Antitumor Activity and Pharmacology of a Selective Focal Adhesion Kinase Inhibitor, PF-562,271

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Abstract
Cancer cells are characterized by the ability to grow in an anchorage-independent manner. The activity of the nonreceptor tyrosine kinase, focal adhesion kinase (FAK), is thought to contribute to this phenotype. FAK localizes in focal adhesion plaques and has a role as a scaffolding and signaling protein for other adhesion molecules. Recent studies show a strong correlation between increased FAK expression and phosphorylation status and the invasive phenotype of aggressive human tumors. PF-562,271 is a potent, ATP-competitive, reversible inhibitor of FAK and Pyk2 catalytic activity with a IC\textsubscript{50} of 1.5 and 14 nmol/L, respectively. Additionally, PF-562,271 displayed robust inhibition in an inducible cell-based assay measuring phospho-FAK with an IC\textsubscript{50} of 5 nmol/L. PF-562,271 was evaluated against multiple kinases and displays >100× selectivity against a long list of nontarget kinases. PF-562,271 inhibits FAK phosphorylation \textit{in vivo} in a dose-dependent fashion (calculated EC\textsubscript{50} of 93 ng/mL, total) after p.o. administration to tumor-bearing mice. \textit{In vivo} inhibition of FAK phosphorylation (>30%) was sustained for >4 hours with a single p.o. dose of 33 mg/kg. Antitumor efficacy and regressions were observed in multiple human s.c. xenograft models. No weight loss, morbidity, or mortality were observed in any \textit{in vivo} experiment. Tumor growth inhibition was dose and drug exposure dependent. Taken together, these data show that kinase inhibition with an ATP-competitive small molecule inhibitor of FAK decreases the phospho-status \textit{in vivo}, resulting in robust antitumor activity. [Cancer Res 2008;68(6):1935–44]

Introduction
A hallmark of cancer is the ability for cells to proliferate regardless of adhesion to a substrate (1). Mounting evidence suggests that focal adhesion kinase (FAK) plays an essential role in the ability of cells to grow in an anchorage-independent manner (2). FAK is a nonreceptor tyrosine kinase that transduces signaling from a diverse group of stimuli (e.g., integrins, cytokines, chemo- kines, and growth factors) to control a variety of cellular pathways and processes, including cell proliferation, migration, morphology, and cell survival (3, 4). Consistent with these cellular functions and implicating the importance of FAK in tumor progression, exogenous expression of the tumor suppressor gene PTEN results in dephosphorylation of FAK (5). FAK is found at elevated levels in most human cancers, particularly as cancers transform into highly invasive metastases (6). This makes disruption of FAK activity and function an exciting and novel approach to targeted anticancer therapy against virtually any solid tumor (7).

FAK is localized at sites of contact with the extracellular matrix (ECM; focal adhesions) and serves to transduce signals from integrin receptors and enhance signaling by growth factor receptors (8, 9). The transmembrane integrin receptors are important for linking the ECM proteins with the cellular actin cytoskeleton and the nucleus to regulate cell morphology, tissue architecture, and attachment-induced gene expression (10). FAK activity enhances signaling of many receptor tyrosine kinases that are themselves viable anticancer targets, such as insulin-like growth factor receptor (IGF-IR) (11), the erbB family (12), and angiogenesis receptors (13). Given that integrin signaling is mediated through FAK catalytic activity (14), a small molecule inhibitor of FAK could be considered a nonantibody approach to inhibit integrin signaling. Interestingly, loss of FAK activity using conditional knockout mice disrupted angiogenesis in late-stage embryos but did not affect vasculogenesis (15), suggesting that FAK inhibition may also result in an antiangiogenic effect (16). These data are consistent with the role of FAK in signal transduction of known angiogenic integrins as well as the fact that overexpression of FAK in endothelial cells promotes angiogenesis (17, 18).

Pyk2 is the only other member of the FAK family with 48% amino acid identity (19). However, the role of Pyk2 in tumorigenesis is not well-established. Although FAK is expressed in most tissues and cell types, Pyk2 seems to have more limited tissue distribution (hematopoietic cells, vascular smooth muscle, endothelium, spleen, kidney, and central nervous system; ref. 19). Unlike the lethal FAK knockout (20), Pyk2 knockout mice develop normally except that they exhibit defective macrophage migration and have increased bone density (21). Interestingly and importantly for prostate and breast cancer patients with or without bone metastasis, dual FAK/Pyk2 inhibitors resulted in prevention of bone resorption and increased bone formation while inhibiting the growth of bone metastases (22, 23).

