we showed that FAK was constitutively activated in SCLC cell lines, with high levels of FAK phosphorylation at Tyr397, and that the pharmacologic inhibition of FAK with PF-228 decreased cell adhesion and modified cell phenotype (25). Here, we explored the role of FAK in cellular functions relevant for cancer progression and showed for the first time that inhibition of FAK activity with PF-228 decreased proliferation, induced cell-cycle arrest,

**Figure 4.**

Effect of FAK shRNA and FAK-related nonkinase (FRNK) transduction on FAK expression/activity, cell proliferation, cell cycle, and apoptosis in SCLC cell lines. **A**, Two SCLC cell lines were stably transduced with FAK shRNA or no-target (NT) shRNA as control, and submitted to puromycin selection for 2 weeks. 1. FAK expression and activity evaluation by Western blot analysis. Whole-cell lysates from these two cell lines were resolved with SDS-PAGE, and blots were incubated with antibodies against total FAK (125 kDa), phospho-FAK (Tyr397; 125 kDa), and β -actin (45 kDa) for normalization. Significant decrease of FAK expression and phosphorylation (Tyr397) was observed by Western blot analysis in SCLC cell lines transduced with FAK shRNA as compared with those transduced with NT shRNA. Western blot densitometric quantification is available in Supplementary Fig. S1. 2. Cell proliferation evaluation by WST-1 assay. SCLC cell lines were cultured for 3 days. No significant difference in cell proliferation was observed by WST-1 between cells transduced with FAK shRNA and those transduced with NT shRNA. Optical density (OD) in y-axis reflects the proportion of metabolically active cells. Error bars, mean $\pm$ SD ($n = 5$). All the graphs represent one of five independent experiments with similar results. ***, $P \leq 0.001$. 3. Cell-cycle evaluation by flow cytometry. SCLC cell lines were stained with anti-BrdU antibody and PI, and the staining was quantified by FACS. No significant difference in cell cycle was observed between cells transduced with FAK shRNA and those transduced with NT shRNA transfection. Error bars represent mean $\pm$ SD from three independent experiments. ***, $P \leq 0.001$. **B**, NCI-H446 cell lines were stably transduced with doxycycline-inducible FRNK-expression plasmid or empty vector (pCLX) as control, and submitted to blasticidin-selection for 2 weeks. Cells were treated with doxycycline for 48 hours before the experiments. 1. FAK and PARP p85 expression/activity evaluation by Western blot analysis. Whole-cell lysates from SCLC cell lines were resolved with SDS-PAGE, and blots were incubated with antibodies against total FAK (125 kDa), FRNK (41 kDa), phospho-FAK (Tyr397; 125 kDa), PARP p85 (85 kDa), and β -actin (45 kDa) for normalization. Significant increase of FRNK expression was confirmed by Western blot analysis in SCLC cell lines transduced with FRNK and treated with doxycycline as compared with those not treated with doxycycline or transduced with pCLX empty vector. Significant increase of PARP p85 expression, a marker of apoptosis, was also observed by Western blot analysis in cells expressing FRNK, while total FAK and phospho-FAK (Tyr397) expression remained unchanged. 2. Cell proliferation evaluation by WST-1 assay. SCLC cell lines were cultured for 5 days. Inhibition of proliferation was observed by WST-1 in cell lines expressing FRNK as compared with those not expressing it. Optical density (OD) in y-axis reflects the proportion of metabolically active cells. Error bars, mean $\pm$ SD ($n = 5$). All graphs represent one of five independent experiments with similar results. ***, $P \leq 0.001$. 3. Cell-cycle evaluation by flow cytometry. SCLC cell lines were stained with anti-BrdU antibody and PI, and the staining was quantified by FACS. DNA synthesis was decreased in cell lines expressing FRNK as compared with those not expressing it. Error bars represent $\pm$ SD from five independent experiments. ***, $P \leq 0.001$.

