Identification of RIPK3 Type II in-hibitors using high-throughput mechanistic studies in hit triage.

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Method development for time dependency and mode of inhibition measurements:

High throughput time-dependence and mode of inhibition experiments were conducted using modified HTRF binding competition assay formats. The assay requires His-tagged RIPK3, fluorescent active site probe acceptor, and terbium-labeled anti-His fluorescent antibody donor. The highthroughput assays were carried out in 384- or 1536-well plate format, where reagents were added to the plates using multidrop liquid dispensers. After reactions times elapsed, the HTRF signals (the 520/495 ratio), ratio of fluorescence intensities at emission wavelengths for fluorescein acceptor (520 nm) and terbium donor (495 nm), were measured on the Envision Plate reader. All reactions were conducted using 50 mM Hepes buffer containing 10 mM MgCl2, 100 mM NaCl, 1 mM dithiothreitol (DTT), 0.025 mg/ml bovine serum albumin (BSA), and 0.015% Brij 35 at pH 7.4 and room temperature.

TD formats: Two formats were tested for TD measurements. For probe displacement at single concentration of compound, 1 nM RIPK3 , 0.2 nM Anti-His Tb antibody, and compound at 10 x IC_{50} were preincubated for 1 hour, after which HTRF probe at 30 x Kd was added and HTRF signals measured at 5 min and 60 min. Percent probe displacement was calculated as $F_{Cmpd,\ time}$- /$F_{DMSO,time}$. For IC_{50} vs incubation time method, ternary complex between 1 nM RIPK3, 0.2 nM anti-His Tb antibody, and HTRF probe (Kd) was preformed for 1 hour. Following this, dose response of inhibitor was added (11 pt concentration response curve , 3 fold dilution) to reactions. HTRF signals were measured at 5 min and 60 min post compound addition, and IC_{50} calculated from the 520/495 ratio generated by the no protein control reactions for 100% inhibition and vehicle-only reactions for 0% inhibition.

Methods for high-throughput screen, counter screens and mechanistic screens:

HTS Method: The high-throughput screen was conducted using a HTRF-based probe displacement method to measure the activity of test compounds. An Enzyme Mix consisting of a GST-tagged full length human protein expressed in Sf9 cells (at 1 nM) was mixed with a proprietary small molecule probe (at the Kd 40 nM) that was labeled with fluorescein. In addition an anti-GST antibody labeled with Tb (Cis-Bio) was added at 0.2 nM. 5 nL Test compounds were transferred from 3 mM DMSO stocks using non-contact acoustic dispensing (Labcyte ECHO 555) into white polystyrene 1536-well plates (Corning). 2 uL of the Enzyme mix was added to the first 46 columns of the plate using a Combi (Thermo). A blank reagent to measure background fluoroscence was added to the last two columns of the plate; this blank mixture was identical to the Enzyme mix except that the enzyme was omitted. In an effort to reduce waste, the two compounds/well were added to the test plates from two separate master compound plates; this reduced the number of test plates by 50%. The reaction was incubated at room-temperature for 60 minutes to allow the system to come to equilibrium. The fluorescence resulting from FRET was measured in an Envision plate reader (Ex 337 nm, Em 495 nm and 520 nM) and the ratio of the 520nm/495nm emmissions were calculated. These data were normalized using high values (no test compound) and low values (blank wells) to a % inhibition.

Compounds that were active above a threshold % inhibiton value were re-ordered from wet DMSO stocks and re-tested using the identical method. Compounds that confirmed activity were then triaged using cheminformatics methods to a shorter list and the IC50 values were determined using a 10-point dilution series in triplicate.

Counter screens: All compounds that had IC_{50} values determined were also screened in an similar assay using the RIPK1 and RIPK2 enzymes to determine selectivity.

Time Dependence: The assays were set up identically as described above. The plates were read in the plate reader at 5 minutes, 10 minutes and then at 10 minute intervals for 60 minutes. Each time point was treated as a separate data set for curve fitting.

HTRF Assay for RIPK3, RIPK1, RIPK2 and Met kinases:

A time resolved FRET-based competition binding assay was used to assess test article binding to the kinase of interest. His-tagged or GST-tagged kinase at a concentration of 1 nM was incubated with 0.2 nM Tb-labeled detection antibody (anti-His or anti-GST), test compound, and fluorescein-labeled ATP competitive probe at a concentration corresponding to the probe's equilibrium dissociation constant for one hour. Fluorescence at 495 nm and 520 nm was measured using an EnVision microplate reader to quantify FRET between Tb-labeled detection antibody and fluorescein-labeled probe. Background subtracted FRET ratios were normalized to the maximum signal obtained in the absence of test compound. These values were converted to a percent inhibition. Percent inhibition was determined for test compounds at 11 concentrations. The IC_{50}, defined as the concentration of competing test compound needed to reduce specific binding of the probe by 50%, was calculated using the 4 parameter logistic equation to fit the data. Standard deviation for the assays was assessed using a control compound. The standard deviations for the control compound in the key assays are as follows: RIPK1: 271 $\pm$ 116 nM, N = 47; RIPK2: 319 $\pm$ 257 nM, N = 46; RIPK3 667 $\pm$ 461 nM, N = 51; c-Met standard deviation 809 $\pm$ 342 nM, N = 45.