PF-562,271 represents an unprecedented approach to treating cancer through FAK inhibition. Moreover, FAK inhibition is

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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expected to affect both tumor and endothelial cells. With its novel target and dual antitumor and antiangiogenesis mechanisms of action, PF-562,271 offers the potential to enhance the current armamentarium of cancer therapeutics as a single agent or in combination with cytotoxics or other targeted agents.

Materials and Methods

Chemical synthesis. PF-562,271 was identified through a combination of rational design, high throughput kinase activity screens, and structure-activity relationships as previously described (24).

Expression. The FAK catalytic domain (FAKcd) protein used for crystallization experiments was expressed with an NH2-terminal 6XHis-tag and comprises residues 410 to 689 after thrombin cleavage (FAK in aliquots at 0.08 mmol/L. Crystals grew to 0.08 mm3 over 5 d). The reaction mixture was loaded back onto the Ni-NTA column equilibrated with buffer A and the flow-through was collected. The flow-through was concentrated down to 1.5 mL and loaded directly onto a Superdex 200 HiLoad 16/60 prep grade column (GE Healthcare Bio-Sciences Corp.) equilibrated with buffer C [10 mmol/L HEPES (pH 7.5), 200 mmol/L NaCl, 1 mmol/L ammonium sulfate, and 0.1 mmol/L TCEP]. The protein was stored frozen in aliquots at –80°C in buffer C.

Crystallization. The FAKcd/PF-562,271 complex was crystallized using hanging drop vapor diffusion in 24-well VDX plates (Hampton Research). The protein was mixed 1:1 with reservoir solution containing 15% polyethylene glycol 8K, 0.1 mmol/L HEPES (pH 7.5), 0.2 mmol/L (NH4)2SO4. The concentration of PF-562,271 was 10 μmol/L. Crystals grew to 0.08 × 0.08 × 0.02 mm over 3 to 5 d.

Data collection and structure determination. X-ray diffraction data for FAKcd/PF-562,271 collected on a FRE rotating anode X-ray generator outfitted with a HTC image plate detector system (Rigaku/MSC, Inc.). After transfer to a cryoprotectant consisting of 80% reservoir solution + 20% ethylene glycol + 10 μmol/L compound, crystals were flash cooled in liquid nitrogen. All images were processed using HKL2000 (25).

The FAKcd/PF-562,271 structure was determined by the molecular replacement method, using the program MOLREP (26), using as a search model a FAKcd monomer structure derived from an ongoing structure-based drug design program. Refinement of the structures was performed using the program REFMAC (27). Arc/Warp (28, 29), and Xfit (30). Various data, coordinate, and map manipulations were performed using programs from the CCP4 suite (29).

Recombinant kinase assay and enzyme kinetics. All in vitro assays used for identification of a FAK inhibitor have been previously described (31). Briefly, purified-activated FAK kinase domain (amino acid 410–689) was reacted with 50 μmol/L ATP and 10 μg per well of a random peptide polymer of Gln and Tyr, p(Glu/Tyr), in kinase buffer [50 mmol/L HEPES (pH 7.5), 125 mmol/L NaCl, and 48 mmol/L MgCl2] for 15 min. Phosphorylation of p(Glu/Tyr) was challenged with serially diluted compound at 1/2-log concentrations starting at a top concentration of 1 μmol/L. Each concentration was tested in triplicate. Phosphorylation of p(Glu/Tyr) was detected with a general anti-phospho-tyrosine (PY20) antibody followed by horseradish peroxidase (HRP)-conjugated goat antirabbit IgG and HRP substrate was added, and absorbance readings at 450 nm were obtained after addition of stop solution (2 mol/L H2SO4).

IC50 values were determined using the Hill-Slope Model. Broad kinase selectivity profiling was performed in house and by using the KinaseProfiler Selectivity Screening Service available through UpState Biotechnology.