increased apoptosis, and decreased motility and invasion. All these important antitumoral effects of PF-228 suggest that FAK is important in SCLC biology and may have a therapeutic potential. The inhibitory effect of PF-228 on SCLC motility and invasion was similar to the results reported in other cancer types or in normal cells (20). Of note, we tested migration and invasion only in the two adherent cell lines as these functions are difficult to evaluate in suspension cell lines. Interestingly, we observed an effect of PF-228 on proliferation and apoptosis already at low drug concentrations. The first study that tested PF-228 showed an effect on migration and focal adhesion turnover but failed to demonstrate an effect on proliferation and survival in prostate cancer cell line PC3, fibroblastic cell line REF52, and canine kidney cell line MDCK (20). Another study showed that PF-228 inhibited proliferation and induced

apoptosis in endometrial cancer cell lines but, as opposed to our study, much higher concentrations of PF-228 were used (50 μ mol/L). The fact that low concentrations of PF-228 inhibited proliferation and survival in SCLC cell lines suggest the specificity of the drug and the importance of FAK in proliferative and prosurvival signaling pathways. Other FAK inhibitors induced inhibition of proliferation or survival *in vitro* in various cell types but were not specific of FAK (e.g., TAE-226 inhibits FAK, PYK2, and IGF-1R; refs. 33–35). In this study, we additionally tested two other FAK inhibitors, Inh14 and PF-271 (18, 36), and observed that they also inhibited proliferation and induced apoptosis in SCLC. This strengthened us in the idea that PF-228's effects were related to FAK, even though Inh14 and PF-271 are both less FAK-specific than PF-228, known to have the highest FAK specificity among FAK inhibitors.

**Figure 5.**

Effect of FRNK transduction on FAK expression/activity, proliferation, apoptosis, and Rac1 expression in SCLC cell lines transduced with FAK shRNA. NCI-H446 cell lines were first stably transduced with FAK shRNA or no-target (NT) shRNA and then stably transduced with doxycycline-inducible FRNK-expression plasmid or pCLX empty vector as control. After transduction, they were submitted to puromycin and blasticidin-selection for 2 weeks. Finally, they were treated with doxycycline for 48 hours before the experiments. **A**, FAK and PARP p85 expression/activity evaluation by Western blot analysis. Whole-cell lysates from SCLC cell lines were resolved with SDS-PAGE, and blots were incubated with antibodies against total FAK (125 kDa), FRNK (41 kDa), phospho-FAK (Tyr397) (125 kDa), PARP p85 (85 kDa), and β-actin (45 kDa) for normalization. Decreased FAK and phospho-FAK (Tyr397) expression was observed by Western blot analysis in cell lines double-transduced with FAK shRNA and FRNK as compared with those transduced with NT shRNA and pCLX. Increased FRNK expression was observed in cell lines double-transduced with FAK shRNA and FRNK when treated with doxycycline. Increased PARP p85 expression, a marker of apoptosis, was observed in cells expressing FRNK. Western blot densitometric quantification is available in Supplementary Fig. S1. **B**, Cell proliferation evaluation by WST-1 assay. SCLC cell lines were cultured for 4 days. Inhibition of proliferation was observed in cell lines double-transduced with FAK shRNA and FRNK and expressing FRNK after treatment with doxycycline. Optical density (OD) in y-axis reflects the proportion of metabolically active cells. Error bars, mean $\pm$ SD ($n = 5$). All graphs represent one of five independent experiments with similar results. **C**, Effects of FRNK on Rac1 activity. NCI-H446 SCLC cell lines were double-transduced with FAK shRNA and a doxycycline-inducible FRNK vector or with no-target (NT) shRNA and pCLX empty vector as control. **1**, FAK and FRNK expression evaluation by Western blot analysis. Whole-cell lysates from SCLC cell lines were resolved with SDS-PAGE, and blots were incubated with antibodies against total FAK (125 kDa), FRNK (41 kDa), and β-actin (45 kDa) for normalization. Decreased FAK expression was observed by Western blot analysis in cell lines double-transduced with FAK shRNA and FRNK as compared with those transduced with NT shRNA and pCLX. Increased FRNK expression was observed in cell lines double-transduced with FAK shRNA and FRNK when treated with doxycycline. Western blot densitometric quantification is available in Supplementary Fig. S1. **2**, Rac1 activation evaluation by Rac pull-down assay for activated GTPases. Whole-cell lysates from SCLC cell lines were enriched in activated GTPases using a GST-PAK affinity assay (GTPases pull-down assay). Enriched eluates were resolved with SDS-PAGE, and blots were incubated with antibodies against Rac1 (21 kDa) and β-actin (45 kDa) for normalization. In control cells double-transduced with NT shRNA and pCLX, Western blot analysis revealed low activated Rac1 expression at baseline, while treating them with GTP significantly increased its expression. In cells double-transduced with FAK shRNA and FRNK, activated Rac1 expression was present in the absence of doxycycline, while doxycycline-induced FRNK expression significantly decreased its expression. Western blot densitometric quantification is available in Supplementary Fig. S1.

