Supplemental Data

Small Molecule Inhibitors of the c-Fes Protein-tyrosine Kinase

Sabine Hellwig, Chandra V. Miduturu, Shigeru Kanda, Jianming Zhang, Panagis Filippakopoulos, Eidarus Salah, Xianming Deng, Hwan Geun Choi, Wenjun Zhou, Wooyoung Hur, Stefan Knapp, Nathanael S. Gray and Thomas E. Smithgall

Figure S1. Identification of Fes inhibitors using the Z-LYTE™ in vitro kinase assay and a recombinant Fes SH2-kinase domain (SH2-KD) protein (related to Figure 1). Results for two 384-well plates containing a total of 586 small molecule inhibitors at final concentrations of 1 μM are shown. Black diamonds indicate inhibition scores below the cutoff of 80% (dotted line). Crosses represent compounds producing > 80% inhibition of SH2-KD. Replicate wells containing DMSO (empty circles) served as negative controls. TAE684, a compound that showed virtually 100% inhibition of SH2-KD in a test screen, was included on both plates as a positive control (black triangles).

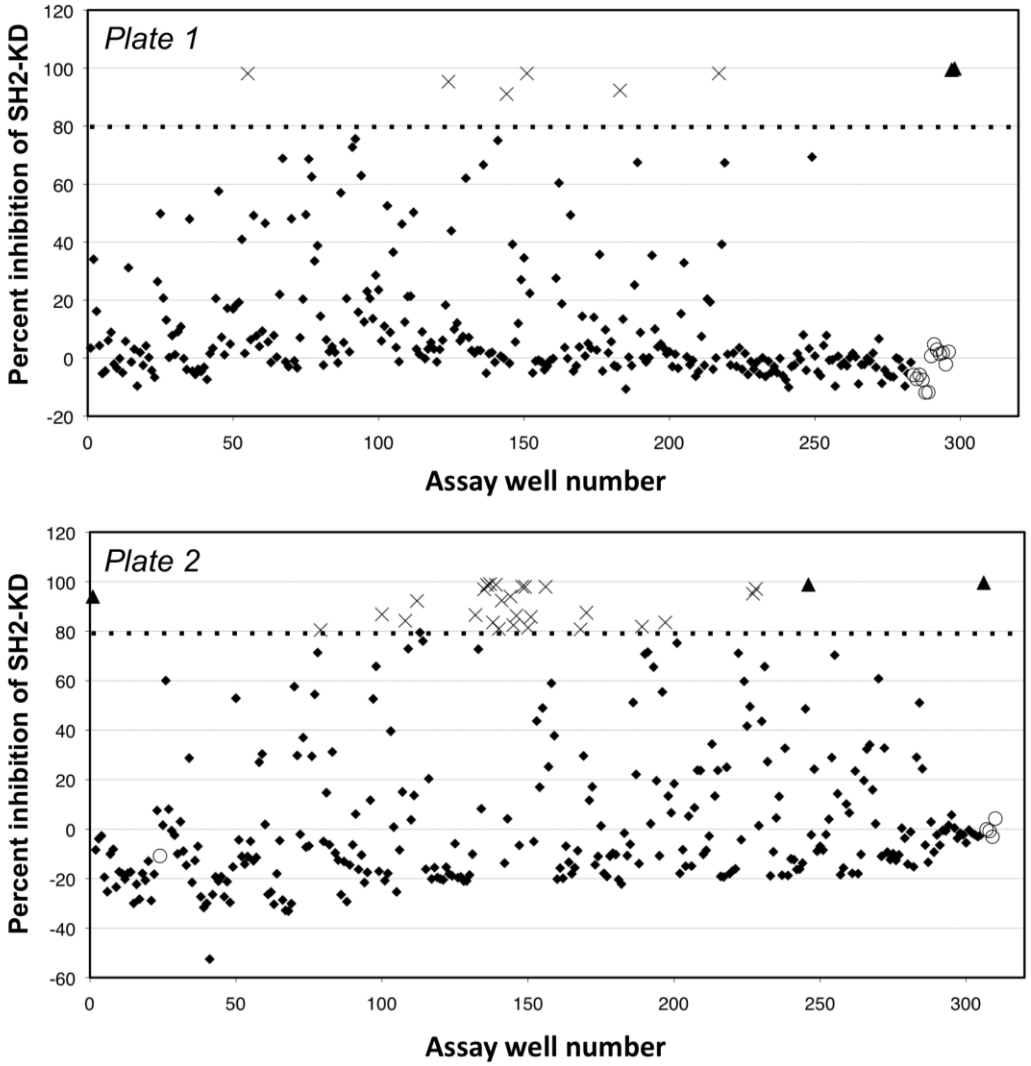
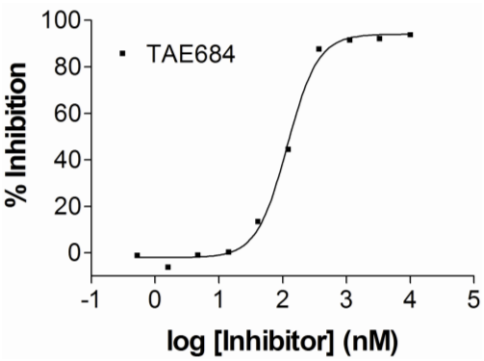
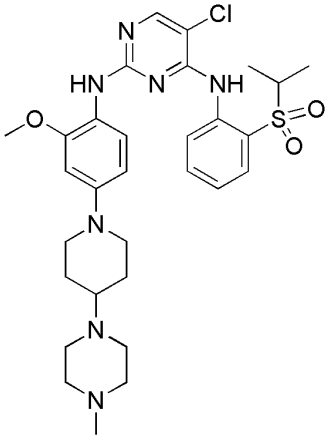


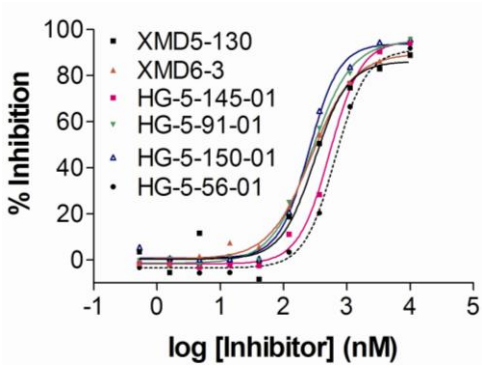
Figure S2. Concentration-response curves for Fes kinase inhibitor candidates (related to Table 1). Twenty-one compounds that inhibited peptide substrate phosphorylation by recombinant Fes SH2-KD to > 80% in the primary screen at 1 μM were retested over a concentration range of 50 nM to 10 μM using the Z-Lyte assay. Results were grouped by chemical scaffold and are presented below, along with the chemical structures. IC_{50} values were determined for each compound by curve fitting and are presented in Table 1 of the main text.

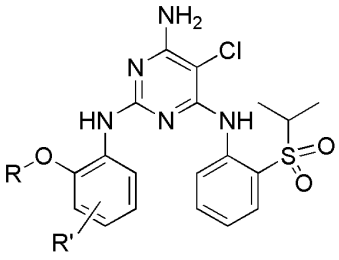
Diaminopyrimidine





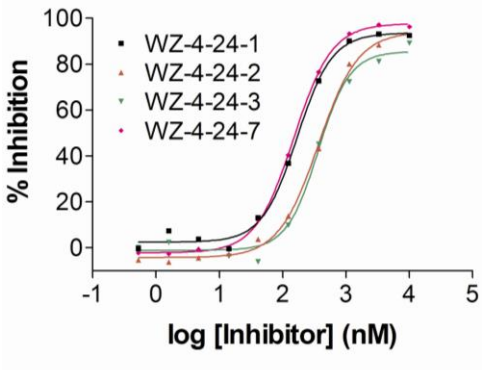
Triaminopyrimidines

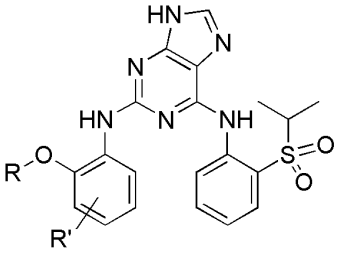




R= Me or iPr

Diaminopurines

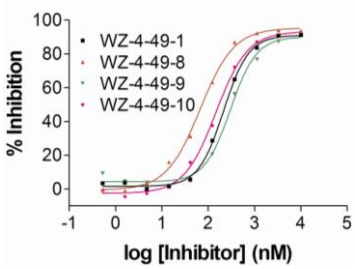


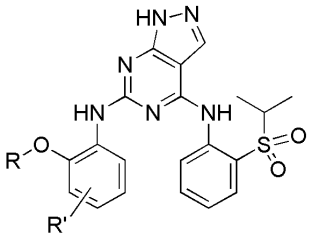


R= Me, Et or iPr

Figure S2, continued.