Cell-based FAK phosphorylation assay. The GeneSwitch Inducible System from Invitrogen was used for the cell assay (32). Stable A431 epithelial carcinoma clones were generated to express either wild-type V5-tagged FAK protein or mutant FAKY397F V5-tagged protein under the inducible regulation of mifepristone. Stable clones were grown in DMEM 10% fetal bovine serum, 750 μg/mL Zeocin, and 50 μg/mL Hygromycin. One day before running the FAK cell ELISA, A431 FAK wild-type cells were seeded in growth medium in 96-well U-bottomed plates. After 4 to 6 h at 37°C, 5% CO2, FAK expression was induced with 0.1 μmol/L mifepristone. Uninduced controls were included. Anti-V5- or anti-FAK–coated plates were blocked in 3% bovine serum albumin (BSA)/0.5% Tween for 1 h at room temperature. Cells were treated with 1/2-log serial dilutions starting at a top concentration of 1 μmol/L for 30 min at 37°C, 5% CO2. Lysates from cells treated with indicated concentrations of compound were prepared in lysis buffer [50 mmol/L Tris (2-carboxy-ethyl)-phosphine-HCl (pH 7.4), 1% NP40, 0.25% Na-deoxycholate, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L Na3VO4, 1 mmol/L NaF; and protease inhibitors] and transferred to the anti-V5- or anti-FAK–coated plates to capture total induced or total FAK protein. Antiphosphospecific FAKY397 was used to detect autophosphorylated FAKY397 followed by secondary reporter antibody. HRP substrate was added, and plates were read at 450 mm/L. IC50 values were determined using the Hill-Slope Model. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays were performed to determine compound cytotoxicity.

Animals for in vivo studies. Athymic female mice (CD-1 Nu/Nu, ~20 grams) were used for all in vivo studies. Mice were obtained from Charles River Laboratories and housed in specific pathogen-free conditions, according to the guidelines of the Association for the Assessment and Accreditation for Laboratory Animal Care, International. All in vivo studies were carried out under approved institutional experimental animal care and use protocols.

Biotinylated ELISA. Female athymic mice were injected with 1 × 106 U87MG human glioblastoma cells on day 1. On day 9, when tumors were ~300 mm3, the mice received compound or vehicle (5% Gelucire 44/14 in sterile water; Gaetoffé) p.o. For pharmacokinetic/pharmacodynamic (PK/ PD) analysis, blood and tumor samples were collected from each animal (n > 4 mice per group per time point) into heparinized vacutainers and liquid N2, respectively, at the indicated times postdose. Blood and tumors were harvested for evaluation of drug levels and tumor-associated phospho-FAK and total FAK. Plasma concentrations of PF-562,271 were determined using reverse phase high performance liquid chromatography with mass spectrometric (MS/MS) detection. Tumors were homogenized in 1 mL lysis buffer per 200-mg tumor [lysis buffer: 50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 1.5 mmol/L MgCl2, 1 mmol/L EDTA, 1% Glycerol, 1% Triton X-100, 1.6 mol/mL Na2VO4, 10 mmol/L NaF, 25 mg/L Soy Bean Trypsin Inhibitor, EDTA-free complete Protease Inhibitor Tablets, spun 5 min at 14,000 rpm, and the supernatant were aliquoted to 96-well polypropylene plates on dry ice. Total protein concentration was determined using BSA protein assay (Pierce). Ninety-six–well goat-anti-rabbit ReactiBind plates (Pierce) were blocked with 100 μL per well cold blocking buffer (TBS, 0.1% Tween 20, and 3% BSA) for 60 min on a plate shaker at room temperature. The blocking buffer was replaced with 0.5 μg anti-phosphoFAK397 in 100 μL cold blocking buffer per well and incubated for 60 min at room temperature with...
agitation. Plates were washed with TBS-T (TBS, 0.1% Tween 20) before addition of tumor lysate (500 µg total protein in lysis buffer without protease inhibitors) and incubated 2 h at room temperature with agitation. Captured pFAK was detected with anti-FAK Ab and then incubated with 15 ng HRP-anti-IgG per well (in blocking buffer) for 30 min at room temperature. The plates were washed as above and phosphotyrosine quantitated using 3, 5', 5'-tetramethylbenzidine as described above. All phospho-FAK inhibition data are analyzed by comparing PF-562,271-treated tumors to vehicle-treated tumors.