To better address the specificity of PF-228's effects on proliferation and survival in SCLC cell lines, we evaluated the consequence of FAK inhibition by a genetic method, namely FAK shRNA stable transduction. Surprisingly, the physical loss of FAK did not impact on proliferation and cell cycle. But interestingly, treatment of FAK shRNA-transduced cells with PF-228 did not induce apoptosis and had only a limited effect on proliferation. The absent/limited effect of PF-228 in cells with no/low FAK expression also suggests the drug's specificity.

To address the apparent discrepancy between PF-228's effects and those of FAK shRNA transduction, we used a second genetic

approach to inhibit FAK, namely the stable transduction of doxycycline-inducible FRNK vector leading to the overexpression of FRNK, a truncated protein including only FAK's carboxy-terminal noncatalytic domain and a well-known physical repressor of FAK signaling (28, 37). We observed that, as with PF-228, FRNK overexpression inhibited cell proliferation and DNA synthesis and increased apoptosis in SCLC cell lines. At this step, we hypothesized that the opposite results obtained with the two genetic approaches we used to inhibit FAK were related to FRNK, absent in cells transduced with FAK shRNA while present in those transduced with doxycycline-inducible FRNK vector and

expressing FRNK. Our hypothesis was confirmed in double-transduced SCLC cell lines, first with FAK shRNA and then with FRNK, which revealed antitumoral effects in cells overexpressing FRNK. In a similar way, it has previously been reported that different methods of FAK inhibition result in different functional outcomes in endothelial cells: approaches inhibiting FAK phosphorylation at Tyr397 (such as FAK small-molecule inhibitors or FRNK transduction) inhibited proliferation and migration, while those abolishing FAK expression (such as FAK shRNA or siRNA) did not impact on these cellular processes (29). Also supporting the importance of FRNK in the regulation of proliferation, a previous report showed that expressing FRNK with a C1034S mutation disrupted focal adhesion binding but had no effect on proliferation (38).

In endothelial cells, FAK has been proposed as a phospho-regulated repressor of the activation of Rac (29), a pro-proliferative GTPase present in focal adhesions (31, 39). This was based on the observation that FRNK expression, FAK Tyr397F mutation (simple substitution of Tyr397 with a nonphosphorylated residue), or treatment with a FAK kinase inhibitor decreased Rac activation induced by complete growth medium, while the physical loss of FAK following FAK shRNA transduction did not affect it (29). Similarly, in cells double-transduced with FAK shRNA and doxycycline-inducible FRNK vector, we found high level of activated Rac1 in cells overexpressing FRNK, whereas it was low in the absence of FRNK expression. On the basis of these results, we propose the following model in SCLC, schematized in Fig. 6: (i) In normal conditions (absence of FAK inhibition) in SCLC, FAK constitutive activation results in FAK phosphorylation at Tyr397, leading to the activation of downstream phosphorylation-dependent signaling and to changes in the conformation of FAK and/or its binding partners, which allow Rac1 activation in the focal adhesion complex. (ii) PF-228 and FRNK overexpression both inhibit FAK phosphorylation at Tyr397, leading to the inhibition of downstream signaling and the absence of change in conformation of FAK and/or its binding partners, which prevents Rac1 activation. This results in antitumoral effects, as observed in our experiments. (iii) In contrast, the physical loss of FAK after FAK shRNA transduction induces an inhibition of FAK phosphorylation-dependent signals but allows the activation of Rac1 because of the absence of repression by FAK. This last event results in protumoral effects counterbalancing the antitumoral effects of FAK phosphorylation inhibition, explaining why FAK shRNA transduction did not affect proliferation and survival in the SCLC cell lines we tested.