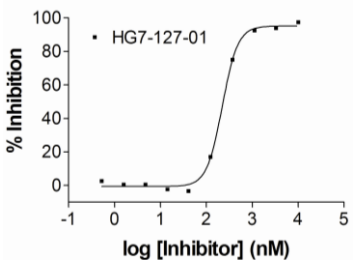
Pyrazolopyrimidines

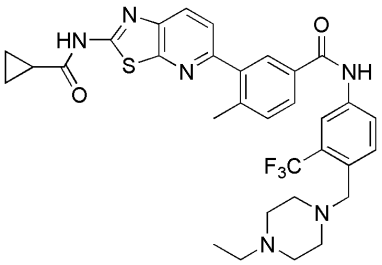




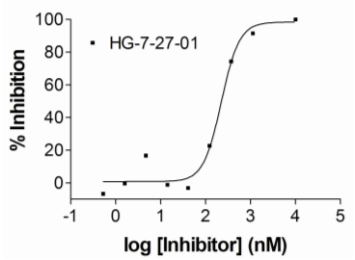
R= Me, Et or iPr

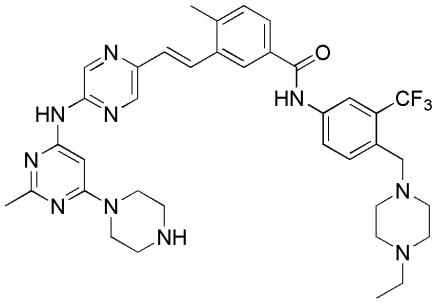
Thiazolepyridine



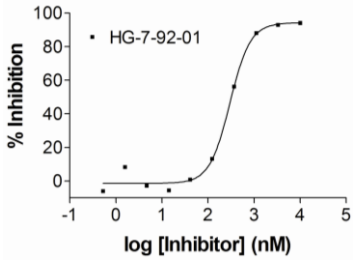


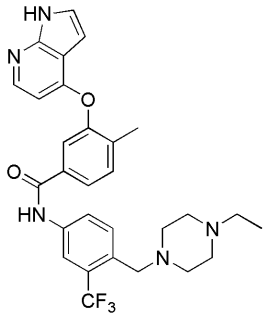
Pyrazine



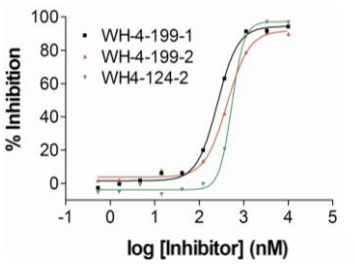


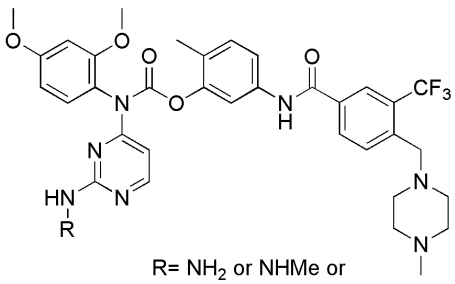
Pyrrolopyridine





Pyrimidine carbamates





R= NH_2 or NHMe or substituted aniline

Figure S3. Kinase selectivity of lead compounds.

A. Kinase interaction maps for lead compounds. The kinase selectivity of four structurally distinct c-Fes inhibitors (see Figure 1) was assessed using KINOME*scan*™ screening technology (DiscoveRx Corporation), a kinome-wide method for screening kinase inhibitors against a panel of 353 kinases (for TAE684 and HG-7-92-01) or 442 kinases (for WZ-4-49-8 and HG-7-27-01). Selectivity profiling was performed at relatively high inhibitor concentrations (10 μM) to identify the full spectrum of possible targets. Kinome interaction maps were generated from the relative inhibitor binding data, and the kinase dendrograms shown were produced using the program TREEspot™. The circle size represents the relative binding score for each kinase (legend at right). Only kinases with a binding score of < 1% relative to the DMSO control are shown. Fes is represented by the blue dot. (A list of all kinases targeted by each inhibitor is provided in Supplemental Table S1.)

B, C. TAE684 and WZ-4-49-8 show selective inhibition of c-Fes over Erk2. B) Phosphorylation of tubulin by recombinant purified Fes SH2-KD (left panel) and phosphorylation of myelin basic protein (MBP, dephosphorylated, Millipore) by recombinant purified active Erk2 (right panel, Millipore) in the presence of TAE684, WZ-4-49-8 or the Erk inhibitor FR180204 (EMD Biochemicals) was evaluated by in vitro kinase assay. Kinase assays were performed as described in Experimental Procedures. Prior to inhibition experiments, a kinase titration was performed to verify linear reaction conditions, and 25 ng of purified Fes SH2-KD and 40 ng of Erk2 were used per reaction. The final ATP concentration was 0.1 mM in all reactions. Incorporation of radiolabeled phosphate was monitored by autoradiography. Kinase input was verified by immunoblot. C) IC_{50} values of kinase inhibition were determined from the experiments shown in A by plotting percent phosphorylation compared to uninhibited control and curve fitting. Calculated values are listed.

Figure S3, continued.

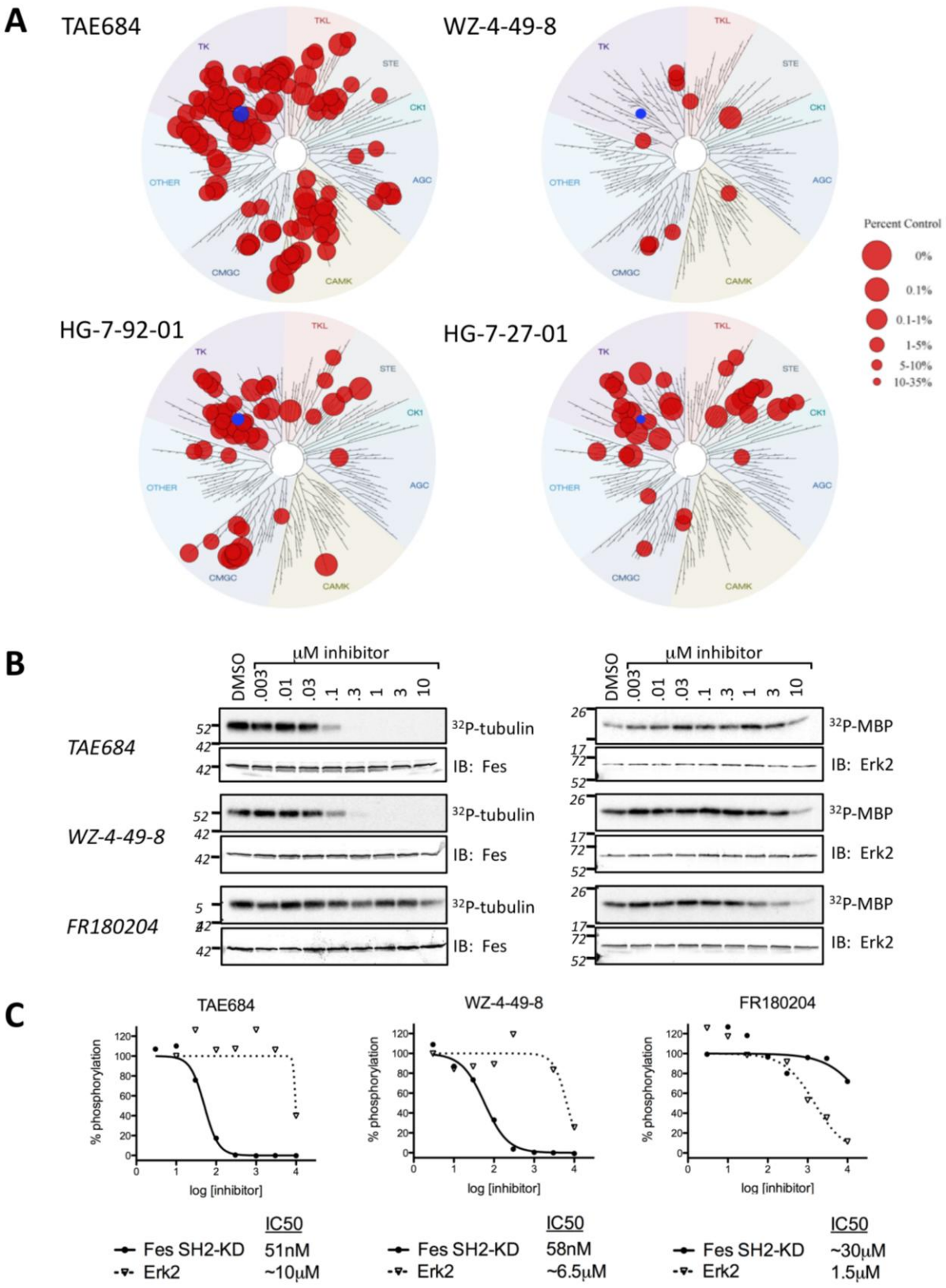


Figure S4. Additional features of the Fes SH2-KD TAE684 co-crystal structure (related to Figure 2)

A,B. Structural comparison of the Fes SH2-KD and Alk TAE684 complexes. A) Overlay of c-Fes (grey) and ALK (orange) complexes with TAE864 (compound carbons are yellow in the c-Fes structure and green in ALK. Part B provides a closer view of the binding site. See main text for details.

C,D. **Water-mediated interactions in the c-Fes:TAE684 complex**. A) Water-mediated interaction of TAE684 with the hinge region of the c-Fes kinase domain. Water molecules represented as caged red spheres. B) Binding to the c-Fes P-loop is also stabilized by water molecules that link the inhibitor to the DFG motif and the conserved active site lysine residue (K590).