**Prediction of efficacious concentration.** Blood and tumor samples were collected at each time point postadministration of PF-562,271 for determination of blood drug concentration and FAK phosphotyrosine reduction. The relationship between compound concentration and FAK phosphotyrosine reduction has been explored in pharmacologic models (tumor-bearing athymic mice) with pooled experimental data from multiple individual studies. FAK phosphotyrosine reduction correlates well with blood concentrations of PF-562,271 in athymic mice and follows a simple E\text{max} pharmacodynamic model:

\[ E = \frac{E_{\text{max}} \times C}{EC_{50} + C} \]

where \( E \) is the measured response (FAK phosphotyrosine reduction), \( E_{\text{max}} \) is the maximum response, \( EC_{50} \) is the concentration of PF-562,271 required for half-maximal reduction of FAK phosphotyrosine, and \( C \) is the blood concentration of PF-562,271.

**Tumor growth inhibition studies.** All tumor cell lines were obtained from American Type Culture Collection (ATCC) and propagated by standard tissue culture procedures in the medium suggested by ATCC. Exponentially growing cells were trypsinized and resuspended in sterile PBS and inoculated s.c. (1 × 10⁵ cells per mouse in 200 µL) into the right flank of mice. Animals bearing tumors of ~150 mm³ in size were divided into groups receiving either vehicle (5% Gelucire) or PF-562,271 (diluted in 0.5% methylcellulose). Animal body weight and tumor measurements were obtained every 2 d. Tumor volume (mm³) was calculated as previously described (33). For all tumor growth inhibition studies, the addition of tumor lysate (500 µg total protein in lysis buffer without protease inhibitors) and incubated 2 h at room temperature with agitation. Captured pFAK was detected with anti-FAK Ab and then incubated with 15 ng HRP-anti-IgG per well (in blocking buffer) for 30 min at room temperature. The plates were washed as above and phosphotyrosine quantitated using 3, 5', 5'-tetramethylbenzidine as described above. All phospho-FAK inhibition data are analyzed by comparing PF-562,271-treated tumors to vehicle-treated tumors.

**Inhibition of FAK tyrosine kinase activity, selectivity, and autophosphorylation in intact cells.** PF-562,271 is a potent ATP-competitive, reversible inhibitor of FAK and Pyk2 kinase, with an IC₅₀ of 1.5 nmol/L (0.7 ng/mL) and 13 nmol/L (7 ng/mL), respectively (Table 1). This compound shows Michaelis-Menten kinetics, consistent with it being an ATP-competitive and reversible inhibitor of the enzyme (Fig. 1D). PF-562,271 was evaluated in a number of kinase screens and panels and displays >100× selectivity against all tested enzymes, except for some cyclin-dependent kinase (cdk) cyclin complexes (Table 1). Although PF-562,271 was shown to be a 30- to 120-nmol/L (15.2 to 60.1 ng/mL) inhibitor of cdk2/E, cdk5/p35, cdk1/B, and cdk3/E in recombinant enzyme assays, in cell-based assays evaluating the role of cdk5, a 48-hour exposure of 3.3 µmol/L PF-562,271 was required to alter cell cycle progression (Fig. 2). Furthermore, confirmatory studies failed to detect any PF-562,271 inhibitory activity against cdk5/p35 enzyme. The cell-based assay exploits the mechanism of the GeneSwitch system to exogenously control the expression and subsequent phosphorylation of FAK and the kinase-dependent autophosphorylation site at residue Y397. PF-562,271 is potent in an inducible cell-based assay measuring phospho-FAK with a IC₅₀ of 5 nmol/L (2.5 ng/mL; Table 1). Comparative cell assays suggest that PF-562,271 is ~4-fold less potent for Pyk2 than FAK (data not shown). These cell-based data show an even greater selectivity for FAK (and Pyk2) relative to other kinase targets (e.g., cdk5). Therefore, PF-562,271 is an excellent molecule to inhibit FAK activity in animal models of disease.