Production and purification of mRIPK3 crystallography construct:

Production of mRIPK3 crystallography construct

Baculovirus was generated for the construct using the Bac-to-Bac baculovirus expression system (Invitrogen) according to the manufacturer's protocol. Briefly, recombinant bacmid was isolated from transformed DH10Bac E.coli competent cells (Invitrogen) and used to transfect Spodoptera frugiperda (Sf9) insect cells (Invitrogen). Baculovirus was harvested 72 hours post transfection and a virus stock was prepared by infecting fresh Sf9 cells at a 1/1000 (v/v) ratio for 66 hours.

For large scale protein production, Sf9 cells (Expression System, Davis, CA) grown in ESF921 insect medium (Expression System) at 2 × 106 cells/ml were infected with virus stock at a 1/200 (v/v) ratio for 67 hours. The production was carried out either at a 10 L scale in a 22 L cellbag (GE Healthcare Bioscience, Pittsburgh, PA) or at a 20 L scale in a 50 L cellbag using WAVE-Bioreactor System 20/50 (GE Healthcare Bioscience). The infected cells were harvested by centrifugation at 2000 rpm for 20 min at 4 ºC in a SORVALL® RC12BP centrifuge. The cell pellets was stored at -70 ºC before protein was purified.

Purification of mRIPK3(1-313)-C111A-His10

RIPK3 containing cell paste was resuspended in 25mM Tris pH 8.0, 150mM NaCl, 1mM $MgCl_2$, 25U/ml Benzonase (Sigma-Aldrich, St. Louis, MO), and Complete Protease Inhbitor tablets (1/50ml, Roche Diagnostics, Indianapolis, IN). The cells were lysed by nitrogen cavitation using an unstirred pressure vessel @525 PSI (Parr Instrument Company, Moline, IL). The suspension was clarified by centrifugation at 35,000 RPM for 30min, at 4°C. The lysate was decanted from the pellet and passed through a 5ml NiNTA Superflow cartridge (Qiagen,Valencia, CA) using an AKTA Pure (GE Healthcare, Chicago IL). Column was eluted with linear 10CV linear gradient into 25mM Tris 8.0, 150mM NaCl, 500mM imidazole. Peak fractions were pooled and loaded directly onto 6ml ResourceQ column (GE Healthcare). Column was washed with 25mM Tris 8.0, 150mM NaCl and eluted in 10CV linear gradient into 25mM Tris 8.0, 150mM NaCl, 1M NaCl. The flowthrough and fractions identified by SDS-PAGE as containing RIPK3 were pooled and concentrated using 30kDa MWCO spin concentrators (Amicon Ultra-15, Millipore, Billerica, MA) and loaded onto a HiLoad 26/600 Superdex 200 column (GE Healthcare) equilibrated in 25mM Tris 8.0, 150mM NaCl, 5mM DTT.

The yield was ~10mg/L with a purity >95% as determined by Coomassie stainedd SDS-PAGE gel analysis. LCMS analysis of the protein showed that the protein had lost the N-terminal methionine, and was partially phosphorylated up to 3 times.

mRIPK3 Crystal Structure Determination:

Crystals of mRIPK3(1-313)-C111A-His10 were prepared by the sitting drop vapor diffusion method. The protein stock solution consisted of 12.95 mg/ml (0.358 mM based on the calculated MW of 36,160 Da) in 150mM NaCl, 5mM DTT buffered by 25 mM Tris at pH 8.0. Protein was incubated overnight with 1.8mM of ligand. A 100mM ligand stock solution dissolved in 100% DMSO was used for complexing. Sample was clarified by centrifugation prior to crystallization.

Crystallization screens were prepared using a Mosquito Crystal (TTP Labtech Ltd., Melbourn, United Kingdom) on MRC 2 well crystallization plates. The optimized conditions consisted of a reservoir solution with 18-22% PEG 3350 (w/v) and 10-15 mM L-Proline. Drops were formed from 0.3 μl of the protein solution and 0.3 μl of the reservoir solution (total initial volume of 0.6 μl), mixed and placed at 20 ºC to equilibrate. Crystals appeared within 3 days and grew to dimensions of 100 μm X 50 μm X 50 μm in 7 days.

Crystals were cryo-protected in a solution consisting of 13.5% PEG (w/v), 8.25mM L-Proline, 10% PEG 400 (v/v) and 10% glycerol (v/v). Crystals were flash frozen in liquid nitrogen and stored prior to data collection. Diffraction from these crystals yielded unit cell parameters a = 144.9, b = 52.8, c = 103.8 Å, $\alpha = \gamma = 90$, $\beta = 130.8$ and space group C 1 2 1. Data was collected at IMCA-CAT at the Advanced Photon Source. Data reduction was performed with autoPROC[1]. The mRIPK3 co-structure was solved by molecular replacement with PDB 4M66 as a search model in Phaser[2]. Model building and refinement were performed with Coot[3] and autoBUSTER (Global Phasing Ltd.), respectively.

1. Vonrhein, C. *et al.* Data processing and analysis with the autoPROC toolbox. *Acta Crystallogr. D Biol. Crystallogr.* **67**, 293–302 (2011).
2. Adams, P.D. *et al.* PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr. D Biol. Crystallogr.* **66**, 213–221 (2010).
3. Emsley, P., Lohkamp, B., Scott, W.G. & Cowtan, K. Features and development of Coot. *Acta Crystallogr. D Biol. Crystallogr.* **66**, 486–501 (2010).

