Supporting Information

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SI Materials and Methods

Materials. The following reagents were purchased from Sigma-Aldrich: lactate dehydrogenase, pyruvate kinase, phosphoenolpyruvate, Hepes, MES, NaCl, DTT, $MgCl_2$, ATP, and NADH. Peptide 7 (EVPRRKSLVGTPYWM) and Syntide2 (PLARTLSVAGLPGKK were purchased (CPC Scientific). PAK4, phospho-S474-PAK4, and GEF-H1 were previously described (1). Also, phospho-S474-PAK4 antibodies were purchased (Cell Signaling Technologies).

Chemical Properties of PF-3758309. N-[(1S)-2-(dimethylamino)-1-phenylethyl]-6,6-dimethyl-3-[(2-methylthieno[3,2-d]pyrimidin-4-yl)amino]-4,6-dihydropyrrolo[3,4-c]pyrazole-5(^{1}H)-carboxamide. Molecular formula: $C_{25}H_{30}N_8OS$. Molecular weight: 490.62. Optical rotation: $[\alpha]D = -69.03°$ at 3.77 mg/mL in methanol.

Expression, Purification, and Characterization of Recombinant Human PAK4-Kinase Domain. The kinase domain of PAK4 (amino acids 300–591; GenBank accession no. NM005884) was PCR-cloned into the bacterial expression vector pET28a (EMD Biosciences), which produced a thrombin-cleavable N-terminal His_6-tagged fusion protein. Induced cells (100 μM IPTG, 16 h, 16 °C) were lysed with a microfluidizer (Microfluidics) and clarified ($5{,}000 \times g$ for 1 h). The supernatant was passed twice over a 5-mL HisTrap column (GE Healthcare Life Sciences). Bound protein was washed, eluted (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 14 mM β-mercaptoethanol, and 400 mM imidazole), and dialyzed (20 mM Tris-HCl, pH 8.0, 250 mM NaCl, 14 mM β-mercaptoethanol, and 20 mM imidazole). Proteolytic cleavage was carried out overnight with 20–40 units of thrombin (EMD Biosciences). A reverse-nickel resin binding step was used to remove uncleaved PAK4. The unbound fraction was concentrated (Amicon Ultracel; Millipore) and applied to an S-200 gel-filtration column (GE Healthcare Life Sciences) equilibrated in 25 mM Tris-HCl (pH 7.0), 150 mM NaCl, 10 mM DTT, and 10% glycerol. S-200 fractions containing PAK4 were pooled and concentrated. Purified PAK4 was fully phosphorylated on S474 and typically >95% pure as judged by SDS/PAGE analysis.

Biochemical Kinase Assays. Kinase activity was coupled to NADH oxidation through the activities of pyruvate kinase (PK) and lactate dehydrogenase (LDH) as previously described (2). A typical PAK4 reaction contained 25 nM PAK4, 2 mM phosphoenolpyruvate, 0.33 mM NADH, 10 mM $MgCl_2$, 1 mM DTT, 0.8 mM peptide 7, 0.04 mM ATP, 15 units/mL PK, and 15 units/mL LDH in 50 mM Hepes (pH 7.5). Initial rates were determined by a linear least-squares regression analysis of the change in product formation as a function of time. Inhibition data were fit to the equation for simple competitive binding or tight-binding inhibition (3) as necessary. Kinase assays for PF-3758309 activity toward PAK5- and PAK6-kinase domains were the same as for PAK4 except as follows: PAK5 (0.4 mM peptide 7, 0.08 mM ATP) and PAK6 (0.5 mM peptide 7, 5 mM $MgCl_2$, 0.072 mM ATP). PAK1 assays were performed in a similar manner to PAK4 except with 0.25 mM Syntide2 peptide substrate, 2.5 nM PAK1, 2 mM $MgCl_2$, and 0.2 mM ATP. PF-3758309 (1 μM) was tested at a K_m concentration of ATP against a total of 146 unique kinases at Pfizer, Invitrogen, and the University of Dundee.