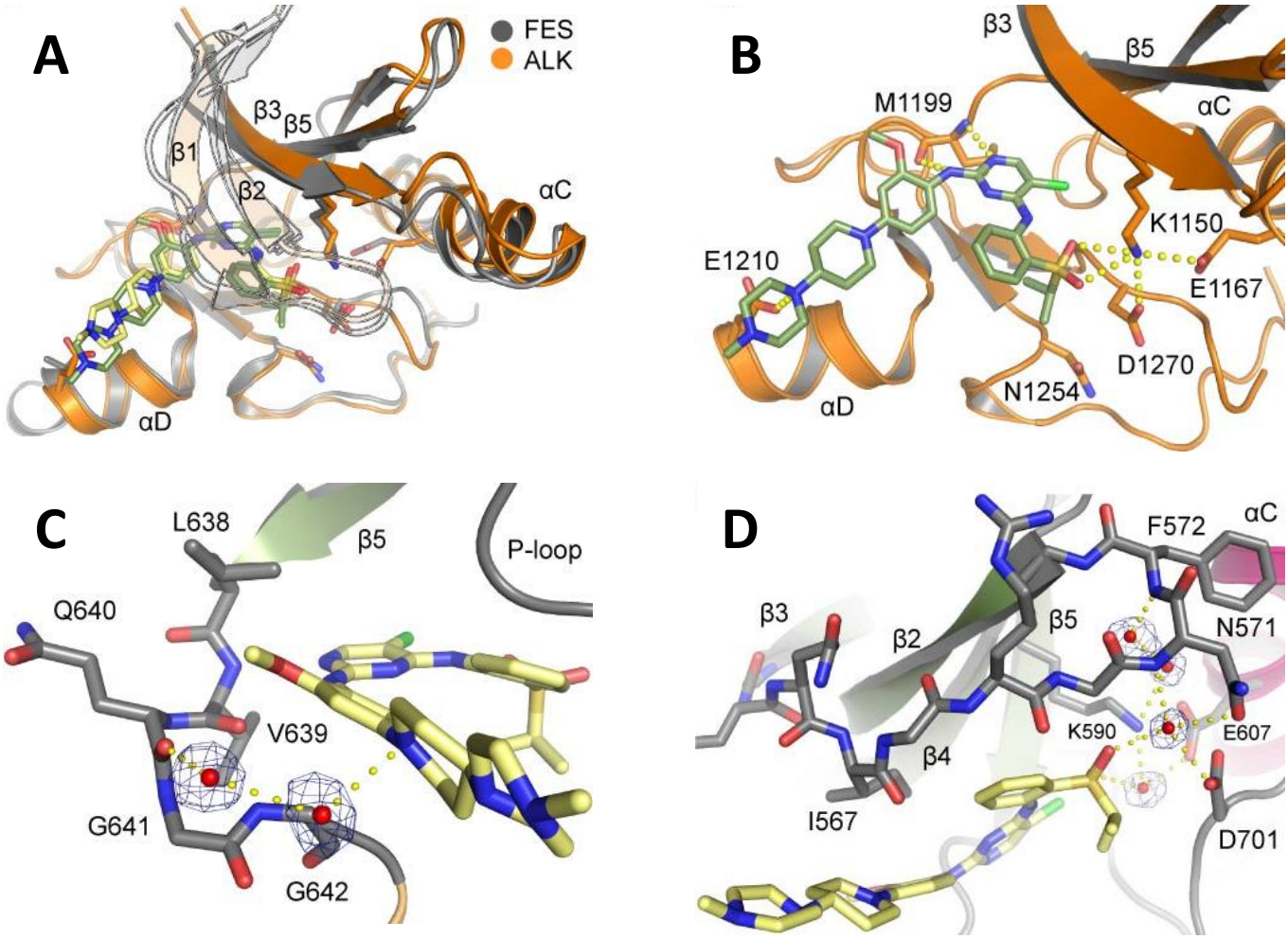


Figure S5. Inhibition of soft-agar colony formation by compounds WZ-4-49-8 and HG-7-92-01 (related to Figure 5). A) Rat-2 fibroblasts were transformed with an active Fes-L145P coiled-coil mutant, tail-activated murine Hck, (mHck-YF), a tail/gatekeeper Hck double-mutant (mHck-TMYF), or the analogous double-mutant form of human c-Yes (hYes-TMYF). Fibroblast transformation was scored in a soft-agar colony assay in the presence of 0.1% DMSO and increasing concentrations of each compound as indicated. The number of transformed colonies observed in the presence of each inhibitor concentration is expressed as the percent of colonies observed for the DMSO-only controls. The experiment was performed in triplicate and data are shown as mean colony number ± S.D. B) Transformed Rat-2 cells were grown in monolayer culture in the presence of the indicated concentrations of Fes inhibitors for 3 days and cell viability was assayed using a fluorometric assay (Cell Titer Blue, Promega).

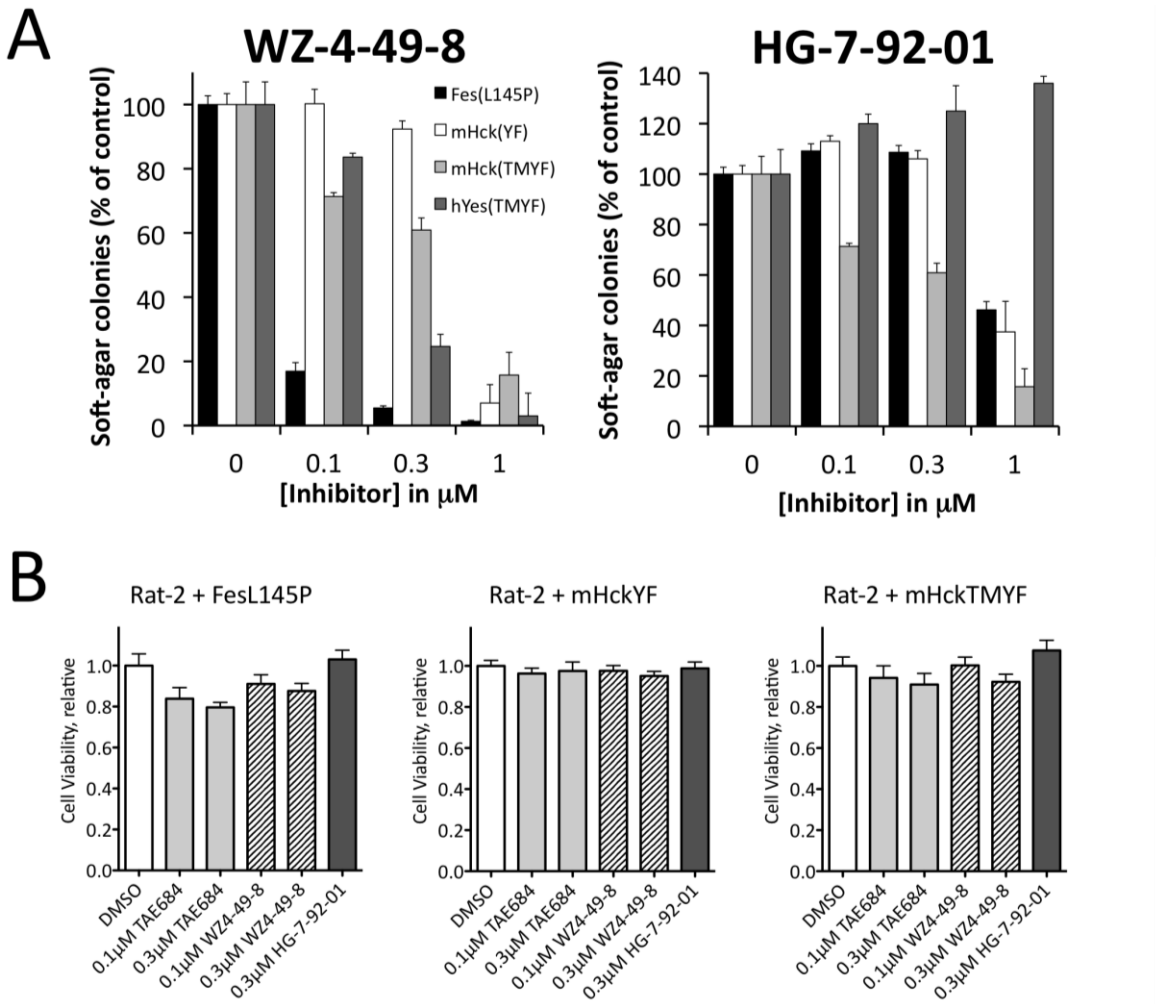


Figure S6. Fes-specific siRNAs block osteoclastogenesis in vitro.

A, B. Transient transfection of RAW 264.7 cells with Fes-specific siRNAs reduces expression of c-Fes but not of Fer. To verify the effect of siRNA-mediated targeting of c-Fes, RAW 264.7 were cultured in 12-well plates at 100,000 cells/well. Following overnight incubation, cells were transfected with each of three different murine c-Fes-specific siRNAs (Mm_Fes_2, Mm_Fes_4, and Mm_Fes_7; FlexiTube siRNAs, Qiagen) at a final concentration of 25 nM using the HiPerFect siRNA transfection reagent (Qiagen) and the manufacturer's protocol. Transfection with AllStars Negative Control siRNA (Qiagen) was included as the negative control. Cells were cultured for 24 h in DMEM plus 10% FBS or for 4 days in medium containing 100 ng/mL VEGF-A and 100 ng/mL RANKL. The 4-day cultures were re-transfected with siRNAs after 48 h and cytokines were renewed at this point.

A. Q-PCR. Total RNA was extracted from the cultures using the RNeasy Mini Kit (Qiagen). cDNA was subsequently generated from 1 μg aliquots of RNA using MLV reverse transcriptase and random decamer priming (RETROscript, Ambion). Real-time quantitative PCR using SYBR Green detection and gene-specific primers were used to determine mRNA expression relative to samples from cells transfected with the negative control siRNA. All samples were normalized to GAPDH expression. The mean ± SEM of three independent transfection experiments is plotted (** P value < 0.01, *** P value < 0.001). Expression analyses revealed that siRNA treatment reduces c-Fes mRNA levels to ~ 50-60% of control levels. This reduction is present after 24 h of treatment in the absence of osteoclast-inducing cytokines (left panel) and is maintained in the prolonged 4-day treatment with siRNAs and VEGF-A/RANKL (right panel). By comparison, mRNA levels of the closely related Fer kinase are not affected.

B. Immunoblot analysis. To confirm c-Fes knockdown by siRNA treatment at the protein level, extracts were prepared from cells transfected with c-Fes-specific and control siRNAs after 24 h. Proteins were resolved by SDS-PAGE, blotted onto PVDF membrane and probed for c-Fes (anti-Fes #2736, Cell Signaling). The membrane was stripped and reprobed for the actin loading control (Chemicon).