Acknowledgement:

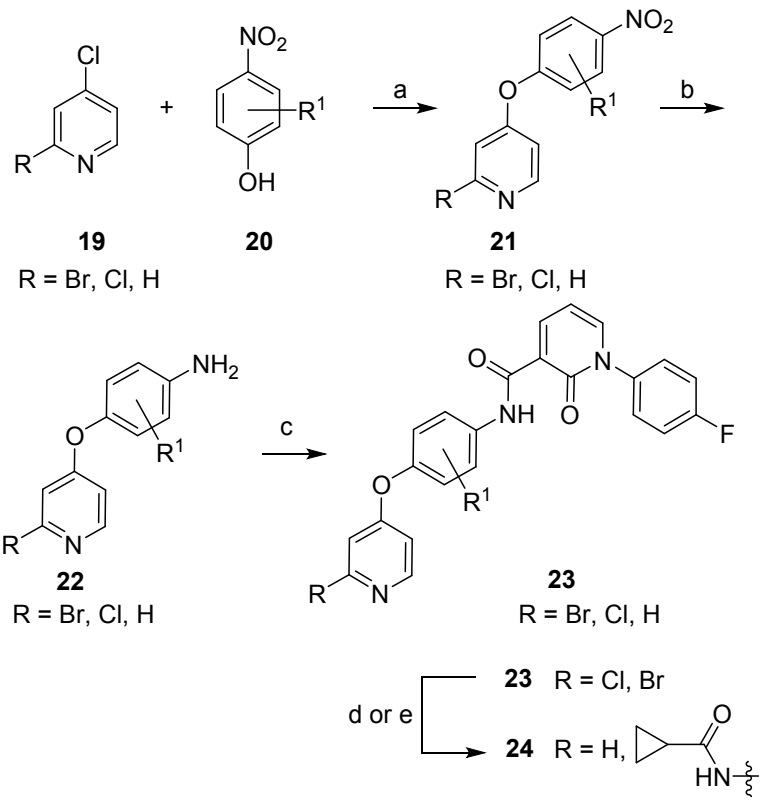
Use of the IMCA-CAT beamline 17-ID at the Advanced Photon Source was supported by the companies of the Industrial Macromolecular Crystallography Association through a contract with Hauptman-Woodward Medical Research Institute. This research used resources of the Advanced Photon Source, a U.S. Department of Energy (DOE) Office of Science User Facility operated for the DOE Office of Science by Argonne National Laboratory under Contract No. DE-AC02-06CH11357.

Experimentals:

General Synthetic Methods:

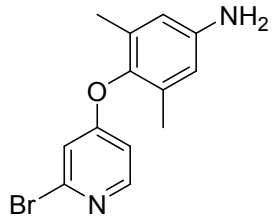
All experiments were carried out under an inert atmosphere and at room temperature unless otherwise stated. Reactions were monitored using thin-layer chromatography on 250 μm plates, or using HPLC and LCMS with UV detection at 220 or 254 nm. Purification was accomplished by medium pressure liquid chromatography on a CombiFlash Companion (Teledyne Isco) with RediSep normal phase silica gel, or by reverse phase preparative HPLC. All compounds for biological testing had purity analyzed with two orthogonal HPLC conditions: Injection 1: a linear gradient using solvent A (5% acetonitrile, 95% water, 0.05% TFA) and solvent B (95% acetonitrile, 5% water, 0.05% TFA); 10-100% of solvent B over 10 min and then 100% of solvent B over 5 min. Column: Sunfire C18 3.5 μm (4.6 x 150 mm). Flow rate was 2 mL/min and UV detection was set to 220 nm; Injection 2: a linear gradient using solvent A (5% acetonitrile, 95% water, 0.05% TFA) and solvent B (95% acetonitrile, 5% water, 0.05% TFA); 10-100% of solvent B over 10 min and then 100% of solvent B over 5 min. Column: Xbridge Phenyl 3.5 μm (4.6 x 150 mm). Flow rate was 2 mL/min and UV detection was set to 220 nm. The columns were maintained at room temperature. LCMS chromatograms were obtained on a Shimadzu HPLC system running Discovery VP software, coupled with a Waters ZQ mass spectrometer running MassLynx version 3.5 software using: method A: a linear gradient using solvent A (10% acetonitrile, 89.9% water, 0.1% of TFA) and solvent B (89.9% acetonitrile, 10% water, 0.1% of TFA); 0-100% of solvent B over 2 min and then 100% of solvent B over 1 min. Column: PHENOMENEX® Luna 3 μm C18 (2.0 x 30 mm). Flow rate was 5 mL/min and UV detection was set to 220 nm; method B: a linear gradient using solvent A (10% methanol, 89.9% water, 0.1% of TFA) and solvent B (89.9% methanol, 10% water, 0.1% of TFA); 0-100% of solvent B over 4 min and then 100% of solvent B over 1 min. Column: PHENOMENEX® Luna 5 μm C18 (4.5 x 30 mm). Flow rate was 4 mL/min and UV detection was set to 220 nm. The LC columns were maintained at room temperature. ^{1}H NMR spectra were recorded on a Bruker spectrometer (400 or 500 MHz) at ambient temperature.