Estimation of Cellular Potency. Percent inhibition values are relative potency rankings that depend on the concentration of ATP, $K_{m,ATP}$, and PF-3758309. More assay-independent potency values (K_i) were estimated. The percent inhibition value (%I) is converted to fractional activity remaining by the following equation: $i = 1 - (\%I/100)$. The fractional activity is equal to the ratio of the velocities of inhibited and uninhibited reactions: $i = v_i/v_o$. Dividing the equation for competitive inhibition (v_i) by the Michaelis–Menten equation (v_o) results in the following equation: $i = v_i/v_o = ([ATP] + K_{m,ATP})/([ATP] + K_{m,ATP}(1 + [PF\text{-}3758309]/K_i))$. If the ATP concentration equals the $K_{m,ATP}$ (K_m – level of ATP) and the inhibitor concentration is 1 μM, the equation simplifies to the following: $K_i = 1/[(2/i) - 2]$. Because the percent inhibition numbers are typically only at one concentration, the transformation from percent inhibition to K_i yields only a crude estimate of the true K_i. The cellular potency of PF-3758309 can be estimated using the cellular ATP concentration, the kinase $K_{m,ATP}$ value, and the inhibitory potency (K_i). Although the concentration of ATP in a cell should range from 1 to 5 mM, to be conservative, 2 mM was selected as the cellular concentration of ATP. The Cheng–Prusoff equation was used to estimate the biochemical potency at a physiological level of ATP: cellular $IC_{50} = K_i(1 + ([ATP]/K_{m,ATP}))$. Using this approach, the biochemical potency can be used to rank the potential for off-target kinase effects in a cellular context. Of course, there are other extremely important considerations as to why a biochemical hit does not translate into cellular potency.

Isothermal Titration Calorimetry. Isothermal titration calorimetry (ITC) experiments were carried out on a VP ITC instrument (GE Healthcare Life Sciences) at 25 °C in a buffer containing 150 mM NaCl, 25 mM Hepes (pH 7.5), and 5 mM $MgCl_2$. The protein concentration was determined spectrophotometrically (ε_{280} of 27,055 M^{-1} cm^{-1}). The compound solution in the syringe was diluted from a 100% DMSO stock into a buffer without DMSO and an equivalent amount of DMSO was added to the protein in the cell before the titration. PF-3758309 injections (15 μL, 50 μM) were made into a 5 μM PAK4 catalytic domain solution. Experiments were carried out in duplicate and data were corrected for heat of dilution and fit to a simple 1:1 binding model in ORIGIN software (GE Healthcare Life Sciences).

Surface Plasmon Resonance Assay Protocol. Using a Biacore 3000 instrument (GE Healthcare), a CM5 sensor chip was derivatized with streptavidin through standard amine coupling chemistry. PAK4 protein was minimally biotinylated in vitro using sulfo-NHS-LC-LC-biotin. The biotin-PAK4 was captured on the CM5 chip by the immobilized streptavidin. A six-concentration dilution series starting at 1 μM PF-3758309 was made. The samples were injected from lowest concentration to highest concentration. The data were analyzed and fit to a mass transport 1:1 binding model using Scrubber software (BioLogic Software).

Phospho-GEF-H1 Cellular Assay. An engineered cell line was used to measure PAK4-dependent phosphorylation of its substrate GEFH1 on S810. TR-293-KDG cells were constructed from HEK293 cells stably transfected with tetracycline-inducible PAK4-kinase domain (amino acids 291–591) and constitutively expressed HA-tagged GEFH1ΔDH (amino acids 210–921). TR-293-KDG cells (20,000 cells/well in 100 μL) were plated to polylysine-coated tissue-culture 96-well plates in low-serum media and treated with 1 μg/mL doxycycline except for negative-control wells. PF-3758309 was diluted in low-serum media containing 1 μg/mL doxycycline and added to the cells. After a 3-h incubation with PF-3758309, media were removed. $HNTG^{plus}$ is the following: 50 mM Hepes (pH 7.5), 150 mM NaCl,

1% Triton X-100, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM EGTA, plus the following inhibitors: 20 mM β-glycerophosphate, 10 mM NaF, 1 mM Na_3VO_4, and protease inhibitor mixture (Roche Applied Science). $HNTG^{plus}$ was added to the cell plate (100 µL/well), shaken (4 °C, 10 min) with 50 µL cell lysate, and transferred to an anti-HA antibody-coated plate (Invitrogen). Plates were shaken for 60 min (room temperature), the cell lysate was removed, and then washed (three times). Anti-phospho-S810-GEF-H1 antibody was added to the ELISA plates (100 µL per well) and plates were shaken for 60 min at room temperature. Anti-phospho-S810-GEF-H1 antibody was detected by a horseradish peroxidase-goat anti-rabbit antibody conjugate with 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) solution using the manufacturer's protocols (Invitrogen).