C. Transient transfection of RAW 264.7 cells with Fes-specific siRNAs suppresses osteoclast differentiation. Osteoclast differentiation assays with siRNA-transfected cells were performed as described in Wang and Grainger, *Pharm Res*. 27: 1273-1284 (2010). In brief, RAW 264.7 cells were seeded in 24-well plates at 40,000 cells/well in DMEM/Ham's F-12 containing 10% FBS. Cells were allowed to attach overnight and transfected with individual murine c-Fes-specific siRNAs, as well as a negative control siRNA, as described in above. To induce osteoclast differentiation, VEGF-A and RANKL were added to the culture medium at a final concentration of 100 ng/mL each. To maintain targeted knockdown of c-Fes, the transient transfection protocol was repeated after 2 and 4 days of culture. Fresh cytokines were also added at those time points. After 6 days, cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP) and counterstained with hematoxylin to visualize nuclei. Wells were inspected microscopically (100X magnification) and osteoclasts (TRAP-positive, ≥ 5 nuclei) were scored in 24 microscopic fields per well. Representative fields of cells are shown and the average numbers of osteoclasts/field ± SEM are graphed. In the case of wells transfected with Fes-specific siRNAs a clear reduction in both the number and size of TRAP-positive polykaryons is observed.

D. Fes inhibitors TAE684 and WZ-4-49-8 but not the Erk inhibitor FR180204 suppress osteoclast differentiation from RAW 264.7 cells. RAW 264.7 cells were seeded into 48-wells at 2000 cells/well in DMEM + 10% FBS. The medium was adjusted to contain DMSO (0.1%), Fes inhibitors TAE684 (0.1 μM) and WZ-4-49-8 (0.3 μM) or the Erk inhibitor FR180204 (1 μM and 10 μM). VEGF-A and RANKL were added to a final concentration of 100 ng/mL each. Cells were fixed, stained for TRAP, counterstained with hemotoxylin and analyzed by microscopy (100X magnification) 6 days later. Representative microscopy fields are shown. The number of multinucleated TRAP-positive osteoclasts was counted and plotted in D as the mean ± S.E.M. for three independent trials. In contrast to treatment with the Fes inhibitors, Erk inhibition did not result in detectable reduction in the number of osteoclasts.

Figure S6, continued.

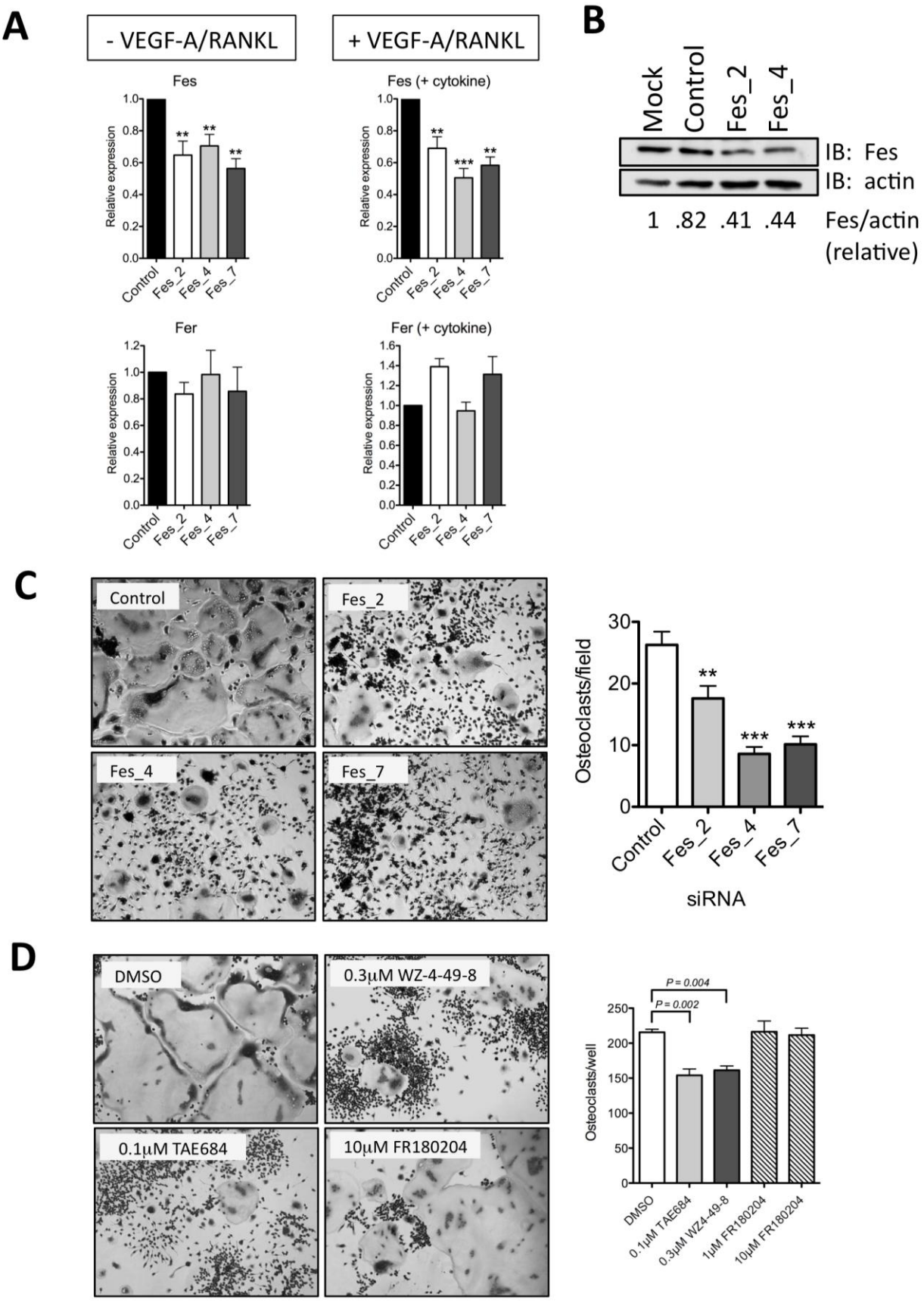


Table S1. KINOME*scan* data. Shown is a complete listing of all of the kinases tested in the KINOME*scan* experiment with four structurally distinct inhibitors of c-Fes (see Figure 1 in main text). Each inhibitor was tested at 10 μM, and the values represent the binding of each inhibitor as a percent of the DMSO control. Values range from 0 (complete displacement of the tagged probe compound for a given kinase) to 100 (no displacement; indistinguishable from the DMSO control). Results with c-Fes are highlighted in blue. For more information on this kinome-wide binding assay, see *Nature Biotechnology* 26: 127, 2008. *See Excel file (uploaded separately due to large size).*

Table S2. Crystallographic data and refinement statistics for the Fes SH2-kinase region in complex with TAE684.

Space group:	$P2_1$
Unit cell dimensions (Å):	a = 54.97, b = 35.33, c = 100.72
Unit cell dimensions (°):	α = 90.00, β = 96.58, γ = 90.00
Resolution (Å):	1.84 (1.94-1.84)
Unique observations:	33,290 (4701)
Completeness (%):	98.0 (95.9)
Redundancy:	4.8 (4.7)
R_{merge}:	0.084 (0.721)
I/σI:	12.5 (2.0)
Refinement	
R_{work}/R_{free} (%):	19.8/24.7
Number of atoms (protein/other/water):	2793/42/249
B-factors (protein/other/water, $Å^2$):	12.47/19.23/14.61
R.m.s.d. bonds (Å):	0.016
R.m.s.d. angles (°):	1.634
Ramachadran favored, allowed, disallowed (%):	96.33, 3.11, 0.56

Supplemental Experimental Procedures - Chemistry

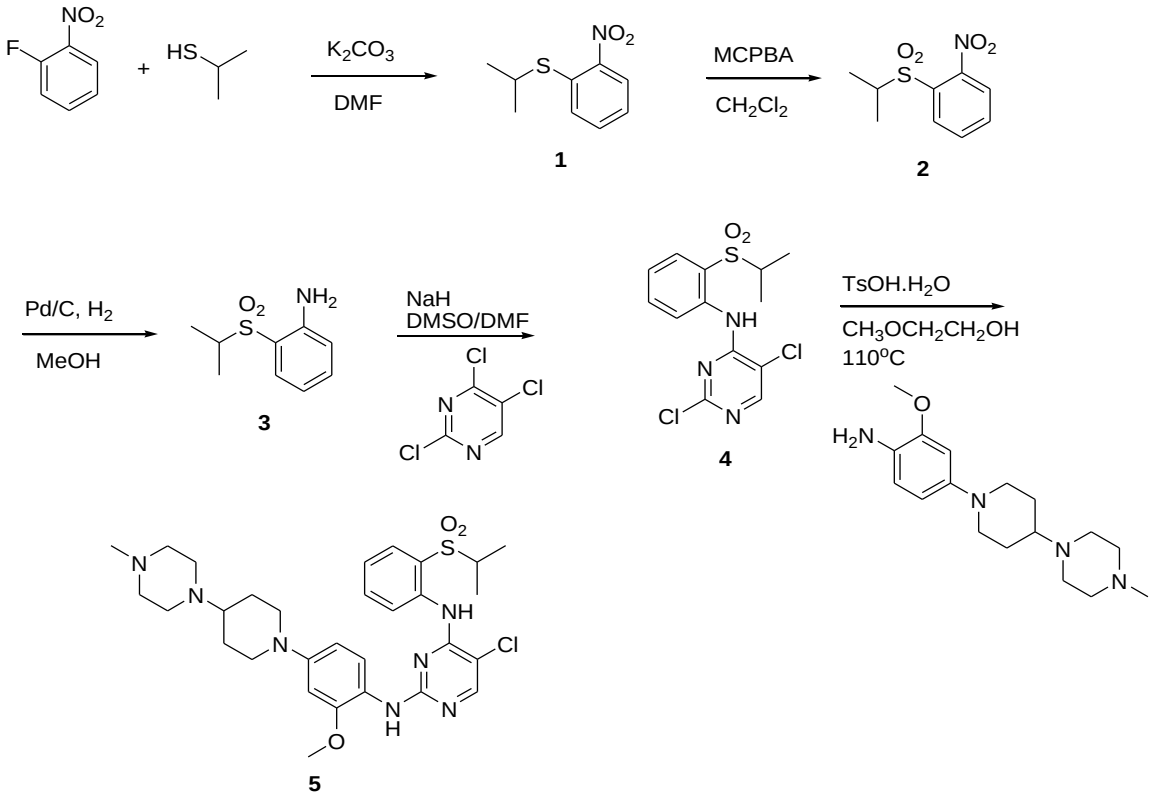
Small Molecule Inhibitors of the c-Fes Protein-tyrosine Kinase

Sabine Hellwig, Chandra V. Miduturu, Shigeru Kanda, Jianming Zhang, Panagis Filippakopoulos, Eidarus Salah, Xianming Deng, Hwan Geun Choi, Wenjun Zhou, Wooyoung Hur, Stefan Knapp, Nathanael S. Gray and Thomas E. Smithgall

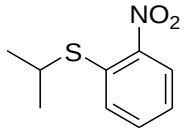
Unless otherwise noted, reagents and solvents were obtained from commercial suppliers and were used without further purification. ^{1}H NMR spectra were recorded at 600 MHz (Varian AS600), and chemical shifts are reported in parts per million (ppm, δ) downfield from tetramethylsilane (TMS). Coupling constants (*J*) are reported in Hz. Spin multiplicities are described as s (singlet), brs (broad singlet), t (triplet), q (quartet), and m (multiplet). Mass spectra were obtained on a Waters Micromass ZQ instrument. Preparative HPLC was performed on a Waters Symmetry C18 column (19 mm × 50 mm, 5 µM) using a gradient of 5−95% acetonitrile in water containing 0.05% trifluoacetic acid (TFA) over 8 min (10 min run time) at a flow rate of 30 mL/min. Purities of assayed compounds were in all cases greater than 95%, as determined by reverse-phase HPLC analysis.