General Scheme:



Reagents and conditions: (a) *i*Pr_2NEt, NMP, microwave, 210 °C; (b) $SnCl_2$, EtOAc, 80 °C; (c) 1-(4-fluorophenyl)-2-oxo-1,2-dihydropyridine-3-carboxylic acid, BOP, Et_3N, DMF; (d) cyclopropanecarboxamide, $Pd_2(dba)_3$, Xantphos, Cs_2CO_3, dioxane, 100 °C; (e) $H_{2(g)}$, Pd/C, MeOH, HCl.

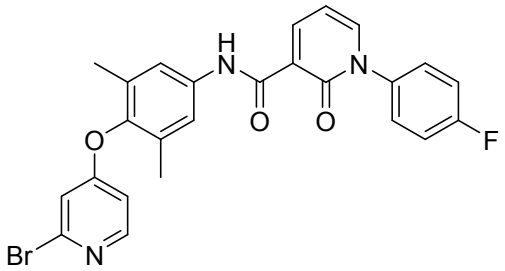
Synthesis of N-(4-((2-(cyclopropanecarboxamido)pyridin-4-yl)oxy)-3,5-dimethylphenyl)-1-(4-fluorophenyl)-2-oxo-1,2-dihydropyridine-3-carboxamide (17):



A solution of 2-bromo-4-fluoropyridine (0.1 g, 0.568 mmol), 4-amino-2,6-dimethylphenol, HCl (0.113 g, 0.653 mmol) and Cs_2CO_3 (0.555 g, 1.705 mmol) in DMF (2.185 ml) was heated to 120 °C for 30 min under microwave. The reaction mixture was diluted with EtOAc and washed with water, 10% LiCl solution and brine. The organics were dried over anhydrous sodium sulfate, filtered and concentrated *in vacuo* to yield a crude product. The crude oil was purified by column chromatography on the Isco system (12 g, 0-30% EtOAc/CH2Cl2). 4-((2-Bromopyridin-4-yl)oxy)-3,5-dimethylaniline was isolated as a brown sticky solid and a regioisomer ratio of 3:1. Material used as is in subsequent chemistry.

MS ESI m/z 292.9 (M+H)

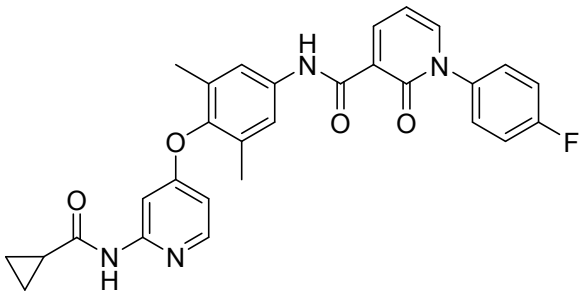
^{1}H NMR (400 MHz, $CDCl_3$) δ 8.16 (d, J=5.7 Hz, 1H), 6.91 - 6.85 (m, 1H), 6.72 (dd, J=5.7, 2.1 Hz, 1H), 6.44 (s, 2H), 2.01 (s, 6H).



To a round bottom flask charged with 4-((2-bromopyridin-4-yl)oxy)-3,5-dimethylaniline (0.133 g, 0.454 mmol) in DMF (0.907 ml) and acetonitrile (0.907 ml) and cooled to 0 °C was added 1-(4-fluorophenyl)-2-oxo-1,2-dihydropyridine-3-carboxylic acid (0.106 g, 0.454 mmol), Hünig's base (0.238 ml, 1.361 mmol) and TBTU (0.160 g, 0.499 mmol). The reaction mixture was allowed to slowly warm to rt 1 h. Water was added and the mixture stirred at rt 20 min. The solid product was isolated by vacuum filtration, rinsing with water. After drying under vacuum, N-(4-((2-bromopyridin-4-yl)oxy)-3,5-dimethylphenyl)-1-(4-fluorophenyl)-2-oxo-1,2-dihydropyridine-3-carboxamide (0.188 g, 0.370 mmol, 82 % yield) was isolated as a light orange solid.

MS ESI m/z 507.8 (M+H)

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.57 (dd, *J*=7.3, 2.1 Hz, 1H), 8.28 - 8.20 (m, 1H), 8.11 (dd, *J*=6.6, 2.1 Hz, 1H), 7.66 - 7.55 (m, 4H), 7.47 - 7.37 (m, 2H), 7.01 (d, *J*=2.2 Hz, 1H), 6.90 (s, 1H), 6.82 (dd, *J*=5.7, 2.2 Hz, 1H), 6.72 (t, *J*=7.0 Hz, 1H), 2.04 (s, 6H).