Colony Formation Cellular Assay (Anchorage-Independent Growth). A suspension of 1% agar (wt/vol) was heated in a microwave until molten and a bottom layer (0.5% agar) was prepared by coating a sterile 96-well flat-bottom black with clear bottom plate with 50 µL of a 1:1 mixture of 2× DMEM (45 °C) and 1% agarose. A top layer of agar was prepared (0.35% agar) by adding 50 µL of a 1:1 mixture of 2× DMEM (45 °C) and 0.7% agarose containing 4,000 cells. Both layers of agarose were allowed to harden at room temperature for 20–30 min before 100 µL of 1× DMEM containing the appropriate concentration of PF-3758309 was added to the wells. Plates were then incubated for 6 days before addition of 10 µL of Alamar blue and quantifying data on a fluorescent plate reader at 540 nm.

Cellular Proliferation Assays. A panel of cancer cell lines was used to test the potency of PF-3758309. On day 1, cells were plated on 384-well plates. On day 2, compounds were added to the cell culture. The cells were incubated with the compound for 3 days. On day 5, all media were aspirated from the wells. Dose-dependent effects of PF-3758309 on cell proliferation were quantified via the CellTiter-Glo Luminescent Cell Viability Assay(a,b,c) (Promega), which is a homogeneous method of determining the number of viable cells in culture based on quantitation of the ATP present, an indicator of metabolically active cells. Proliferation data displayed in Fig. 2 for HEK293T, HCT116, and SKOV3 cells (all from the American Type Culture Collection) were measured using the Cyquant NF assay as specified by the manufacturer (Invitrogen). Briefly, 24 h after seeding on 384-well plates, cells were treated with vehicle or PF-3758309, Dasatinib, or the positive control, Bleomycin (60 mU/mL). Seventy-two hours after drug treatment, cell proliferation was measured. Results for each drug were normalized to vehicle control and expressed as a percentage of maximum assay inhibition relative to Bleomycin. Error bars represent the SD from at least three experiments.

High-Content and Functional Analysis of Cellular Signaling Pathways. The use of protein-fragment complementation assay (PCA) methods to measure pathway activity and signal transduction complex subcellular localization has been previously described (ref. 4 and references therein). Briefly, HEK293 cells were transfected with expression vectors encoding the indicated proteins expressed as fusions to synthesized fragments of YFP or YFP-variant coding sequences. Transfections were performed in 384-well plates, and cells were incubated for 24 or 48 h (depending on the particular assay) before drug treatment. Cells were treated in duplicate wells with the indicated drug for 8 or 24 h, stimulated with the agonist where indicated, and then fixed and stained with Draq5 (BioStatus) before image acquisition. Cleaved PARP (in U-2 OS cells) was detected with an antibody raised against Asp214 of cleaved PARP (Cell Signaling Technology). For all high-content assays, duplicate wells were used for each drug treatment and time point. A total of 16 images for all drug treatments and 64 images for DMSO controls were acquired on an automated confocal microscopy platform (Opera; PerkinElmer). Fluorescence intensities within each image were quantified via automated image analysis using modules from the Acapella software suite (PerkinElmer). Changes in fluorescence intensity and subcellular localization for each assay were quantified as previously described (4). After normalization to vehicle control (DMSO), compound activities are displayed as percent of maximum assay inhibition or activation relative to assay-specific controls. Error bars represent the SD of at least three independent experiments performed as described above. For NF-κB pathway analysis, HEK293T cells were transfected in 384-well plates with the pNF-κB-TA-Luc luciferase plasmid (Clontech/Takara Bio), which encodes luciferase downstream of an NF-κB-specific enhancer element. After 48 h, cells were treated with vehicle, PF-3758309 (1 µM), or Dasatinib (1 µM) for 7.5 h. Cells were stimulated with 20 ng/mL TNFα (Roche Molecular Biochemicals/Boehringer Mannheim) for the last 7 h of drug treatment. Inhibition of NF-κB-luciferase reporter activation was quantified using the Steady-Glo Luciferase Assay System (Promega) per the manufacturer's recommendations. Results for each drug were normalized to vehicle control and expressed as a percentage of maximum assay inhibition relative to Bortezomib (positive control). Error bars represent the SD of the maximum inhibition in a minimum of three experiments.