Synthesis of TAE684

Synthesis scheme:



Isopropyl(2-nitrophenyl)sulfane; 1

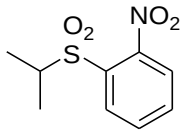


1

To a solution of 1-fluoro-2-nitrobenzene (3.0 g, 21.2 mmol) in DMF (60 mL) was added 2-propane thiol (2.2 mL, 23.40 mmol) and K_2CO_3 (6.8 g, 42.4 mmol). The reaction was heated to 45 ^{o}C for 16 hours. The mixture was then diluted with 300 mL of ethyl acetate, filtered, washed five times with 60 mL of water and brine. The organic phase was dried over sodium sulfate and concentrated *in vacuo*. The resulting yellow solid (4.0 g) was used without further purification.

^{1}H NMR 600 MHz (DMSO-d6) δ 8.09 (m, 1H), 8.07 (m, 1H), 7.68 (m, 1H), 7.36-7.40 (m, 1H), 3.70 (m, 1H), 1.26 (s, 3H), 1.28 (s, 3H).

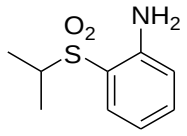
1-(isopropylsulfonyl)-2-nitrobenzene; 2



2

To a solution of isopropyl(2-nitrophenyl)sulfane (4.0 g, 20.3 mmol) in CH_2Cl_2 (100 mL) was added MCPBA (77%) (11.2 g, 50.0 mmol) at 0 ^{o}C. The mixture was stirred for 4 hours. The solution was diluted with CH_2Cl_2 and washed with saturated sodium bicarbonate solution three times. The organic layer was dried with sodium sulfate and concentrated *in vacuo* to give the title compound (4.0 g) which was used without further purification.

2-(isopropylsulfonyl)benzenamine; 3

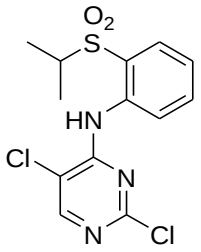


3

Palladium on activated carbon (400 mg) was added to a solution of 1-(isopropylsulfonyl)-2-nitrobenzene (4.65 g, 23.4 mmol) in methanol (100 mL). The reaction was stirred under hydrogen for 5 hours. The mixture was filtered through celite. The filtrate was concentrated *in vacuo* to give the title compound (4.0 g) which was used without further purification.

^{1}H NMR 600 MHz (DMSO-d6) δ 7.42 (m, 1H), 7.31-7.36 (m, 1H), 6.84 (dd, 1H), 6.64-6.70 (m,1H), 5.82-6.16 (s, 2H), 3.28-3.36 (m, 1H), 1.14 (s, 3H), 1.16 (s, 3H).
MS m/z : 200.20 (M + 1)

2,5-dichloro-N-(2-(isopropylsulfonyl)phenyl)pyrimidin-4-amine; 4

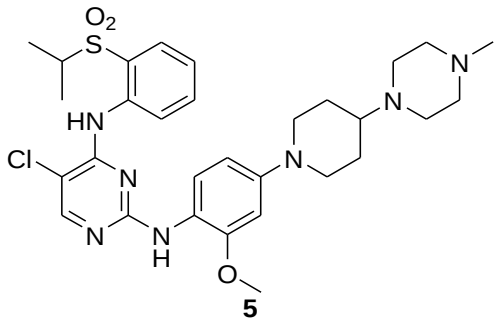


4

To a solution of 2-(isopropylsulfonyl)benzenamine (1.04 g, 5.22 mmol) in DMF/DMSO (10 mL, 9:1 v/v) was added sodium hydride (0.42 g, 10.44 mmol) at 0 ^{o}C, followed by addition of 2,4,5-trichloropyrimidine (1.20 mL, 10.44 mmol). The mixture was warmed to room temperature and stirred overnight. The reaction was quenched with water and diluted with 50 mL of ethyl acetate. The separated organic layer was dried and concentrated. The crude residue was purified by flash chromatography with 1:1 hexane-ethyl acetate to yield yellow solid (0.90 g, 50% yield).

^{1}H NMR 600 MHz ($CDCl_3$) δ 10.06 (s, 1H), 8.63 (d, 1H), 8.30 (s, 1H), 7.92 (dd, 1H), 7.720-7.75 (m, 1H), 7.32 (m, 1H), 3.21 (m, 1H), 1.35 (d, 6H).
MS m/z : 347.01 (M + 1)

5-chloro-N4-(2-(isopropylsulfonyl)phenyl)-N2-(2-methoxy-4-(4-(4-methylpiperazin-1-yl)piperidin-1-yl)phenyl)pyrimidine-2,4-diamine; 5



5

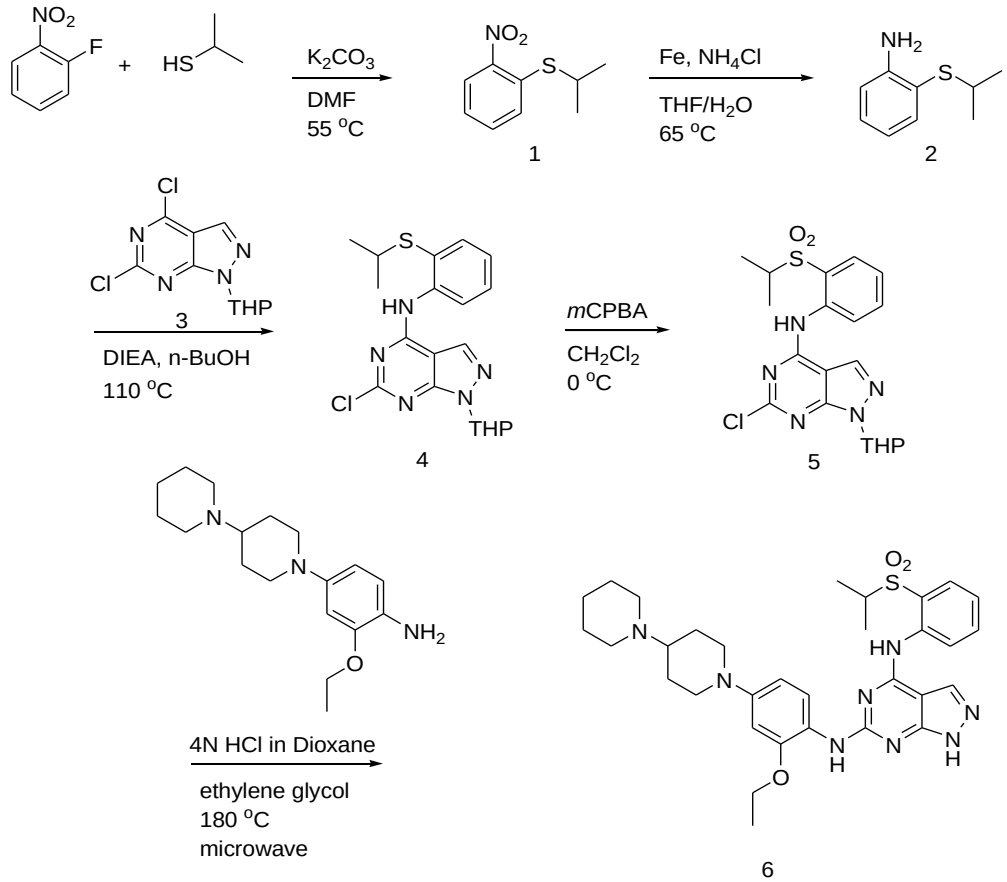
To a suspension of 2,5-dichloro-N-(2-(isopropylsulfonyl)phenyl)pyrimidin-4-amine (0.90 g, 2.60 mmol) in 1-methoxyethanol (10 mL) were added toluene sulfonic acid (1.49 g, 7.8 mmol), and 2-methoxy-4-(4-(4-methylpiperazin-1-yl)piperidin-1-yl)benzenamine (0.79 g, 2.60 mmol). After the reaction was stirred at 110 ^{o}C for 24 hours and cooled to room temperature, the mixture was neutralized with

saturated sodium bicarbonate and extracted with ethyl acetate (3 x 30 mL). The organic layer was collected, dried with sodium sulfate and concentrated *in vacuo*. The crude residue was purified by flash chromatography with 10:1 (v/v) methylene chloride-ammonium (7N in methanol) to afford a light yellow solid as the title product (0.63 g, 39% yield).