Altogether our results suggest that, to induce proliferation and survival in SCLC cell lines, the physical presence of FAK is not required because the physical loss of FAK release the repressive signal on Rac and allows its activation, which induces proliferation and survival. In contrast, when the FRNK region of the FAT domain is present, FAK phosphorylation at Tyr397 seems necessary to induce proliferation and survival. Importantly, in a natural setting, there is no FAK shRNA; normal or cancer cells express total FAK (including FRNK) and FAK phosphorylation at Tyr397 is required for their proliferation and survival. Therefore, we can conclude that FAK plays an important role in various protumoral properties of SCLC through its kinase domain and that inhibiting FAK phosphorylation at Tyr397 may have a therapeutic potential. Even though the discoveries made with FAK shRNA correspond to an artificial setting, they suggest that FAK small-molecule

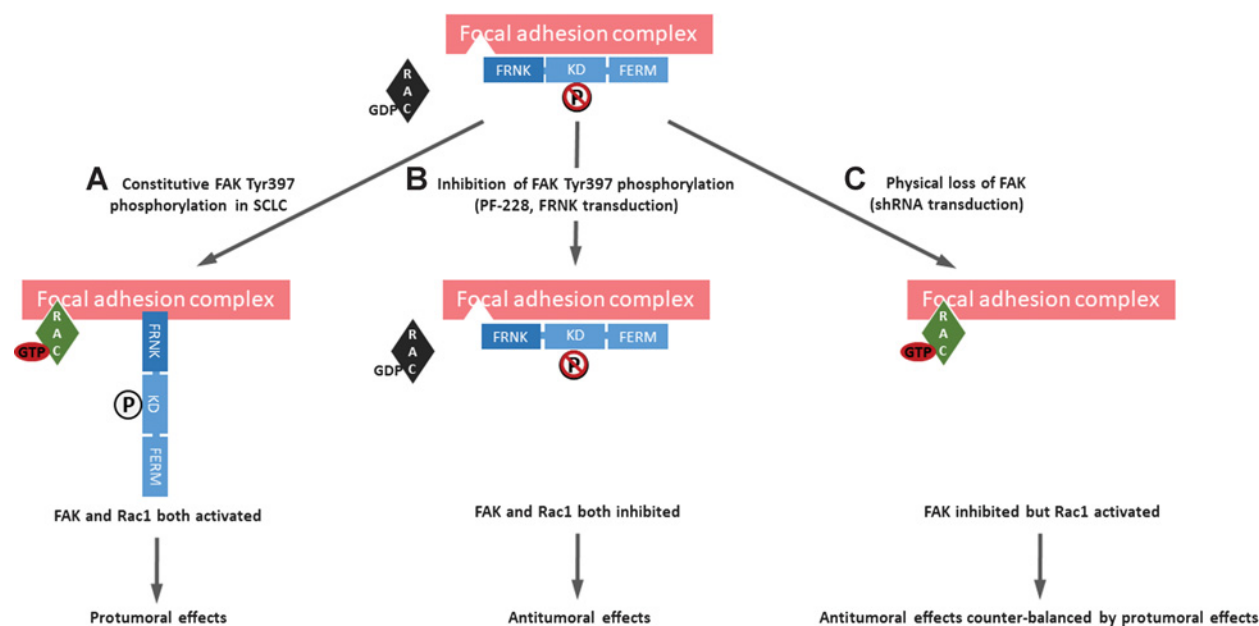


Figure 6.

A model of FAK as a regulator of Rac1 activation in SCLC. **A**, "Normal" conditions in SCLC with FAK constitutively activated. FAK phosphorylation at Tyr397 leads to the activation of downstream phosphorylation-dependent signaling and to changes in the conformation of FAK and/or its binding partners, which allow Rac1 activation in the focal adhesion complex. These two events result in protumoral effects. **B**, FAK inhibition by PF-228 and FRNK overexpression. These two methods inhibit FAK phosphorylation at Tyr397, leading to the inhibition of downstream signaling and the absence of change in conformation of FAK and/or its binding partners, which prevents Rac1 activation. These two events result in antitumoral effects. **C**, FAK inhibition by FAK shRNA transduction. The physical loss of FAK induces an inhibition of FAK phosphorylation-dependent signals but allows the activation of Rac1 because of the absence of repression by FAK. The antitumoral effects related to the absence of FAK are counterbalanced by the protumoral effects related to Rac1 activation.

inhibitors should target the kinase domain but not FAK's regions which play a repressive role on pro-proliferative proteins, such as FRNK on Rac. Recently, small-molecule inhibitors targeting different FAK scaffolding protein-protein interactions have been developed and shown to induce antitumoral effects in preclinical studies (16), but further development of such inhibitors should take into account the complexity of FAK to be successful.