In Vivo Function: Tumor Growth Inhibition, Histopathology, and [^{3}H] FLT Uptake. To evaluate the efficacy of PF-3758309 in vivo, human xenograft models were used. Tumor cells (e.g., 2×10^6 HCT116 cells/0.15 mL in 1:1 Matrigel:PBS) were s.c. implanted into the right flank of nude mice (female nu/nu, CRL breed 6–8 weeks old). For tumor growth inhibition (TGI) studies, when tumors were 8 days postimplant (170 mm^3 average, >22 g animals), animals were randomized into treatment groups. PF-3758309 formulated in 0.5% methylcellulose was dosed via oral gavage (p.o.). Tumor size measurements were made with a caliper two to three times per week until the tumor volume became ≥2,000 mm^3. For histopathology and FLT-uptake studies, mice bearing HCT116 tumors in the range between 300 and 350 mm^3 were randomized into three groups of five each. Mice were administered PF-3758309 orally twice daily (vehicle, 15 mg/kg PF-3758309, 25 mg/kg PF-3758309). Tumors were harvested on days 2, 3, and 6 after the initial dosing to perform IHC analysis. Xenograft tumors were fixed overnight in paraformaldehyde followed by dehydration in 70% ethanol. The antibody for caspase 3 and anti-Ki67 was ordered from Cell Signaling (9661; dilution 1:200) and Lab Vision (RM-9106; dilution 1:800), respectively. Stained slides were analyzed using the Chromovision automated cell-imaging system. The expression level of three serial tumor sections was evaluated according to a semi-quantitative method: (Ki67-positive cells) 0 < 40%, 1 = 40–50%, 2 = 50–60%, 3 = 60–70%, 4 > 70% and (caspase 3-positive cells) 0 = <2%, 1 = 2–3%, 2 = 4–5%, 3 = 6–7%, 4 > 7%. [Methyl-^{3}H(N)]-3′-deoxy-3′-fluorothymidine ([^{3}H]-FLT) was purchased from Moravek Biochemicals with a specific activity of 2.2 Ci/mmol. For the [^{3}H]FLT uptake test, the experiment was performed similarly to the histopathology study except that isofluorane-anesthetized mice were administered [^{3}H]FLT (15 nCi/g, 100 µL/mouse, i.p.) 2 h before tumor harvest. Two hours after radiotracer injection, the mice were killed and tumor and liver were rapidly harvested, weighed, and solubilized with Ecolume liquid scintillation fluid (VWR). Radioactivity in the samples was measured using an LS6500 multipurpose scintillation counter (Beckman Coulter). Standardized uptake values (SUV) were calculated using the following formula: $SUV = C \div (ID/W)$, where C is the measured activity of the tissue (in cpm/g), ID is the injected dose in cpm, and W is the mouse body weight (g). [^{3}H]FLT uptake in tumors was normalized and reported as the ratio of tumor-to-liver SUV.

Analysis of PF-3758309 Plasma Concentration. Plasma samples (50 μL) were spiked with 10 mL of internal standard (Buspirone; 0.50 mg/mL) and extracted with 200 μL of acetonitrile/methanol (50:50). Samples were vortexed and centrifuged, and 10 μL of the resultant supernatant was diluted with 190 μL of 10% acetonitrile/90% water and then analyzed by LC-MS/MS. Liquid chromatography was performed using a Shimadzu integrated HPLC system. The mobile phase consisted of (A) water with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid. The samples were eluted using a linear gradient of 10–90% B over 0.3 min, followed by 90% B for an additional 1.7 min. Mass spectrometry was performed using a Sciex API 4000 in the TurboIon spray positive-ion mode. Multiple reaction monitoring transitions were 491.3–284.2 for PF-3758309 and 386.3–122.2 for Buspirone. The lower limit of quantitation was 1 ng/mL PF-3758309.