**Evaluation of in vivo efficacy, \( C_{\text{max}} \) or \( C_{\text{ave}} \).** In an effort to better understand the pharmacologic inhibition of FAK and the relationship to tumor growth inhibition, experiments were designed to evaluate different dosing regimens, i.e., daily dosing, twice daily dosing, and continuous coverage using osmotic mini-pumps. These experiments allow the comparison between \( C_{\text{ave}} \) and \( C_{\text{max}} \).
Cave, and Cmin and the relationship to attaining maximal efficacy, providing the critical PK/PD linkage of FAK inhibition to antitumor activity in these preclinical models. A direct comparison between daily and twice daily dose while keeping the total daily dose equivalent provides either a high Cmax with 4 hours of >50% target inhibition or a lower Cmax but extended target inhibition (Fig. 3C; Table 2). Whereas the 50 mg/kg daily dose resulted in a Cmax of 157 ng/mL (free), the corresponding tumor growth inhibition was only 45% with 37% inhibition of pFAK after 15 days of dosing in the PC-3M human prostate xenograft model. Contrast these results to the 61% tumor growth inhibition and 27% pFAK inhibition observed in the same model for the same number of dosing days while splitting the dose to provide two daily doses of 25 mg/kg (still 50 mg/kg/day) and only reaching a Cmax of 25 ng/mL.

Breadth and amount of antitumor activity with p.o. dosing. The pharmacokinetics, inhibition of phosphorylated FAK, and antitumor efficacy of PF-562,271 was evaluated in the following human s.c. xenograft models: PC-3M (prostate), BT474 (breast), BxPc3 (pancreatic), LoVo (colon), U87MG (glioblastoma), and H125 and H460 (lung; Table 2). Dose-dependent tumor growth inhibition was observed in all models. Maximum tumor inhibition for PC-3M, BT474, BxPc3, and LoVo only maintained 0.7 ng/mL (free) blood levels but did so continuously for 24 hours. This results in a lower overall inhibition of FAK phosphorylation in tumors but for extended periods of time compared with p.o. dosing (Table 2). Importantly, the tumor growth inhibition using mini-pumps was identical to the 50 mg/kg twice daily dosing regimen that resulted in a Cmax of 273 ng/mL (free; Fig. 3D).

Figure 1. A, chemical name: N-Methyl-N-(3-[2-(2-oxo-2,3-dihydro-1H-indol-5-ylamino)-5-trifluoromethyl-pyrimidin-4-ylamino]-methyl]-pyridin-2-yl)-methanesulfonamide; molecular formula: C21H2F3N7O3S • C6H6O3S; molecular weight: 507.50 (free base); 665.68 (besylate salt). B, the crystal structure of PF-562,271 (green) bound to the active site of FAK (cyan). Dashed lines, canonical backbone hydrogen bonds to Cys-502, as is the hydrogen bond between the oxindole O atom and Arg-426. Glu-506 and Asp564 are labeled for reference. Residues 428 to 435, which fold over the active site upon substrate or ligand binding, have been omitted for clarity. C, closeup view of the methyl-sulfonamide interaction with the DFG region of FAK. Orange dashed lines, hydrophobic interactions between Leu-567 and the pyridinyl moiety of PF-562,271. Note the hydrogen bond between the sulfonamide O atom and the backbone NH of Asp-564. D, a Double-Reciprocal Plot (Lineweaver-Burke) of velocity versus ATP concentration (50 to 200 nmol/L) at varying PF-562,271 concentrations (1 pmol/L to 9 nmol/L) demonstrating competitive inhibition. PF-562,271 competitively inhibits FAK at equivalent Vmax rates over the concentration range tested: VM, ATP = 0.059 nmol/L min; VM, 1 pmol/L = 0.060 pmol/L min; VM, 1 nmol/L = 0.049 pmol/L min; VM, 3 nmol/L = 0.037 nmol/L min; and VM, 9 nmol/L = 0.032 nmol/L min.
Regressions were observed in PC-3M, BT474, BxPc3, and LoVo models at doses of 25 to 50 mg/kg twice daily, corresponding to $C_{\text{max}}$ (free) ranges of 78 to 885 ng/mL, $C_{\text{ave}}$ (free) of 14 to 40 ng/mL, and inhibition of phospho-FAK of 31% to 76% for $>4$ hours. No weight loss, morbidity, or death was observed in any tumor growth inhibition (TGI) experiment (up to 50 mg/kg twice daily × 29 days or 100 mg/kg daily × 25 days). All data are based on 6 to 10 animals per dose, and experiments were completed at least twice. After dosing, animals were euthanized, blood and tumor were analyzed for drug concentration (PK), and tumors were evaluated for phospho-FAK (PD).