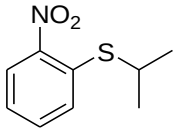
^{1}H NMR 600 MHz ($CDCl_3$) δ 9.54 (s, 1H), 8.60 (d, 1H), 8.12 (s, 1H), 8.01 (dd, 1H), 7.95 (dd, 1H), 7.61 (m, 1H), 7.33-7.23 (m, 1H), 6.55 (d, 1H), 6.47 (d, 1H), 6.11 (dd, 1H), 3.87 (s, 3H), 3.24 (m, 1H), 3.16-3.23 (m, 4H), 2.97-3.15 (m, 4H), 2.71-2.79 (m, 4H), 2.27-2.43 (m, 2H), 1.95-2.17 (m, 5H), 1.83-1.92 (m, 1H), 1.32 (s, 3H), 1.30 (s, 3H).
MS m/z : 614.30 (M + 1)
^{13}C NMR 600MHz (DMSO-d6) δ 15.8 28.2 46.2 48.5 48.7 56.0 61.8 101.0 104.0 108.1 120.0 123.8 124.0 126.2 131.8 138.8 150.0 154.0 155.8 156.0 160.0

Synthesis of WZ-4-49-8

Synthesis scheme



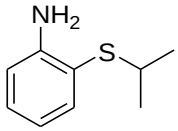
Isopropyl(2-nitrophenyl)sulfane; 1



1

To a solution of 1-fluoro-2-nitrobenzene (3.0 g, 21.2 mmol) in DMF (60 mL) was added 2-propane thiol (2.2 mL, 23.4 mmol) and K_2CO_3 (6.8g, 42.4 mmol). The reaction was heated to 45 ^oC for 16 hours. The mixture was then diluted with 300 mL of ethyl acetate, filtrated, washed five times with 60 mL of water and brine. The organic phase was dried over sodium sulfate, filtered, concentrated. The resulting yellow solid (4.0 g, 95% yield) was used without further purification.

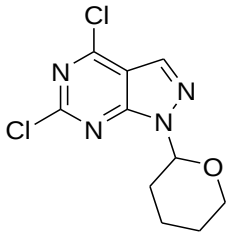
(isopropylthio)benzenamine; 2



2

To a solution of isopropyl (2-nitrophenyl)sulfane (4.0 g, 20.3 mmol) in methanol (200 mL) was added Pd/C (0.40 g). The 500 mL flask was evacuated and charged with high purity hydrogen gas. The reaction was stirred for 20 hours. The mixture was filtrated through celite. After concentration, the residue was purified by flash chromatography using 10:1 methylene chloride-ethyl acetate as solvent to afford the title compound (3.36 g, 99% yield) as brown oil.

4,6-dichloro-1-(tetrahydro-2H-pyran-2-yl)-1H-pyrazolo[3,4-d]pyrimidine; 3

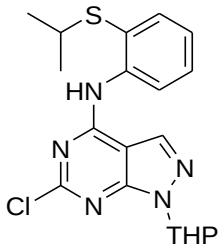


3

To a 100 mL flask was added 4,6-dihydroxylpyrazolo(3,4-d)pyrimidine (2.0 g, 13.15 mmol), phosphorus oxychloride (12 mL, 131.5 mmol) and N,N-diethylaniline (4.0mL, 26.3 mmol). The reaction mixture was heated to 110 ^oC for 2 hours. After removal of phosphorus oxychloride, the dark residue was poured onto a mixture of crushed ice and water. The cold aqueous solution was

extracted with diethyl ether (3 x 100 mL). The organic layer was washed with water and brine, dried over sodium sulfate, concentrated to afford 1.30 g of a crude tan product, which was used without further purification. To the solution of 4,6-dichloro-1H-pyrazolo[3,4-d]pyrimidine (1.30 g, 6.88 mmol) in methylene chloride (20 mL) and THF (20 mL) was added TsOH (0.13 g, 0.688 mmol) and 3,4-dihydro-2H-pyran (0.93 mL, 10.32 mmol). The solution was stirred for 4 hours. After removal of solvent, the residue was dissolved in methylene chloride (100 mL), washed with twice with a saturated aqueous solution of Na_2CO_3, water and brine. The methylene chloride solution was dried over sodium sulfate and concentrated. The resulting yellow oil was treated with 1 mL of ethyl acetate and 10 mL of hexane to induce solidification. The resulting white solid (1.2 g) was used without further purification.

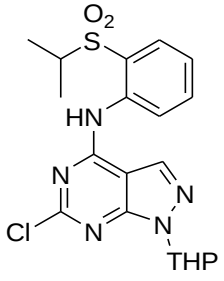
6-chloro-N-(2-(isopropylthio)phenyl)-1-(tetrahydro-2H-pyran-2-yl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine; 4



4

To a 25 mL of flask charged with 4,6-dichloro-1-(tetrahydro-2H-pyran-2-yl)-1H-pyrazolo[3,4-d]pyrimidine (0.955 g, 3.50 mmol) and (isopropylthio)benzenamine (1.168 g,7.0 mmol) in n-butanol (3 mL) was added DIEA (0.69 mL, 4.2 mmol). The reaction mixture was heated to 110 ^{o}C for 2 hours. After removal of solvent, the residue was purified by flash chromatography using 3:1 hexane-ethyl acetate as solvent to afford title compound (1.20 g, 85% yield) as a white solid.

6-chloro-N-(2-(isopropylsulfonyl)phenyl)-1-(tetrahydro-2H-pyran-2-yl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine; 5

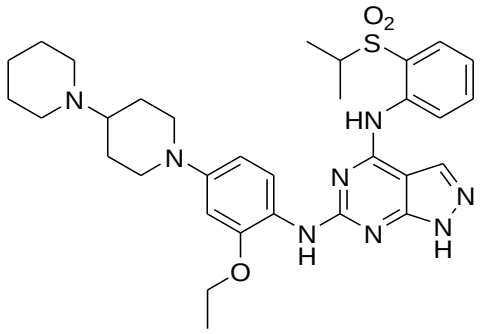


5

To a solution of 6-chloro-N-(2-(isopropylthio)phenyl)-1-(tetrahydro-2H-pyran-2-yl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (1.20 g, 2.97 mmol) in dichloromethane (80 mL) was added *m*CPBA (2.00 g, 8.92 mmol). The reaction mixture was stirred for 4 hours after which, it was diluted with dichloromethane

(100 mL). The organic layer was washed with saturated solutions of $NaHCO_3/Na_2S_2O_3$ and $NaHCO_3$, and then washed with brine. The organic layer was dried over Na_2SO_4, filtered and concentrated. The resulting light yellow solid (1.20 g) was used without further purification.

N6-(4-([1,4'-bipiperidin]-1'-yl)-2-ethoxyphenyl)-N4-(2-(isopropylsulfonyl)phenyl)-1H-pyrazolo[3,4-d]pyrimidine-4,6-diamine; 6



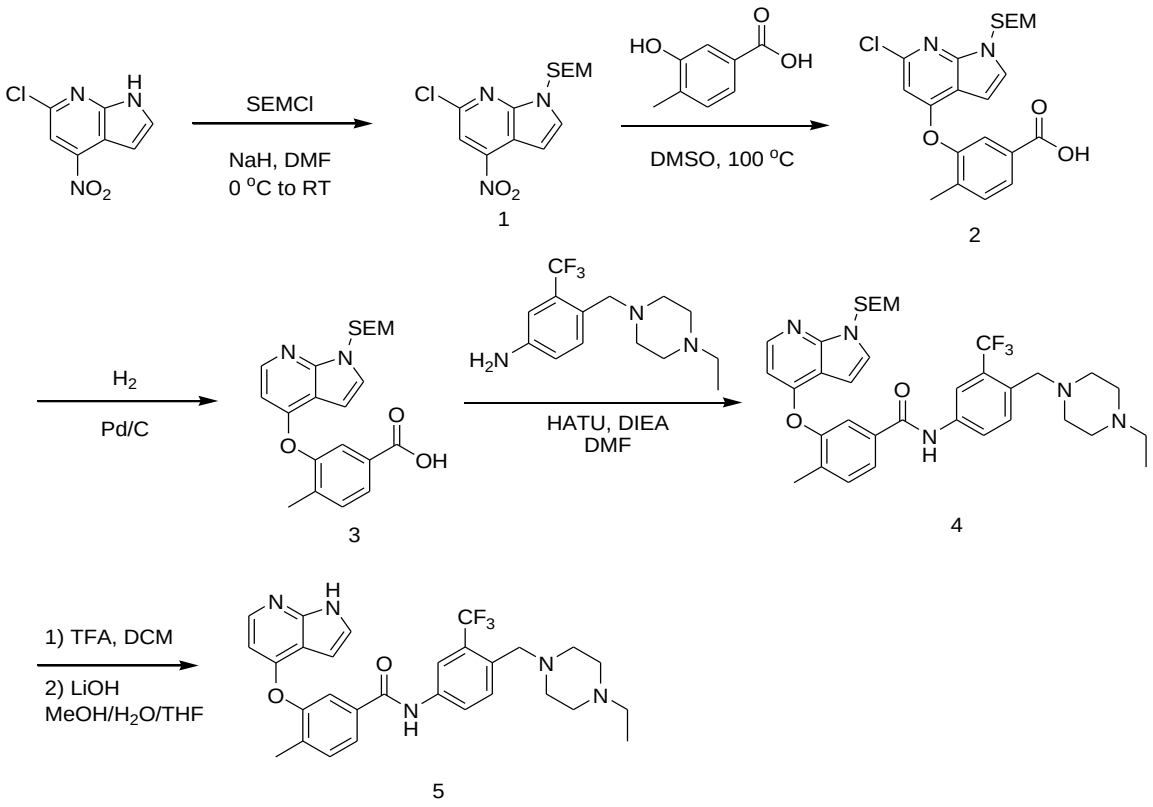
6

A microwave tube was charged with 6-chloro-N-(2-(isopropylsulfonyl)phenyl)-1-(tetrahydro-2H-pyran-2-yl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (40 mg, 0.0917 mmol), 4-([1,4'-bipiperidin]-1'-yl)-2-ethoxyaniline (82.8 mg, 0.275 mmol), 4N HCl in Dioxane (0.069 mL, 0.276 mmol) and ethylene glycol (1.5 mL). The tube was sealed and the reaction mixture was stirred at 180 ^{o}C for 30 min in a microwave reactor. The reaction mixture was purified by reverse-phase preparative HPLC. The crude TFA salt of product was neutralized with a saturated aqueous solution of $NaHCO_3$ and extracted with ethyl acetate. The organic layer was dried over sodium sulfate, filtered and concentrated. The residue was purified by flash chromatography using 30:1:0.3 methylene chloride-methanol-triethylamine as solvent to afford the title compound (20.0 mg, 43% yield).