PK/PD Modeling of PF-3758309 Tumor Growth Inhibition Data. Alzet minipumps were implanted in athymic nude mice bearing HCT116 tumors when the tumors reached 200 mm^3. The pump delivery of PF-3758309 (3.3, 1.1, 0.36, and 0.12 $mg \cdot kg^{-1} \cdot day^{-1}$ for 9 days) could achieve plasma concentrations of 37, 5.6, 1.7, and 0.56 ng/mL resulting 114, 104, 78, and 16% TGI, respectively. A PK/PD model was created to describe the time course of TGI of HCT116 tumor-bearing mice following pump administration. Tumor dynamics were described by an exponential function (as suggested by the data from the control group of the study) that includes cell-proliferation and cell-death rate constants. The model assumes that the concentration of compound in the central (plasma) PK compartment effectively inhibits the rate of growth of the HCT116 tumor by increasing the tumor cell death rate. The model parameters k_{pro}, k_{death}, and φ were fitted using tumor growth data from control parameters E_{max}, EC_{50}, and γ using the data from the and treated groups via naïve pool analysis. The modeled TGI IC_{50} for PF-3758309 was estimated to be 0.40 nM (unbound concentration).

$$\frac{dA_c}{dt} = -k_{el} \cdot A_c \qquad C_p = \frac{A_c}{V_c}$$
$$\frac{dTV}{dt} = \left(k_{\text{pro}} \cdot f(C_p) - k_{death}\right) \cdot TV \qquad f(C_p) = 1 - \frac{E_{\max} \cdot C_p^{\gamma}}{EC_{50}^{\gamma} + C_p^{\gamma}}$$

Reverse-Phase Protein Array Data Analysis. The relative amount of protein detected by the specific antibody (log2 scale) was plotted against PF-3758309. *P* values were used to measure the significance level of whether a given gene changes protein levels between dosages. The R statistical approach maSigPro (microarray Significant Profiles in time courses; ref. 5) was adapted for protein array data by using antibody values instead of gene probe values and grouping tumor samples by dosage instead of time. The maSigPro method uses regression to find significantly changed antibodies between dosage groups. We used a *P*-value cutoff of 0.05, which corresponds to hits 1–27. Another regression model was used to fit each antibody across doses. Antibodies with an R^2 value ≥ 0.5 are retained (a value of 1 means a perfect correlation with the regression line) so that the resulting hits 1–13 show more of a linear dosage-dependent effect.

1. Callow MG, Zozulya S, Gishizky ML, Jallal B, Smeal T (2005) PAK4 mediates morphological changes through the regulation of GEF-H1. *J Cell Sci* 118:1861–1872.
2. Murray BW, Padrique ES, Pinko C, McTigue MA (2001) *Biochemistry* 40:10243–10253.
3. Morrison JF (1969) Kinetics of the reversible inhibition of enzyme-catalysed reactions by tight-binding inhibitors. *Biochim Biophys Acta* 185:269–286.
4. MacDonald ML, et al. (2006) Identifying off-target effects and hidden phenotypes of drugs in human cells. *Nat Chem Biol* 2:329–337.
5. Conesa A, Nueda MJ, Ferrer A, Talon M (2006) maSigPro: A method to identify significantly differential expression profiles in time-course microarray experiments. *Bioinformatics* 22:1096–1102.

A

C

B

Fig. S1. Enzymatic (*A*), kinetic (*B*), and thermodynamic (*C*) characterization of PF-3758309 binding to PAK4 catalytic domain.

Fig. S2. DNA damage increases PAK4 activity and decreases p53 levels in PAK4-sensitive cells (HCT116) treated with camptothecin for 20 h. PAK4 inhibitors block PAK4 activity. PAK4 inhibitors block p53 accumulation and function.