**In vivo mechanism of action.** The primary mechanism by which FAK inhibits tumor growth is believed to be through the induction of anoikis (i.e., anchorage-dependent apoptosis). To test whether inhibition of FAK activity in tumors induces apoptosis, mice bearing H125 lung xenografts were treated with PF-562,271 twice daily for 3 or 10 days to evaluate apoptosis or tumor growth inhibition, respectively. Apoptosis cannot be evaluated at the end of TGI experiments because tumors are too necrotic to obtain reliable staining data. At a dose of 25 mg/kg, apoptosis was 2-fold greater in treated tumors compared with vehicle-treated control tumors on day 3 (Fig. 4). There was no increase in apoptosis at doses of 12.5 mg/kg at day 3, although tumor growth was inhibited 44% by day 10. FAK inhibition may also result in an antiangiogenic effect due to its linkage to αv integrin signal transduction. As shown in Fig. 4D, U87MG tumors treated with a PF-562,271 derivative (50 mg/kg, twice daily, p.o.) resulted in a ~50% decrease in microvascular density after only 3 days of treatment.

**Table 1. In vitro profile of PF-562,271**

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC$_{50}$ nmol/L</th>
<th>IC$_{50}$ ng/mL</th>
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<tr>
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<td>Pyk2</td>
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Abbreviations: JNK, c-Jun-NH$_2$-kinase; PDGFR, platelet-derived growth factor receptor.

**Discussion**

In this report, we characterize the pharmacology of a selective small molecule inhibitor of FAK, PF-562,271. These preclinical pharmacology studies helped determine the appropriate utility of this compound resulting in the advance to human clinical trails for the treatment of cancer. PF-562,271 has been administered to >60 patients in the phase I setting. Although no objective responses were reported, several patients have experienced prolonged disease stabilization of 6 months or longer, including some beyond 1 year. These include patients with a variety of tumor types including sarcoma, renal, lung, lacrimal, and prostate cancers. Moreover, patients have shown a metabolic response by 18 F-fluoro-2-deoxyglucose–positron emission tomography (PET) scan criteria. For example, one patient with stage IV ovarian cancer showed a metabolic response by PET scan criteria, with a reduction in the standardized uptake value signal of a splenic metastasis by 46%, 2 weeks after taking PF-562,271 (34).

Crystallographic analysis of PF-562,271 with the catalytic domain of FAK combined with the Scatchard analysis confirms that the compound binds in the ATP-binding pocket consistent with it being an ATP-competitive and reversible inhibitor of FAK (35). The most remarkable feature of the crystal structure of FAKcd/PF-562,271 is the conformation of the residues comprising the conserved "DFG motif," which adopt a helical conformation. This helical conformation is driven primarily by interactions between the methane sulfonamide moiety and the backbone NH of Asp 564, as well as hydrophobic interactions between the side chain of Leu 567 and the pyridinyl moiety of PF-562,271. In addition to perturbing the conformation of the activation loop, this helix forces the side chain of Asp 564 upwards and away from the ATP-binding cleft, where it interacts with the conserved Lys 454-Glu 471 salt bridge. This Lys-Glu salt bridge is a key feature of activated protein kinases, correctly orienting the "C-helix" of the kinase and establishing the proper orientation of the NH$_2$- and COOH-terminal loops for catalysis. Because Asp 564 is believed to play an important role in catalysis by coordinating a Mg$^{2+}$ ion and helping to stabilize the β- and γ-phosphates of Mg-ATP (36), the repositioning of the Asp 564 side chain would presumably abolish ATP binding by removing this critical interaction. In addition to increased potency, this sulfonamide interaction pocket may explain to some extent the exquisite selectivity of PF-562,271 for FAK because the activation loop region is the most plastic and idiosyncratic structure in the otherwise well-conserved protein kinase topology. Finally, although helical segments in the activation loop region have been seen in other protein kinases, including CDK2, Src, Hck, MAP/ERK kinase (MAPK), and epidermal growth factor receptor (EGFR; refs. 37–39), the structure reported here is the first to include directly the catalytically important "DFG motif." It will be interesting to determine if the DFG helix observed here functions in an analogous manner to these other kinases in contributing to an autoinhibited state of the enzyme.