To a degassed solution of N-(4-((2-bromopyridin-4-yl)oxy)-3,5-dimethylphenyl)-1-(4-fluorophenyl)-2-oxo-1,2-dihydropyridine-3-carboxamide (50 mg, 0.098 mmol) and cyclopropanecarboxamide (12.56 mg, 0.148 mmol) in dioxane (492 µl) was added Xantphos (5.69 mg, 9.84 µmol), $Pd_2(dba)_3$ (18.01 mg, 0.020 mmol) and Cs_2CO_3 (51.3 mg, 0.157 mmol). The reaction mixture was heated to 80 °C for 4 h and cooled to rt. The reaction mixture was filtered through a syringe filter and diluted to a total volume of 1.5 mL with DMF. The crude material was purified via preparative LC/MS with the following conditions: Column:

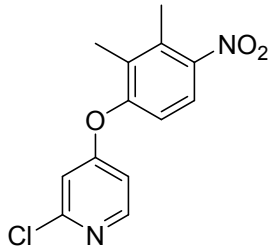
XBridge C18, 19 x 200 mm, 5-µm particles; Mobile Phase A: 5:95 acetonitrile: water with 0.1% trifluoroacetic acid; Mobile Phase B: 95:5 acetonitrile: water with 0.1% trifluoroacetic acid; Gradient: 21-61% B over 20 minutes, then a 4-minute hold at 100% B; Flow: 20 mL/min. Fractions containing the desired product were combined and dried via centrifugal evaporation to yield (N-(4-((2-(cyclopropanecarboxamido)pyridin-4-yl)oxy)-3,5-dimethylphenyl)-1-(4-fluorophenyl)-2-oxo-1,2-dihydropyridine-3-carboxamide (6.6 mg, 0.012 mmol, 13 %).

LRMS ESI m/z calculated for $C_{29}H_{25}FN_4O_4$ 512.5, found 513.3 (M+H)

^{1}H NMR (500 MHz, DMSO-d6) δ 11.93 (s, 1H), 10.76 (s, 1H), 8.61 - 8.53 (m, 1H), 8.14 (d, J=5.8 Hz, 1H), 8.11 - 8.05 (m, 1H), 7.60 (br dd, J=8.5, 4.9 Hz, 2H), 7.53 (s, 2H), 7.49 (s, 1H), 7.41 (br t, J=8.7 Hz, 2H), 6.72 (t, J=6.9 Hz, 1H), 6.55 (br d, J=3.7 Hz, 1H), 2.03 (s, 6H), 1.98 - 1.89 (m, 1H), 0.79 - 0.70 (m, 4H).

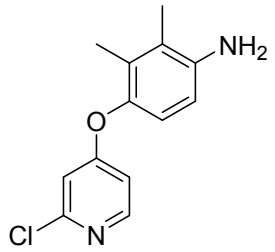
Purity: 98%

Synthesis of N-(4-((2-(cyclopropanecarboxamido)pyridin-4-yl)oxy)-2,3-dimethylphenyl)-1-(4-fluorophenyl)-2-oxo-1,2-dihydropyridine-3-carboxamide (18):



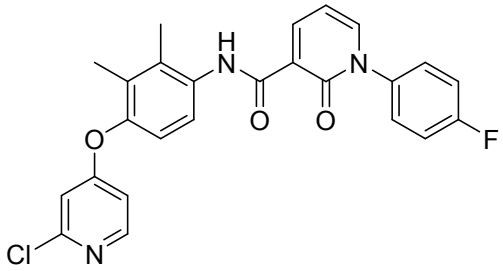
To a microwave vial charged with 2,3-dimethyl-4-nitrophenol (1.741 g, 10.41 mmol) in NMP (11.57 mL) was added Hünig's base (3.03 mL, 17.36 mmol) and 2,4-dichloropyridine (0.75 mL, 6.94 mmol). The vial was capped, and the reaction was heated at 210 °C under microwave for 6 h. The reaction mixture was diluted with ethyl acetate and extracted with water. The organic layer was washed with 10% lithium chloride solution (4 x). The organic layer was dried over anhydrous sodium sulfate, filtered and concentrated *in vacuo*. The crude residue was purified by column chromatography (40 g, 0-50% EtOAc/Hex). 2 peaks with correct mass observed. The desired 4-chloro-2-(2,3-dimethyl-4-nitrophenoxy)pyridine (second peak) was carried forward as is (moderate purity).

MS ESI m/z 279.0 (M+H).



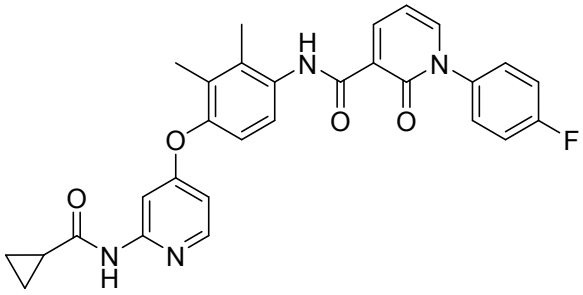
To a vial charged with 2-chloro-4-(2,3-dimethyl-4-nitrophenoxy)pyridine (0.29 g, 1.041 mmol) in ethyl acetate (11 mL) was added tin(II) chloride dihydrate (0.939 g, 4.16 mmol). The reaction mixture was warmed to 75 °C 90 min, then allowed to cool to rt. The slurry was transferred to a separatory funnel with ethyl acetate and 1 N NaOH was added. The aqueous layer was washed with ethyl acetate and the combined organics were dried over anhydrous sodium sulfate, filtered and concentrated *in vacuo*. The crude material was purified by flash column chromatography on the Isco system (24 g, 0-10% MeOH in DCM). 4-((2-Chloropyridin-4-yl)oxy)-2,3-dimethylaniline (95 mg, 0.348 mmol, 33.4 % yield) was isolated as a tan solid.

MS ESI m/z 249.3 (M+H).