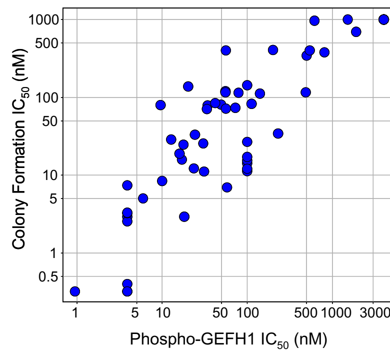
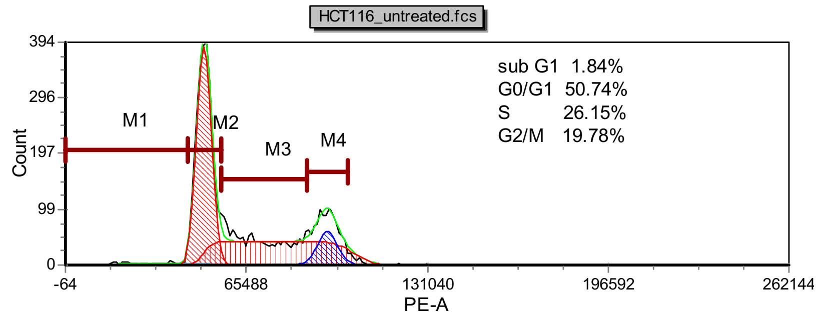


Fig. S3. The biochemical cellular and functional activities of 59 pyrrolopyrazole inhibitors structurally related to PF-3758309 (thienopyrimidine-amino-pyrrolopyrazole) are well-correlated. The phospho-GEF-H1 assay is a cellular readout of the biochemical reaction catalyzed by PAK4. Anchorage-independent growth of HCT116 tumor cells has been previously shown to be PAK4-dependent (1). The trend and absolute values of the pyrrolopyrazole inhibitor potencies in these assays are shown to be well-correlated (R^2 = 0.90). This is a central tenet of establishing causality proposed by Shokat and coworkers (2).

1. Callow MG, et al. (2002) Requirement for PAK4 in the anchorage-independent growth of human cancer cell lines. *J Biol Chem* 277:550–558.
2. Weiss WA, Taylor SS, Shokat KM (2007) Recognizing and exploiting differences between RNAi and small-molecule inhibitors. *Nat Chem Biol* 3:739–744.

A



B

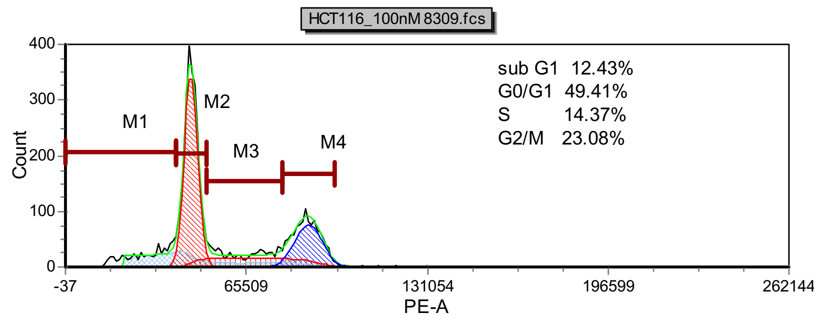


Fig. S4. Fluorescence-activated cell sorter analysis of the cell cycle on untreated (*A*) and 100 nM PF-3758309-treated (*B*) HCT116 colorectal tumor cells.

Other Supporting Information Files

Table S1. PF-3758309 antiproliferative activity toward a panel of colorectal cancer, non-small-cell lung cancer, pancreatic, and breast cell lines

Table S1 (XLS)

Anchorage-independent growth was measured by the inhibition of colony formation in soft agar.

Table S2. List of off-target kinases that have an estimated cellular IC50 < 5,000 nM

Table S2 (XLS)

Table S3. Reverse-phase protein array analysis of PF-3758309 treatment of an HCT116 human xenograft tumor model

Table S3 (XLS)

Analysis of the dose-dependent modulation data from the BayPoint reverse-phase array proteomic study of PF-3758309 in tumor-bearing mice. List of antibodies used in PF-3758309 BayPoint reverse-phase protein array phosphoproteomic study.

SI Appendix (PDF)