^{1}H NMR 600 MHz (CD_3OD) δ 8.26 (*m*, 1H), 8.1 (*m*, 1H), 8.00(*d*, *J* = 7.8 Hz, 1H), 7.80 (*m*, 1H), 7.58 (*m*, 1H), 7.37 (*m*, 1H), 6.68 (*m*, 1H), 6.50 (*m*, 1H), 4.10 (*q*, *J* = 6.6 Hz, *J* = 7.2 Hz, 2H), 3.86 (*m*, 2H), 3.58 (*m*, 2H), 3.35 (*m*, 1H), 3.06 (*m*, 2H), 2.86 (*m*, 2H), 2.42 (*m*, 2H), 2.00 (*m*, 2H), 1.85 (*m*, 6H), 1.34 (*t*, *J* = 6.6 Hz, *J* = 7.2 Hz, 3H), 1.19 (*d*, *J* = 6.6 Hz, 6H), MS m/z : 619.79 (M + 1).

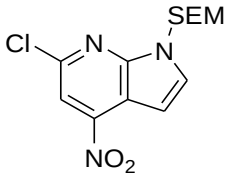
Synthesis of HG-7-92-01

Synthesis scheme



1 2 3 4 5

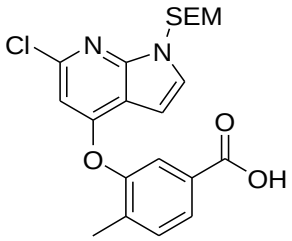
6-chloro-4-nitro-1-((2-(trimethylsilyl)ethoxy)methyl)-1H-pyrrolo[2,3-b]pyridine; 1



1

NaH (420 mg, 10.5 mmol) was added in portions to a mixture of 6-chloro-4-nitro-1H-pyrrolo[2,3-b]pyridine (Schirok, *et al*. *ChemMedChem*. 12: 1893-904, 2008) (1.38 g, 7.0 mmol) in DMF (30 mL) at 0 °C. After warming the reaction mixture to room temperature, SEM-Cl (1.28 g, 7.7 mmol) was added. The reaction mixture was stirred at room temperature overnight. After the reaction was complete as monitored by LC-MS, the reaction mixture was diluted with water and extracted with EtOAc. The organic layer was separated and washed with brine and dried with sodium sulfate. The product **1** was isolated by column chromatography eluting with hexanes and EtOAc (1.5g, 65% yield).

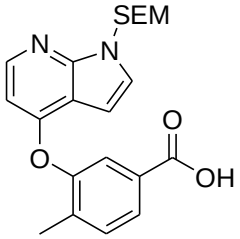
3-(6-chloro-1-((2-(trimethylsilyl)ethoxy)methyl)-1H-pyrrolo[2,3-b]pyridin-4-yloxy)-4-methylbenzoic acid; 2



2

To a mixture of 6-chloro-4-nitro-1-((2-(trimethylsilyl)ethoxy)methyl)-1H-pyrrolo[2,3-b]pyridine (200 mg, 0.62 mmol) and 3-hydroxy-4-methylbenzoic acid (103 mg, 0.68 mmol) in DMSO (5 mL) was added K_2CO_3 (254 mg, 1.84 mmol). The mixture was heated at 100 °C for 15 hours. After the reaction was complete as monitored by LC-MS, the reaction mixture was acidified with 1N HCl to a pH of ~5-6 and extracted with ethyl acetate. The organic layer was washed with brine and dried with sodium sulfate. The crude material was used for the next step without any further purification.

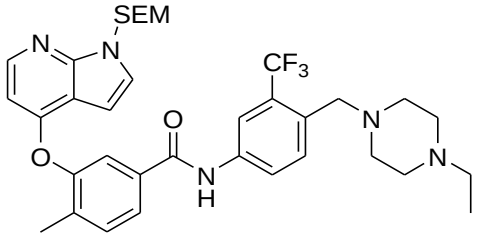
4-methyl-3-(1-((2-(trimethylsilyl)ethoxy)methyl)-1H-pyrrolo[2,3-b]pyridin-4-yloxy)benzoic acid; 3



3

To a mixture of 3-(6-chloro-1-((2-(trimethylsilyl)ethoxy)methyl)-1H-pyrrolo[2,3-b]pyridin-4-yloxy)-4-methylbenzoic acid (0.61 mmol) in MeOH was added 10% Pd-C (0.15 g). The reaction flask was fitted with a H_2 gas balloon and the mixture stirred at room temperature for 36 hours. After the reaction was complete as monitored by TLC and LC-MS, the resulting solution was filtered through a pad of celite and washed with ethyl acetate. The crude material was concentrated *in vacuo* and used for the next step without any further purification.

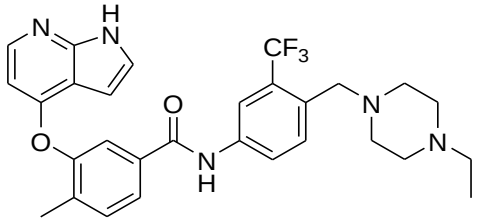
N-(4-((4-ethylpiperazin-1-yl)methyl)-3-(trifluoromethyl)phenyl)-4-methyl-3-(1-((2-(trimethylsilyl)-ethoxy)methyl)-1H-pyrrolo[2,3-b]pyridin-4-yloxy)benzamide; 4



4

To a mixture of 4-methyl-3-(1-((2-(trimethylsilyl)ethoxy)methyl)-1H-pyrrolo[2,3-b]pyridin-4-yloxy)-benzoic acid (0.31 mmol) in DMF (2.5 mL) was added 4-((4-ethylpiperazin-1-yl)methyl)-3-(trifluoromethyl)benzenamine (130 mg, 0.45 mmol), HATU (0.23g , 0.6 mmol) and DIEA (0.098 mL, 0.6 mmol). After stirring overnight, the reaction mixture was diluted with ethyl acetate and an aqueous saturated $NaHCO_3$ solution. The organic layer was separated, washed with brine and dried with sodium sulfate. The crude material was concentrated and purified by flash column chromatography (1.5 N ammonia methanol in dichloromethane) to afford product **4** (120 mg, 60 % yield).

3-(1H-pyrrolo[2,3-b]pyridin-4-yloxy)-N-(4-((4-ethylpiperazin-1-yl)methyl)-3-(trifluoromethyl)-phenyl)-4-methylbenzamide; 5



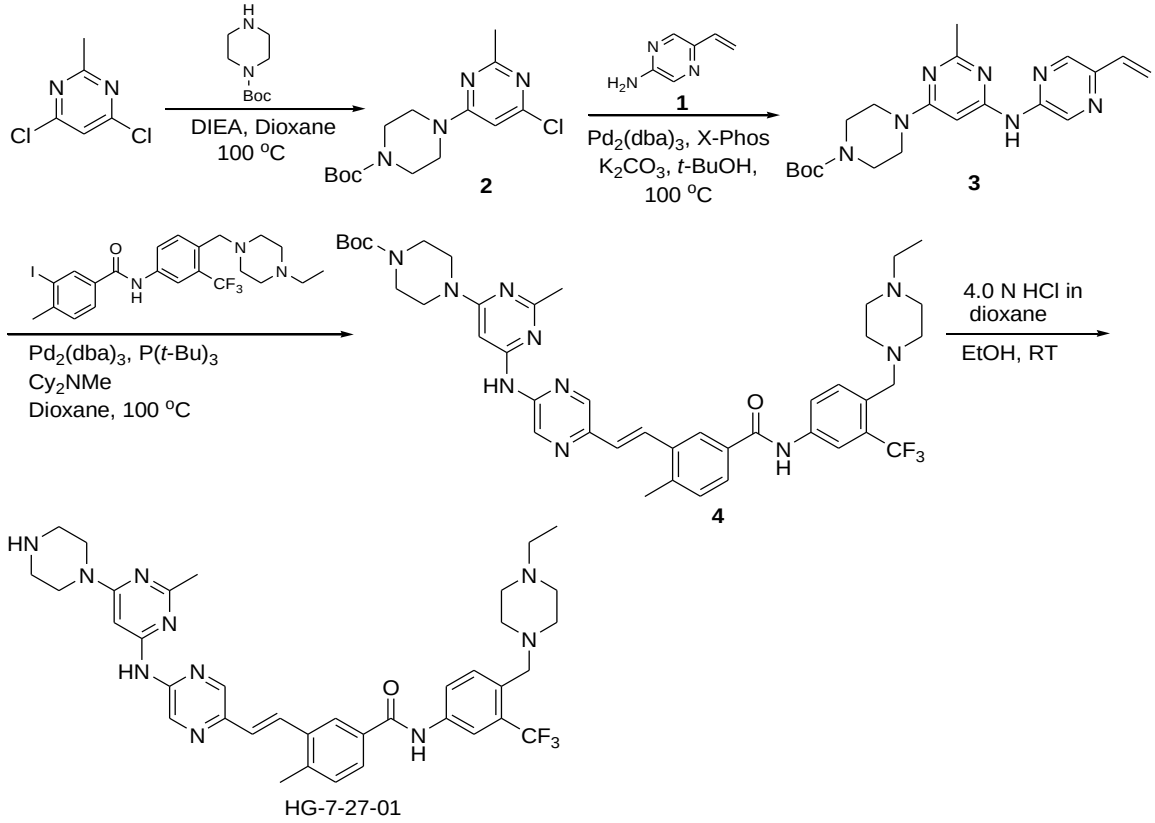
5

A mixture of N-(4-((4-ethylpiperazin-1-yl)methyl)-3-(trifluoromethyl)phenyl)-4-methyl-3-(1-((2-(trimethylsilyl)ethoxy)methyl)-1H-pyrrolo[2,3-b]pyridin-4-yloxy)benzamide (30 mg, 0.045 mmol) in 1/1 (v/v, 1 mL) of TFA and dichloromethane was stirred at room temperature for 3 hours. After the starting material was consumed as monitored by TLC and LC-MS, the reaction mixture was concentrated *in vacuo*. To a mixture of the crude material in 1/1/1 (v/v, 1 mL) of MeOH, THF and H_2O, LiOH (4 mg, 0.099 mmol) was added. The reaction mixture was stirred for 1h at room temperature. The residue was purified by reverse-phase prep-HPLC using a water (0.05% TFA)/acetonitrile (0.05% TFA) gradient to afford the title compound **5** as TFA salt (5 mg, 21% yield).