A solution of 4-((2-chloropyridin-4-yl)oxy)-2,3-dimethylaniline (33 mg, 0.133 mmol), 1-(4-fluorophenyl)-2-oxo-1,2-dihydropyridine-3-carboxylic acid (37.1 mg, 0.159 mmol), Et_3N (0.055 mL, 0.398 mmol) and BOP (70.4 mg, 0.159 mmol) in DMF (0.75 mL) was agitated ON at rt. The reaction mixture was concentrated to an oil, then loaded onto a 12g ISCO column for purification by flash chromatography eluting with 0-10% MeOH in DCM. Afforded N-(4-((2-chloropyridin-4-yl)oxy)-2,3-dimethylphenyl)-1-(4-fluorophenyl)-2-oxo-1,2-dihydropyridine-3-carboxamide (39 mg, 0.082 mmol, 62.1 % yield).

MS ESI m/z 464.4 (M+H).



A solution of N-(4-((2-chloropyridin-4-yl)oxy)-2,3-dimethylphenyl)-1-(4-fluorophenyl)-2-oxo-1,2-dihydropyridine-3-carboxamide (33 mg, 0.071 mmol), cyclopropanecarboxamide (18.16 mg, 0.213 mmol), and Xantphos (8.23 mg, 0.014 mmol) in 1,4-dioxane (1.5 mL) was purged by bubbling nitrogen through the solution in a sealable tube for 5 minutes. Cesium carbonate (93 mg, 0.285 mmol) and $Pd_2(dba)_3$ (13.03 mg, 0.014 mmol) were added, and the vial was then sealed and stirred at 100 °C for 1h. The reaction

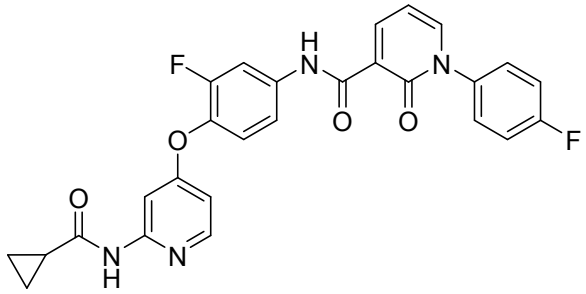
mixture was diluted with DMF and purified via preparative LC/MS with the following conditions: Column: Waters XBridge C18, 19 x 200 mm, 5-µm particles; Mobile Phase A: 5:95 acetonitrile: water with 0.1% trifluoroacetic acid; Mobile Phase B: 95:5 acetonitrile: water with 0.1% trifluoroacetic acid; Gradient: 15-70% B over 20 minutes, then a 5-minute hold at 100% B; Flow: 20 mL/min. Fractions containing the desired product were combined and dried via centrifugal evaporation. AffordedN-(4-((2-(cyclopropanecarboxamido)pyridin-4-yl)oxy)-2,3-dimethylphenyl)-1-(4-fluorophenyl)-2-oxo-1,2-dihydropyridine-3-carboxamide, TFA (21.5 mg, 0.041 mmol, 57.2 % yield).

LRMS ESI m/z calculated for $C_{29}H_{25}FN_4O_4$ 512.5, found 513.2 (M+H)

^{1}H NMR (500MHz, DMSO-d_6) δ 11.85 (s, 1H), 11.05 (br. s., 1H), 8.62 (dd, *J*=7.1, 2.0 Hz, 1H), 8.15 (d, *J*=8.8 Hz, 2H), 8.11 (dd, *J*=6.6, 1.9 Hz, 1H), 7.62 (dd, J=8.8, 4.7 Hz, 2H), 7.44 (t, J=8.8 Hz, 3H), 7.02 (d, J=8.8 Hz, 1H), 6.74 (t, J=6.9 Hz, 2H), 2.23 (s, 3H), 2.06 (s, 3H), 1.93 (br. s., 1H), 0.89 - 0.74 (m, 4H).

Purity: 97%

Data for all analogs:



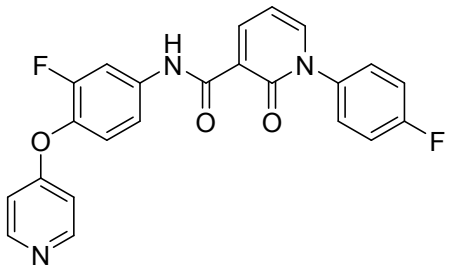
10:

Compound **10** was synthesized following the protocols used in the synthesis of **17** and starting with 2-bromo-4-fluoropyridine and 4-amino-2-fluorophenol.

LRMS ESI m/z calculated for $C_{27}H_{20}F_2N_4O_4$ 502.5, found 503.2 (M+H)

^{1}H NMR (500MHz, DMSO-d_6) δ 12.09 (s, 1H), 10.89 (s, 1H), 8.60 - 8.55 (m, 1H), 8.19 (d, *J*=6.1 Hz, 1H), 8.08 (dd, *J*=6.6, 1.9 Hz, 1H), 8.02 - 7.96 (m, 1H), 7.61 - 7.52 (m, 3H), 7.48 - 7.37 (m, 3H), 7.33 (t, *J*=8.9 Hz, 1H), 6.78 - 6.69 (m, 2H), 1.95 - 1.86 (m, 1H), 0.80 - 0.72 (m, 4H).