Of note, we did not find any phospho-Paxillin (Tyr118) expression modification in SCLC cells where FAK was inhibited with PF-228 or FAK shRNA ± FRNK transduction. Because FAK is known to phosphorylate Paxillin and phospho-Paxillin to activate Rac1 via the adaptor protein CrkII (32), we expected to find an inhibition of Paxillin phosphorylation following FAK inhibition. However, similarly to our observation, previous studies also reported that FAK did not affect Paxillin tyrosine phosphorylation level (40, 41). Further investigations are required to better understand these observations.

To be mentioned, although PF-228 induced cell-cycle arrest in G₂ and S phases, FRNK transduction induced cell-cycle arrest in S-phase only, which was however sufficient to impact on proliferation and apoptosis. We assume that this discrepancy is related to an off-target effect of PF-228, which often happens with small-molecule inhibitors, even the specific ones. Nevertheless, this does not change the conclusion that FAK plays a role in SCLC proliferation and survival, because we showed that PF-228 and FRNK transduction both inhibited cell proliferation and induced apoptosis in SCLC cell lines.

More in-depth investigation of FAK's role in cell cycle, apoptosis, and specifically DDR in SCLC may be relevant. Indeed, a recent study showed that FAK regulates DDR and that FAK inhibition by PF-271, RNA interference, or CRISPR/Cas9 gene editing induces persistent DNA damage and radiosensitizes KRAS-mutated NSCLC cell lines and xenografts (15). In parallel, another study showed that nuclear FAK stimulates gene transcription favoring DDR and that FAK ablation by CRISPR/Cas9 editing induces DNA damage and increased radiosensitivity in NSCLC cells (13). Similarly, it has recently been demonstrated that FAK overexpression is a radioresistance biomarker in locally advanced HPV-negative head and neck squamous cell carcinoma (HNSCC), also a smoking-related malignancy, and that its inhibition with PF-271 radiosensitized HNSCC cell lines, with increased G₂-M arrest and DNA damage (14). In this context, combining FAK small-molecule inhibitor with radiotherapy in SCLC certainly deserves further investigations.

In summary, experiments using PF-228, a FAK small-molecule inhibitor, showed that inhibition of FAK phosphorylation at Tyr397 decreased proliferation, induced cell-cycle arrest, increased apoptosis, and decreased motility and invasion in SCLC cell lines. FAK inhibition by FRNK overexpression after transduction of a doxycycline-inducible FRNK vector also induced inhibition of proliferation and survival, suggesting the specificity of PF-228. In contrast, FAK inhibition by FAK shRNA transduction

did not affect proliferation and survival, probably because the physical absence of FRNK released a repressive signal on Rac, a pro-proliferative protein. Taken collectively, these data demonstrate that FAK is important in SCLC biology and that targeting its kinase domain may have a therapeutic potential in SCLC, while targeting its FAT domain should be avoided or done carefully to prevent pro-proliferative proteins from counter-balancing the antitumoral effects of FAK inhibition. Further studies in SCLC xenograft models are required to better understand the complexity of FAK in SCLC. Ultimately, this may lead to the evaluation of FAK inhibitors in clinical trials of patients suffering from SCLC, a deadly disease that still lacks efficient targeted therapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): F.A. Nana, M. Lecocq, C. Pilette, S. Ocak

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): F.A. Nana, M. Lecocq, M.Z. Ladjemi, S. Dupasquier, S. Ocak

Writing, review, and/or revision of the manuscript: F.A. Nana, M. Lecocq, M.Z. Ladjemi, S. Dupasquier, O. Feron, P.P. Massion, Y. Sibille, C. Pilette, S. Ocak

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Study supervision: S. Ocak

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Molecular Cancer Therapeutics

Therapeutic Potential of Focal Adhesion Kinase Inhibition in Small Cell Lung Cancer

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