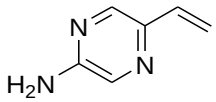
^{1}H NMR (600 MHz, DMSO-d_6) δ 11.88 (s, 1H), 10.55 (s, 1H), 8.27 (d, *J* = 3.0 Hz, 1H), 8.19 (d, *J* = 8.4 Hz, 1H), 8.13 (dd, *J* = 12.0 Hz, 3.0 Hz, 1H), 8.00 (dd, *J* = 12.0 Hz, 3.0 Hz, 1H), 7.88 (d, *J* = 3.0 Hz, 1H), 7.79 (d, *J* = 12.0 Hz, 1H), 7.69 (d, *J* = 12.0 Hz, 1H), 7.48 (dd, *J* = 5.4 Hz, 4.2 Hz, 1H), 6.44 (d, *J* = 7.8 Hz, 1H), 6.33 (dd, *J* = 5.4 Hz, 3.0 Hz, 1H), 3.67 (s, 2H), 3.43 (s, 2H), 2.61 (s, 8H), 2.35 (s, 3H), 1.11 (t, *J* = 10.2 Hz, 3H). MS (ESI) *m/z* 538 $(M+H)^+$.

Synthesis of HG-7-27-01

Synthesis scheme



5-vinylpyrazin-2-amine; 1

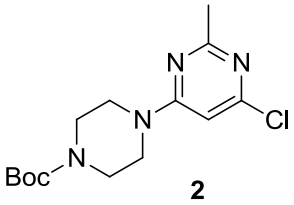


1

To a mixture of 5-bromopyrazin-2-amine (552 mg, 3.19 mmol) and vinyltri-n-butyltin (1.11 g, 3.50 mmol) in DMF(9 mL) were added LiCl (150 mg, 3.50 mmol) and DIEA (0.61 mL). After degassing for 20 minutes, $Pd(PPh_3)_4$ (221 mg, 0.19 mmol) was added to the reaction mixture followed by heating to 100 ˚C for 6 hours under argon. The reaction mixture was cooled to room temperature and stirred with a 10% aqueous solution of potassium fluoride (4.0 mL) for 1 hour. The resulting solution was filtered through a pad of celite and washed with ethyl acetate. The filtrate was washed with brine and

dried over anhydrous Na_2SO_4. The crude product was concentrated and purified by flash column chromatography (ethyl acetate/hexane) to afford the title product **1** (332 mg, 86% yield). MS (ESI) *m/z* 121 $(M+H)^+$.

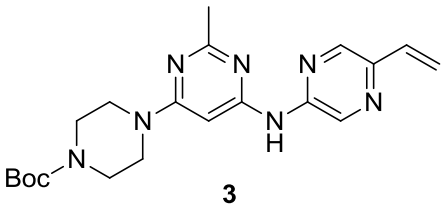
tert-butyl 4-(6-chloro-2-methylpyrimidin-4-yl)piperazine-1-carboxylate; 2



2

To a mixture of 4,6-dichloro-2-methylpyrimidine (1.63 g, 10 mmol) and DIEA (2.61 mL, 15 mmol) in dioxane (12 mL) was added *tert*-butyl piperazine-1-carboxylate (1.86 g, 10 mmol). Then the reaction was stirred at 100 ˚C. After the reaction was complete as monitored by TLC, the resulting mixture was concentrated. The crude product was purified by flash column chromatography (ethyl acetate/ hexane) to give the title compound **2** (2.98 g, 96% yield). MS (ESI) *m/z* 314 $(M+H)^+$.

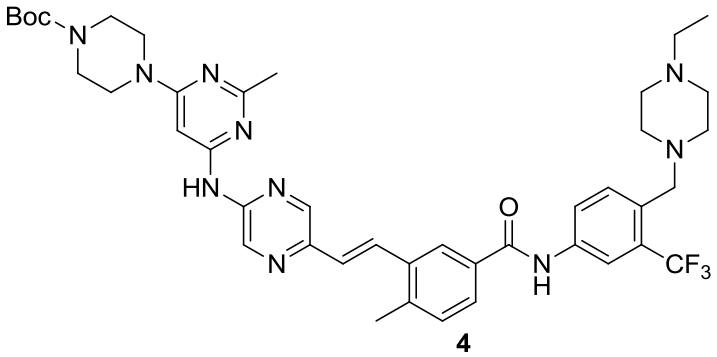
tert-butyl 4-(2-methyl-6-((5-vinylpyrazin-2-yl)amino)pyrimidin-4-yl)piperazine-1-carboxylate; 3



3

A mixture of **1** (52 mg, 0.43 mmol), **2** (135 mg, 0.43 mmol), X-Phos (19 mg), $Pd_2(dba)_3$ (24 mg) and K_2CO_3 (178 mg, 1.29 mmol) in 2.5 mL of *t*-BuOH was heated at 100 ˚C. After the reaction was complete as monitored by LC-MS, the reaction was filtered through celite and eluted with dichloromethane. The residue was concentrated and purified by flash column chromatography (ethyl acetate/hexane) to give the title compound **3** (59 mg, 35% yield). MS (ESI) *m/z* 398 $(M+H)^+$.

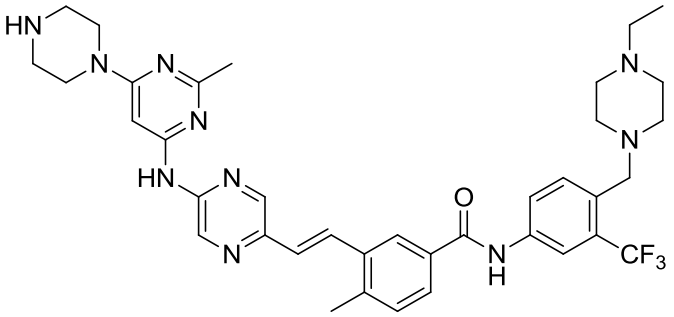
(E)-_tert_-butyl 4-(6-((5-(5-((4-((4-ethylpiperazin-1-yl)methyl)-3-(trifluoromethyl)phenyl)carbamoyl)-2-methylstyryl)pyrazin-2-yl)amino)-2-methylpyrimidin-4-yl)piperazine-1-carboxylate; 4



4

A mixture of **3** (40 mg, 0.1 mmol), N-(4-((4-ethylpiperazin-1-yl)methyl)-3-(trifluoro-methyl)phenyl)-3-iodo-4-methylbenzamide (53 mg, 0.1 mmol), P(*t*-Bu)$_3$(10 µL of 1.0 M solution in toluene), $Pd_2(dba)_3$ (4.6 mg) and N-cyclohexyl-N-methylcyclohexanamine (64 µL, 0.3 mmol) in 2.0 mL of dioxane was heated at 100 ˚C. After the reaction was complete as monitored by LC-MS, the reaction was filtered through celite and eluted with dichloromethane. The residue was concentrated and purified by flash column chromatography (3.5 *N* ammonia methanol solution/dichlorometane) to give the title compound **4** (65 mg, 81% yield). MS (ESI) *m/z* 802 $(M+H)^+$.

(E)-N-(4-((4-ethylpiperazin-1-yl)methyl)-3-(trifluoromethyl)phenyl)-4-methyl-3-(2-(5-((2-methyl-6-(piperazin-1-yl)pyrimidin-4-yl)amino)pyrazin-2-yl)vinyl)benzamide; HG-7-27-01



HG-7-27-01

To a solution of **4** (44 mg, 0.055 mmol) in ethanol (2.0 mL) was added 4 N HCl solution in dioxane (1.0 mL) at room temperature. The reaction was stirred until it was complete as monitored by LC-MS. The resulting mixture was diluted with ethyl acetate and washed with saturated sodium bicarbonate solution. The organic layer was separated and dried over anhydrous Na_2SO_4. The residue was concentrated and purified by reverse-phase prep-HPLC using a water (0.05% TFA)/acetonitrile (0.05% TFA) gradient to afford the title compound **HG-7-27-01** as TFA salt (42 mg, 94%).

^{1}H NMR (600 MHz, CD_3OD) δ 8.40 (d, *J* = 1.2 Hz, 1H), 8.36 (s, 1H), 8.22 (d, *J* = 1.8 Hz, 1H), 8.20 (d, *J* = 2.4 Hz, 1H), 8.00 (dd, *J* = 1.8, 8.4 Hz, 1H), 7.83 (d, *J* = 16.2 Hz, 1H), 7.77 (d, *J* = 8.4 Hz, 2H), 7.34 (d, *J* = 8.4 Hz, 1H), 7.18 (d, *J* = 15.6 Hz, 1H), 6.79 (s, 1H), 4.06 (brs, 4H), 3.80 (s, 2H), 3.70-3.40 (m, 4H), 3.38

(t, *J* = 5.4 Hz, 4H), 3.22 (q, *J* = 7.2 Hz, 2H), 3.20-3.00 (m, 4H), 2.58 (s, 3H), 2.50 (s, 3H), 1.35 (t, *J* = 7.2 Hz, 3H). MS (ESI) *m/z* 702 $(M+H)^+$.