Purity: 99%



11:

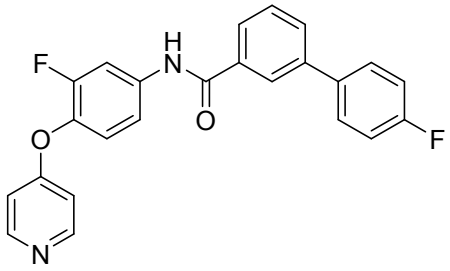
Compound **11** was synthesized following the protocols used in the synthesis of **17** and starting with 4-((2-chloropyridin-4-yl)oxy)-3-fluoroaniline (*J. Med. Chem.* **2009**, *52*, 1251-1254). After the amide coupling step, the final product was obtained via the following protocol:

To a flask charged with N-(3-((2-chloropyridin-4-yl)oxy)-4-fluorophenyl)-1-(4-fluorophenyl)-2-oxo-1,2-dihydropyridine-3-carboxamide (36 mg, 0.079 mmol) in methanol and flushed with nitrogen, was added 10% Pd/C - Degussa Type (5 mg, 0.047 mmol) under nitrogen. Hydrochloric acid (79 µl, 0.079 mmol) was added. The reaction was evacuated and purged with hydrogen (4 x). The suspension was stirred under hydrogen ON. The reaction was diluted with methanol to get all the product into solution, filtered through a pad of Celite, and concentrated under reduced pressure. The crude mixture was purified via preparative LC/MS with the following conditions: Column: Waters XBridge C18, 19 x 200 mm, 5-µm particles; Mobile Phase A: 5:95 acetonitrile: water with 10-mM ammonium acetate; Mobile Phase B: 95:5 acetonitrile: water with 10-mM ammonium acetate; Gradient: 15-100% B over 20 minutes, then a 0-minute hold at 100% B; Flow: 20 mL/min. Fractions containing the desired product were combined and dried via centrifugal evaporation to yield **11** (13.9 mg, 0.033 mmol, 42%).

LRMS ESI m/z calculated for $C_{23}H_{15}F_2N_3O_3$ 419.4, found 420.1 (M+H)

^{1}H NMR (500MHz, DMSO-d_6) δ 12.08 (s, 1H), 8.61 - 8.53 (m, 1H), 8.46 (d, *J*=5.4 Hz, 2H), 8.07 (dd, *J*=6.7, 1.7 Hz, 1H), 8.02 - 7.94 (m, 1H), 7.57 (dd, *J*=8.6, 4.9 Hz, 2H), 7.49 - 7.30 (m, 4H), 6.97 (d, *J*=5.7 Hz, 2H), 6.73 (t, *J*=7.1 Hz, 1H).

Purity: 100%



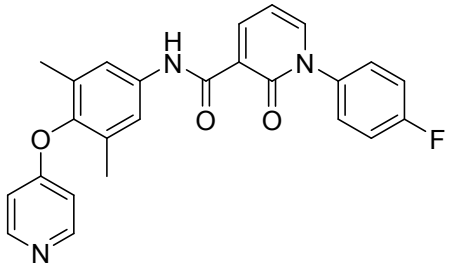
12:

Compound **12** was synthesized following the protocols used in the synthesis of **17** and starting with 2-bromo-4-fluoropyridine and 4-amino-2-fluorophenol. To obtain the final product, a hydrogenation was used as desrcibed for **11**.

LRMS ESI m/z calculated for $C_{24}H_{16}F_2N_2O_2$ 402.4, found 403.1 (M+H)

^{1}H NMR (500 MHz, DMSO-d_6) δ 8.47 (br d, *J*=5.5 Hz, 2H), 8.19 (s, 1H), 8.01 - 7.91 (m, 2H), 7.87 (br d, *J*=7.8 Hz, 1H), 7.83 - 7.77 (m, 2H), 7.70 - 7.60 (m, 2H), 7.41 - 7.29 (m, 3H), 6.94 (d, *J*=5.7 Hz, 2H).

Purity: 100%



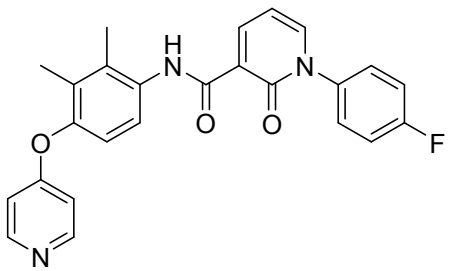
13:

Compound **13** was synthesized following the protocols used in the synthesis of **18** and starting with 4-bromopyridine and 2,6-dimethyl-4-nitrophenol.

LRMS ESI m/z calculated for $C_{25}H_{20}FN_3O_3$ 429.5, found 430.1 (M+H)

^{1}H NMR (500MHz, DMSO-d_6) δ 11.96 (s, 1H), 8.57 (dd, *J*=7.2, 1.9 Hz, 1H), 8.43 (br. s., 2H), 8.11 (dd, *J*=6.4, 2.0 Hz, 1H), 7.67 - 7.53 (m, 4H), 7.42 (t, *J*=8.8 Hz, 2H), 6.80 (d, *J*=5.4 Hz, 2H), 6.72 (t, *J*=6.9 Hz, 1H), 2.03 (s, 6H).