PF-562,271 showed selectivity against a wide panel of kinases, as well as nanomolar potency in a cell-based assay of FAK activity. There have been previous reports of inhibitors with cross-reactivity against FAK, but what distinguishes PF-562,271 from these other inhibitors is the potency for FAK and the selectivity against other kinases, especially IGF-1R, IR, and src (40). The moderate activity of this compound against some cdk's in recombinant kinase assays was insufficient to translate into cellular effects expected of a cdk inhibitor (41). Given that the no-effect concentration for inhibition...
of cell-based cdk activity (>1,100 ng/mL) is far in excess of the FAK in vivo and in vitro IC_{50} (in vivo C_{max}, 69 nmol/L; in vitro C_{max}, 1 nmol/L). PF-562,271 is not considered to be a pharmacologically relevant inhibitor of cdk in vitro or in vivo. When activated, FAK becomes phosphorylated at Y397, which is a binding site for src; thus, both catalytically active molecules are colocalized within focal adhesions (42). Src, FAK, and integrin signaling have long been implicated in cancer biology. Given the catalytic capacity of both FAK and src (43), it is imperative that PF-562,271 is selective for FAK to accurately elucidate the role of FAK and correctly interpret antitumor effects. Because PF-562,271 (and a closely related compound, PF-573,228) showed substantial kinase selectivity for FAK compared with

Figure 2. Cell cycle progression analyzed by fluorescence-activated cell sorting after treatment with PF-562,271. PC3-M cells were maintained in growth medium (A), starved (B), or treated with PF-562,271 for 48 h [in growth medium; 1.1 μmol/L (C); 3.3 μmol/L (D)]. E, compilation of area under the curve for S and G_{2}-M fractions of each experimental group. These data (A–D) are representative of three individual experiments; average and SE are depicted in E.
other kinases, it is now possible to dissect the specific role of FAK ATP catalysis directly (31). More importantly, PF-562,271 has the appropriate pharmacologic properties for clinical testing in humans. This molecule is not only an excellent tool for understanding the role of FAK in vitro and in vivo but also the appropriate molecule to test the hypothesis in the clinical disease setting.

A rigorous in vivo PK/PD evaluation was completed for PF-562,271. The compound is well-absorbed with maximal blood levels occurring between 30 minutes and 2 hours after p.o. administration. Maximal pharmacodynamic modulation occurs simultaneously with maximal pharmacokinetic exposure in the blood regardless of dose or number of repeated doses. Measured PK is accurately modeled using in vitro and in vivo calculation of absorption, distribution, metabolism, and excretion, demonstrating a well-behaved and predictable in vivo pharmacology.

The ability to modulate FAK phosphorylation after p.o. administration of PF-562,271 was evaluated in multiple tumor types, but for routine assessment, the U87MG model was used. This was due to the robust and consistent growth of the tumor, allowing for an efficient drug discovery platform, as well as the elevated expression and activation of FAK in these tumor xenografts. However, inhibition of phospho-FAK was observed in every tumor model to varying degrees. TGI in the nonclinical models was shown with both intermittent and sustained exposures. It is interesting and clinically relevant to note that transient maximal inhibition of FAK activity never provided the greatest antitumor efficacy. This is clearly evident when twice daily dosing is compared with daily dosing and most strikingly shown when mini-pumps are used. Twice daily dosing and mini-pump experiments (steady state concentration) resulted in greater TGI in multiple models compared with daily dosing (equivalent total daily dose), suggesting $C_{\text{ave}}$ and the time above $C_{\text{ave}}$ are more relevant to efficacy than $C_{\text{max}}$. These data clearly show that the area under the curve for target modulation and not maximal inhibition yields maximal antitumor efficacy. This characteristic has been observed with other receptor tyrosine kinase inhibitors (33). The mini-pump model also better mimics the clinical dosing regimen, where repeated dosing will result in a sustained steady-state exposure of the compound over the entire dosing period (34).