Purity: 98%



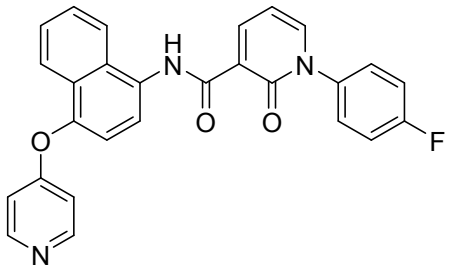
14:

Compound **14** was synthesized following the protocols used in the synthesis of **18** and starting with 4-bromopyridine and 2,3-dimethyl-4-nitrophenol.

LRMS ESI m/z calculated for $C_{25}H_{20}FN_3O_3$ 429.5, found 430.2 (M+H)

^{1}H NMR (500MHz, DMSO-d_6) δ 11.82 (s, 1H), 8.61 (dd, *J*=7.2, 1.9 Hz, 1H), 8.42 (d, *J*=5.0 Hz, 2H), 8.16 - 8.06 (m, 2H), 7.61 (dd, *J*=8.8, 5.0 Hz, 2H), 7.43 (t, *J*=8.8 Hz, 2H), 7.00 (d, *J*=8.8 Hz, 1H), 6.81 (d, *J*=5.7 Hz, 2H), 6.73 (t, *J*=6.9 Hz, 1H), 2.22 (s, 3H), 2.04 (s, 3H).

Purity: 99%



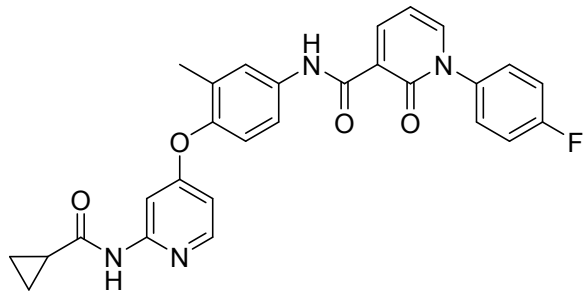
15:

Compound **13** was synthesized following the protocols used in the synthesis of **18** and starting with 4-bromopyridine and 4-nitronapthalen-1-ol.

LRMS ESI m/z calculated for $C_{27}H_{18}FN_3O_3$ 451.5, found 452.2 (M+H)

^{1}H NMR (500MHz, DMSO-d_6) δ 12.69 (s, 1H), 8.68 (d, *J*=5.7 Hz, 1H), 8.54 (d, *J*=8.4 Hz, 1H), 8.45 (d, *J*=4.4 Hz, 2H), 8.14 (d, *J*=8.8 Hz, 2H), 7.88 (d, *J*=8.4 Hz, 1H), 7.75 - 7.63 (m, 3H), 7.63 - 7.56 (m, 1H), 7.46 (t, *J*=8.6 Hz, 2H), 7.41 (d, *J*=8.4 Hz, 1H), 6.95 (d, *J*=5.4 Hz, 2H), 6.79 (t, *J*=6.9 Hz, 1H).

Purity: 100%



16:

Compound **13** was synthesized following similar protocols to that described for **18** and starting with 2-chloro-4-fluoropyridine and 2-dimethyl-4-nitrophenol.

LRMS ESI m/z calculated for $C_{28}H_{23}FN_4O_4$ 498.5, found 499.2 (M+H)

^{1}H NMR (500 MHz, DMSO-d_6) δ 11.99 (s, 1H), 10.81 (s, 1H), 8.59 (dd, *J*=7.2, 1.9 Hz, 1H), 8.17 (d, *J*=5.7 Hz, 1H), 8.11 (dd, *J*=6.6, 1.9 Hz, 1H), 7.71 - 7.64 (m, 2H), 7.61 (dd, *J*=8.7, 4.9 Hz, 2H), 7.55 (d, *J*=1.9 Hz, 1H), 7.43 (t, *J*=8.8 Hz, 2H), 7.08 (d, *J*=9.4 Hz, 1H), 6.73 (t, *J*=6.9 Hz, 1H), 6.62 (dd, *J*=5.7, 2.2 Hz, 1H), 2.08 (s, 3H), 1.95 (quin, *J*=6.1 Hz, 1H), 0.76 (br d, *J*=6.1 Hz, 4H).

Purity: 96%

Kinome Selectivity for 18

Kinome selectivity was assessed against a panel of kinases using the HTRF protocols described herein. Only kinases with an $IC_{50} < 1$ μM are listed here.

Kinase	IC50, uM	Kinase	IC50, uM
ABL	0.37	FYN	0.99
ARG	0.97	GSK3B	0.33
ETK	0.59	HGK	0.97
BRAF/V599E	0.15	KHS1	0.068
BRK	0.18	LCK	0.06
DDR2	0.011	LYN	0.047
EPHA1	0.31	MAP4K3	0.012
EPHA2	0.024	P38A	0.13
EPHA3	0.25	P38B	0.42
EPHA4	0.056	PDE4	0.50
EPHA5	0.014	RAF1	0.011
EPHA6	0.020	RON	0.50
EPHA7	0.13	SKMLCK	0.079
EPHA8	0.026	SRC	0.075
EPHB1	0.035	SRM	0.51
EPHB2	0.010	LOK	0.081
EPHB4	0.13	TIE2	0.42
FES	0.28	TNIK	0.79
FGR	0.012	KDR	0.38
FLT3	0.56	ZAK	0.28
FMS	0.41		
FRK	0.11		