PF-562,271 was evaluated in multiple tumor xenograft models to show breadth of activity, although these models do not predict clinical efficacy nor do they adequately represent the clinical tumor types. The xenograft models were chosen due to increased FAK expression and/or FAK activation (44–46). It is important to note that although FAK expression and activation are elevated in these...
models, tumor growth in these models is not primarily driven by
FAK expression nor due to activating mutations in FAK. Regardless
of the multitude of activated pathways (e.g., EGFR, PDGFR, VEGFR,
and src) and disrupted/inactive tumor suppressors (e.g., PTEN), PF-
562,271 showed robust antitumor activity as a single agent in every
model at well-tolerated doses achieving low to moderate blood
levels. Moreover, regressions were observed in most models with up
to 50% of a dose group resulting in regressions. This level of
antitumor activity was established for repeated dosing of up to 29
days with no decrease in body weight or animal activity,
demonstrating that PF-562,271 is very well-tolerated and potent
in these models.

The function of FAK as a transducer of integrin engagement is
well-established (3). Cells detached from matrix in vitro
undergo
anoikis (attachment-mediated apoptosis) with a concomitant
decrease in FAK activity (1). To evaluate the molecular changes
associated with the inhibition of FAK in vivo
and evaluate a
potential mechanism behind the dramatic antitumor efficacy, we
measured the amount of apoptosis after treatment with PF-
562,271. Consistent with the association of FAK signaling and
apoptosis (47), tumors had a significant increase in apoptotic
bodies after p.o. administration of PF-562,271 when compared
with vehicle-treated animals. Increased apoptosis was observed
with increased individual and cumulative dose, aligned with
pharmacokinetic and pharmacodynamic modulation of FAK
activity. Interestingly, on favorable sections, apoptotic vascular
cells could be identified (data not shown). Moreover, microvas-
cular density was decreased by 50% in U87 tumors after FAK
inhibition, consistent with the linkage of FAK to αvβ3 integrin
signaling and previous reports of FAK expression in angiogenic
blood vessels (48). Therefore, although FAK inhibition is primarily
an antitumor approach, it should also be considered antiangi-
ogenic. Induction of apoptosis occurred as early as 3 days of
dosing. At this time point, there is no obvious distinction in
tumor growth between vehicle- and PF-562,271–treated animals,
suggesting that apoptosis may be one of the earliest functional
changes after FAK catalytic inhibition in vivo
. These results
suggest that inhibitors of FAK activity could limit tumor growth
and metastasis through induction of tumor and endothelial cell
apoptosis and inhibition of migration.

Broadly speaking, pharmacologic anticancer therapy consists of
cytotoxics and targeted agents (small molecules and biologics).
Unfortunately, the majority of patients treated with these agents
eventually progress. In addition, toxicities associated with these
agents often preclude adequate treatment. In this report, the
in vivo
pharmacology of a highly selective and potent inhibitor of

<table>
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<tr>
<th>Tumor growth inhibition studies</th>
<th>EC50 (ng/mL)</th>
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<tr>
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<td>50 BID</td>
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Abbreviations: Conc, concentration; inh, inhibition; QD, daily; BID, twice daily; ND, not determined.

*Calculated from AUC0-t(last)/h.

% inhibition of phosphorylated FAK in the tumor.

Statistically different from vehicle control samples calculated using a Student’s t test with a P value of <0.05.

Steady state concentration.
FAK catalytic activity, PF-562,271, is described. The novel mode of inhibition of FAK, characterized by the inhibitor-induced "DFG helix," resulted in profound antitumor activity across a wide variety of tumor types while being well-tolerated. PF-562,271 showed well-behaved pharmacology in vivo with a robust PK/PD relationship. PF-562,271 shows the selectivity and pharmacology that has allowed it to be a first in class inhibitor presently in clinical testing for the treatment of cancer.

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Antitumor Activity and Pharmacology of a Selective Focal Adhesion Kinase Inhibitor, PF-562,271

Walter Gregory Roberts, Ethan Ung, Pamela Whalen, et al.


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